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The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms

5th International Symposium

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Edited by

Joachim Schiemann

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Dr. Joachim Schiemann
Biologische Bundesanstalt für Land- und Forstwirtschaft
Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit
Messeweg 11/12
38104 Braunschweig, Germany
E-Mail: J.Schiemann@bba.de

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Laplace, F.

Federal Ministry of Education and Research, Bonn, Germany

Ladies and Gentlemen,

It is my pleasure to welcome you on behalf of the Federal Ministry of Education, Science, Research and Technology to the 5th International Biosafety Symposium in Braunschweig.

Following the 2nd International Symposium, which took place in Goslar in 1992, many of you are now for the second time meeting in Germany to discuss the latest results from research involving the analysis of field tests of genetically modified plants and microorganisms.

Like each of the previous symposia, this event on the one hand will also provide an excellent opportunity to analyse the significance of these results for basic research, and on the other hand to extract from them specific approaches to the further development of framework legislation in the field of genetic engineering.

As we are hosting this symposium for the second time, we have the opportunity to demonstrate to what extent biosafety research has influenced the development of framework legislation governing genetic engineering in Germany in the six years since the 2nd Symposium.

While until 1992 our Genetic Engineering Act ensured the strictest conditions in Europe, the amendment of this Act in 1993 allowed the same requirements to be introduced here as in our European neighbouring countries.

The result of this was an impressive biotechnology boom in Germany, which is still going on. The number of new companies is growing constantly, venture capital and direct-investment capital are readily available, foreign biotechnology companies are investing in Germany again, and the Government is promoting promising scientific fields by means of innovative funding programmes.

I am convinced that this development would not have been possible without a high standard of biosafety research. In discussions with parliamentarians, scientists, representatives from industry, regulatory authorities, interested citizens and associations, the results of biosafety research have played an important role. With the help of these scientific data and results, it has proved possible to dispel initial fears of supposed risks which were thought to be connected with the application of genetic engineering procedures. As a result, superfluous bureaucratic burdens have been removed from the framework legislation.

At the same time, all those involved have increasingly come to realise that it is possible to use this technology in a responsible way, and that its extensive use and application in the most diverse areas of research and business is essential for the well-being of mankind and the environment.

It is now a matter of course for any researcher, whether academic or in industry, to be aware from the beginning of the issue of biosafety in the conception of experiments and procedures which make use of genetic engineering.

In this way the results of biosafety research have contributed not only to the successful development of biotechnology but also to the promotion of the acceptance of these technologies in the population, and to the formation of a consensus in society allowing widespread use of these procedures in the most diverse areas.

In order for this development to continue successfully, I believe that there is a need for further international networking in this area of research, for concentration of research on relevant and up-to-date research priorities, and for an international forum to present the results achieved.

I am delighted that many sponsors are supporting this meeting so generously, and my special thanks go to the Federal Biological Research Centre for Agriculture and Forestry, in particular to Rudolf Casper and Joachim Schiemann, for making it possible to organise this symposium in Braunschweig at such short notice. I wish you all success and interesting discussions.

Welcome address

Schulze-Weslarn, K.-W.

Federal Ministry of Food, Agriculture and Forestry

Ladies and Gentlemen,

It is a pleasure for me, as the representative of the Federal Ministry of Food, Agriculture and Forestry, to convey the greetings of Federal Minister Jochen Borchert and to welcome you to the 5th International Symposium on "The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms" here in Braunschweig.

Meanwhile, this symposium has a history of eight years. The first one was held in Kiawah (South Carolina) in 1990, followed by the symposia in Goslar (Germany) in 1992, in Monterey (California) in 1994, and in Tsukuba (Japan) in 1996.

We are proud to host this important meeting for the second time already.

In the public debate on genetic engineering in Germany, people - in general - are very much in favour of applying it in the medical and pharmaceutical field. But as far as its use in agriculture and the production of food is concerned, it frequently is the bad news that get attention. We know from opinion polls: Many citizens in Germany feel uncertain about this way of applying genetic engineering and consider it to be a threat. This is why it is important, in meetings like this, to provide scientifically founded information and to discuss, without any reservation, potential risks of genetic engineering.

For the Federal government, in the assessment of genetic engineering, in accordance with the principle of precaution, the safety of humans, animals and the environment is ranging first. Dr. Laplace has already mentioned the importance of safety research in Germany.

Because of the experience made, the Federal government considers genetic engineering to be a safe method, the application of which can make us expect great benefit in the field of agriculture, too, and which will be indispensable in future. The precondition is responsible handling of this technique.

In our country, safe management is guaranteed by precautionary legal provisions which are applied on a case-to-case basis and a step-by-step procedure for the release of genetically modified organisms to the environment. As already mentioned we know from studies that people have a more positive approach to genetic engineering in the field of health as they expect a high benefit for themselves. In the field of agriculture and nutrition this benefit, in many cases, at present cannot be recognised at first glance or is not directly obvious. There is already now a wide choice of high quality food products at reasonable prices.

So, what is the application of genetic engineering in agriculture about?

Nowadays, the farming sector is facing more competition, both regionally and internationally. At the same time, it has to comply with ever increasing consumer demands in terms of environmentally friendly production and the duality of its output.

Faced with this situation, the farming sector must increase its efforts to produce on an even more environmentally friendly basis and to maintain and increase market shares and income potentials by means of cost reduction, quality output and product innovation. To be able to do so, new scientific findings and the technological progress must be used.

Biotechnology opens up new possibilities of sustainable agriculture by a more target-oriented, more efficient and economical use of natural resources as well as for worldwide food security. Modern biotechnology techniques can be applied in the agricultural and food industries in many ways.

First of all there is the possibility for agriculture of using genetic engineering to improve the cultivation of our crops and the environmental compatibility of plant production. Thus genetic engineering can strengthen the resistance to diseases and pests so that the application, in certain cases, of chemical plant protection products can be reduced in future. Genetic engineering also offers the chance of improving the

tolerance of crops to drought stress and to salinity or other stress factors for soils. This would be an important development, especially for third world countries.

And, today there are already very promising approaches for the use of agricultural products as environmentally friendly renewable resources. By means of genetic engineering, plant varieties could be used in a way that makes them particularly suited to provide the raw material necessary, in terms of substance composition, for processing in the manufacturing industry. These tailor-made crops enlarge the possibilities of the use of agricultural raw materials.

Finally, with regard to food quality aspects there seem to be new possibilities like

- the reduction of the risk of allergies in the use of certain food products;
- the reduction in the nitrate content of certain vegetable varieties;
- the improvement of the nutritional value by means of a change in the combination of nutrients, as well as
- the improvement of the sensory value for consumers.

Furtheron, if certain substances like the ones used to facilitate the processing of food products can be used in genetic engineering through the use of more efficient microorganisms, this means a more considerate use of finite resources since the same output can be produced at a reduced level of energy and raw material input and less waste is produced.

Moreover, breeding targets can be achieved more quickly by molecular analysis of the genetic material of organisms and by marker-assisted selection, even if the classical breeding methods are applied. Genetic markers are an important tool of breeding. This applies to both plant and animal breeding. No genetically modified plants or animals are produced in such procedures. It is only that the findings in the field of molecular genetics research on the presence or absence of certain genes are used by breeders for the purpose of selection.

Last but not least, the impact of genetic engineering on food production for an ever increasing world population must not be underestimated. We are facing a continuous rise in population growth while land resources are finite. The FAO's estimate is that by the year 2030 food production must have increased by 60%. And it is only in a very limited way that land that has not yet been used for the production of food may be put to use for such a purpose. So a major part of the increase in food output must be achieved on the land currently under cultivation. In the light of this fact it will be necessary to improve the productivity of farming per land unit, taking into account, as far as possible, the impact on the environment in a global setting.

Ladies and Gentlemen,

I think Professor Dr. Casper and Dr. Schiemann who have organised this meeting, have succeeded in convincing renowned experts in the field of genetic engineering from all over the world to participate in this symposium. I would like to thank all of you for joining us here today, all organisers for having prepared and staged this event, as well as Mr. Ioannis Economidis from the EU Commission and Dr. Michael Schechtman from the US Department of Agriculture for their support. I hope that this week's meeting provides new findings about risk assessment which can be used by the general public, politicians and the competent agencies as well as the industry to make their decisions. I wish you interesting lectures, lively and substantial discussions as well as a fruitful exchange of ideas. And last but not least I hope you have some time left to enjoy your stay here in Braunschweig.

Thank you.

Welcome

Bartels, U.

State Secretary, Ministry for Food, Agriculture and Forestry of Lower Saxony, Hannover, Germany

As a representative of the government of Lower Saxony, it is a pleasure to welcome you to this important symposium here in our federal state.

After all, biotechnology is known to be one of the key technologies in the future. In the long term it will shape the scientific profile and influence our society.

The industry of Lower Saxony also has discovered the chances of biotechnology which will become an important source of innovation in international competition. This is even more important since agricultural and horticultural economy just as other economic sectors are increasingly confronted with the challenge of the global market. This, in addition, is associated with a continuous reduction of public funding. Recognizing this development it is absolutely necessary that the access of the agricultural and horticultural industry to biotechnology and genetic engineering is supported. Only this will result in a good position in the international market. The options include the improvement of quality in fruit and vegetables, the engineering of new variants of flowers and plants, and new applications of oil seed and potato plants. In addition, the techniques allow the engineering of disease resistant plants. The technique of genetic engineering allows to reach the goals of conventional culture in a significantly reduced time span. Crucial to the actual employment and the future economic success of these crops and flowers, however, is the consumer acceptance of genetically engineered food products.

The application of genetic engineering has to be restricted at a point where consumer health is potentially at risk. Consumers must have free access to any information necessary in order to make their own choice and decisions.

Genetic engineering allows the reduction of fertilizers and pesticides which will contribute to environmentally friendly agriculture. The breeding of resistant and healthy lifestock is possible. We appreciate the extensive authorization and application procedures required for laboratory work and field testing as a precaution to avoid potential risks. The vandalism of field testing areas or genetic research facilities or plants by self-appointed “saviours” is in no way acceptable. There is no justification for such criminal activities.

I mentioned at the beginning that biotechnology is one of the leading technologies of the future. Still, economic risks will be involved since it can be foreseen that biotechnology will be profitable only with a significant delay of time. All the more commendable, therefore, is the future-orientated mutual effort of industry and science. In 1997, only 4,013 jobs were provided by biotech companies within Germany; in Europe there were 39,000, and 140,000 in the USA.

In order to support recognizable positive tendencies within Europe, it is essential to provide political stability and continuity for scientific and developmental activities and technology transfer projects. Therefore, the government of Lower Saxony will make political efforts to maintain already existing structures and to support further development. BioRegioN is a first starting point.

In addition, I feel that it is necessary for society to get involved with this subject such that a constant dialogue between people, politics, and science can develop. This meeting is a good example.

I wish you will gain a lot of information and have interesting discussions which will have a positive impact on our society’s view of biotechnology.

New developments in science for biosafety evaluation

Beringer, J.E. (Moderator)

School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK

In order for biotechnology to be adopted wisely and with public confidence it is necessary to ensure that releases will be safe. For this to be done effectively it is necessary for regulatory committees to have the latest information from experience and appropriate research. This session provided valuable information about both.

For all risk assessments it is important to be able to evaluate risks with knowledge of the impact of scale because risk is scale-related. In this respect it was particularly beneficial for Europeans to be made aware of the extent of releases in the world beyond the borders of the European Union. The paper by Clive James demonstrates how rapidly GM crops have been adopted in many different countries. The 28 million hectares of such crops grown outside China in 1998, together with extensive area of such crops in the proceeding few years, have provided some comfort to those who have argued that GM crops per se are not dangerous. From the prospective of countries in which the commercialisation of GM crops has been slow there is the added advantage for regulatory authorities that data from experience with the cultivation of equivalent crops in the USA and elsewhere can be used to assist risk assessments.

It is particularly important for all regulatory authorities to be aware of these releases and of any problems that may, or may not, arise. However, it is equally important to remember that environmental conditions differ, as also will the prevalence of cross-pollinating relatives that might inherit genes. It is also important for critics of the technology to recognise that the exceptionally rapid uptake of GM crops in the USA has been driven by farmers purchasing seed on an open market, indicating that to date any problems have been minor.

An important potential problem with GM crops is the pollination of neighbouring crops and weedy relatives. It is thus very valuable to have information about gene flow from crops; some is provided in the Chapters by Allison Snow and Glynnis Giddings. It is clear from their work, and other studies, that pollen can travel long distances and that at least within a radius of five kilometres there is a finite chance that inter-fertile relatives could become pollinated. The frequency with which such pollinations will occur will be dependent on many parameters.

While it has been valuable to learn about possible cross-pollination distances, of more value to risk assessments will be good data on the probability of introgression of such genes within populations of plants that receive the pollen. Unfortunately data of this nature are limited. However, Allison Snow made us aware of work demonstrating that weedy species had inherited rust resistance from cross-pollinating non-GM crop species. How important such events will be must be determined by the benefit to the recipient that is conferred by the transferred genes. It is essential that we learn much more about how selection operates and thus how to assess the risks that could arise from the introduction of genes that could enhance environmental fitness.

Allison Snow's talk makes it clear that, as one might expect, non-GM crops growing in centres of origin and diversity of crop plants will have been exchanging genes with their wild relatives. There appears to be little rational reason, therefore, to assume that GM crops should be excluded from such areas to prevent introgression of genes into wild populations.

There have been reports that the long-term stability of transgenes could affect the viability of GM crops, and particularly compromise the chance of usefully manipulating trees. It was particularly useful to receive information from Dave Ellis about work on gene expression in transgenic conifers in the field. While observing variation in expression among clones, the general observation was that expression was stable for up to seven years in spruce. There is clearly a need for much more data on the stability of gene expression to be obtained from the very large number of transgenic cultivars now in cultivation. This information will be particularly valuable for future risk assessments of organisms in which a given amount of gene expression is required to make them safe in the environment.

A further observation from work with trees was that long-term natural forests are strongly buffered against single genes conferring a selective advantage. The logic of this observation in terms of the known

stability of forests is clear, but how widely applicable is this observation for populations of other types of perennial plants?

William Schneider reported on the way in which EPA had handled the regulatory approval of *Bt* crops. The EPA is not giving unrestricted approval for the commercialisation of such crops because there are inadequate data to prove that the anticipated risks of harm to the environment will not be greater than expected. An interesting aspect of the EPA approval process was that risk management is needed to ensure that the development of resistance to *Bt* toxin does not compromise the use of this pesticide; something that has not been required for other pesticides including other „natural“ products such as pyrethroids. While I accept that there is logic in controlling the development of resistance in pests, I can see none in treating *Bt* toxin as uniquely different. If the EPA is setting a precedent for future GM pest resistant crops it could seriously impede the development of transgenic crops in place of traditional chemical control of insect pests.

We seldom hear much about the release of transgenic bacteria because such releases have been rare to date. Alf Pühler reported on the release of a $RecA^-$ derivative of *Sinorhizobium meliloti*. Surprisingly, despite having a replication time nearly 1/3 longer than the $RecA^+$ parent the population of both remained approximately the same 160 weeks after introduction into the soil. The populations of both strains initially declined by about 99%, but thereafter remained fairly constant. It is perhaps typical of our present knowledge of microbial ecology that we cannot be sure that the bacteria were metabolically active and that what was being recorded was viable, but relatively inactive, bacteria dying very slowly.

This and other sessions helped to demonstrate that the direct impact of GM crops on the environment is unlikely to be greater than that of the varieties they replace. It is also clear that GM crops can reduce the amounts of pesticides used in agriculture, which is an undoubted benefit in terms of public perception of advantages. However, we should realise that herbicide tolerance offers the chance for crops that are presently difficult to keep weed-free to become much less weedy with obvious benefits in terms of yield. However, benefits of this nature are potentially very harmful to wildlife because the weeds are no longer available as food for wild animals, birds and insects; let alone their role as part of the natural diversity of plants in the environment. I believe that properly handled GM crops need be no more harmful to wildlife than conventional crops, and could indeed help to develop farming systems that allow increased diversity of plants and animals. It is perhaps time that we thought more about the whole agricultural system and how we can use technology to maximise all the different returns we want from agriculture.

Global review of commercialized transgenic crops: 1998 (preliminary executive summary and principal tables)

James, C.

Chair, ISAAA Board of Directors

Executive summary

The adoption of commercialized transgenic crops globally, excluding China, in 1998 is characterized. The global data base is analyzed by country, crop and trait, and the economic benefits to growers of selected transgenic crops in 1996 and 1997 in the USA and Canada are estimated. The data presented on the current global status of commercialized transgenic crops is complemented with a discussion of some of the important issues, including global food security and the potential benefits for developing countries in a rapidly evolving global market for transgenic crops.

Between 1996 and 1998, eight countries, five industrial and three developing, have contributed to more than a 15 fold increase in the global area of transgenic crops. Adoption rates for transgenic crops are some of the highest for new technologies by agricultural industry standards. High adoption rates reflect grower satisfaction with the products that offer significant multiple benefits ranging from more flexible crop management, higher productivity and a safer environment through decreased use of conventional pesticides, which collectively contribute to a more sustainable agriculture. In 1998, the global area of transgenic crops increased by 16.8 million ha to 27.8 million ha from 11.0 million ha in 1997. Five principal transgenic crops were grown in eight countries in 1998, three of which, Spain, France and South Africa, grew transgenic crops for the first time in 1998. Data for China has not been included in the global data base because only tentative estimates were available which suggest that 100,000 ha of transgenic crops were grown in 1998, representing <1% of global transgenic area with *Bt* cotton being the principal crop.

The countries listed in descending order of transgenic crop area on a global basis in 1998 are: USA 20.5 million ha representing 74% of the global area, Argentina with 4.3 million ha equivalent to 15% of global area; Canada 2.8 million ha representing 10%; Australia with 0.1 million ha equivalent to 1% and finally Mexico, Spain, France and South Africa each with <0.1 million ha, equivalent to less than 1% of the global area of transgenic crops in 1998. The proportion of transgenic crops grown in industrial countries was 84%, about the same as 1997 (86%) with 16% grown in the developing countries, with most of that area in Argentina, and the balance in Mexico and South Africa. As in 1997, the largest increase in transgenic crops in 1998 occurred in the USA (12.4 million ha) where there was a 2.5 fold increase, followed by Argentina (2.9 million ha) with a 3.0 fold increase, and Canada (1.5 million ha) with a 2.1 fold increase. The USA continued to be the principal grower of transgenic crops in 1998 and its share of global area was the same (74%) in 1997 and 1998. Argentina's transgenic crop area increase was the largest relative change, increasing 3.0 fold from 1.4 million ha in 1997 to 4.3 million ha in 1998; thus Argentina's global share of transgenic crop area increased from 13% of global area in 1997 to 15% in 1998. Canada's share of global transgenic crop area decreased marginally from 12% in 1997 to 10% of global area in 1998.

The five principal transgenic crops grown in 1998 were, in descending order of area, soybean, corn/maize, cotton, canola/rapeseed, and potato. Transgenic soybean and corn continued to be ranked first and second in 1998, accounting for 52% and 30% of global transgenic area respectively. Cotton and canola shared third ranking position in 1998 each occupying 9% of global area. The relative ranking of the principal transgenic traits were the same in 1997 and 1998, with herbicide tolerance being by far the highest, increasing from 63% in 1997 to 71% in 1998. Insect resistant crops decreased from 36% in 1997 to 28% in 1998. Stacked genes for insect resistance and herbicide tolerance increased from <0.1% in 1997 (<0.1 million ha) to 1% or 0.3 million ha in 1998 with quality traits being subject to no change, occupying less than 1% and <0.1 million ha in both 1997 and 1998.

In reviewing the shift in global share of transgenic crops for the respective countries, crops and traits, the major changes between 1997 and 1998 were related to the following trends: growth in area of transgenic crops between 1997 and 1998 in the industrial countries continued to be significant and almost five times

greater than in developing countries (13.9 million ha versus 2.9 million ha); in terms of crops, soybean contributed the most (56%) to global growth of transgenic crops, equivalent to 9.4 million ha between 1997 and 1998, followed by corn at 30% (5.1 million ha), canola at 7% (1.2 million ha) and cotton at 6% (1.1 million ha). There were three noteworthy developments in terms of traits, herbicide tolerance contributed the most (77% or 12.9 million ha) to global growth, and insect resistance contributed 22% equivalent to 3.7 million ha; the multiple or stacked traits of insect resistance and herbicide tolerance increased by 0.2 million ha in 1998 representing 1% of global area with significant prospects for further growth in future. Of the five major transgenic crops grown in eight countries in 1998, the two principal crops of soybean and corn, represented 82% of the global transgenic area. In 1998 herbicide tolerant soybean was the most dominant transgenic crop (52% of global transgenic area) followed by insect resistant corn (24%), herbicide tolerant canola (9%), and insect resistant/herbicide tolerant cotton at 9% and herbicide tolerant corn at 6%. The three major factors that influenced the change in absolute area of transgenic crops between 1997 and 1998 and the relative global share of different countries, crops and traits were: firstly, the enormous increase in herbicide tolerant soybean in the USA from 3.6 million ha in 1997 to 10.2 million ha in 1998 (equivalent to 36% of the US national soybean area) coupled with a similar increase in herbicide tolerant soybean in Argentina from 1.4 million ha in 1997 to 4.3 million in 1998 and equivalent to approximately 55% of the Argentinean national soybean area; secondly, the significant increase of insect resistant corn in the USA from 2.8 million ha in 1997 to 6.5 million ha in 1998, equivalent to 22% of the US national corn area in 1998; and thirdly, the large increase of herbicide tolerant canola in Canada from 1.2 million ha in 1997 to 2.4 million ha in 1998, equivalent to 45% of the Canadian canola area. The combined effect of these three factors resulted in a global area in 1998 that was 16.8 million ha higher and 2.5 fold greater than 1997. It is noteworthy that 1998 was the first year for a commercialized transgenic crop to be grown in the countries of the European Union. Initial estimates suggest that introductory quantities of insect resistant maize were grown primarily in Spain (20,000 ha) and France (2,000 ha); this is judged to be potentially a very significant development because it could have important implications for the further adoption of transgenics in countries of the European Union.

Estimated benefits from transgenic crops

More information on the benefits associated with new transgenic crops is now becoming available following the substantial area of transgenic crops planted in the USA and Canada in 1997. Multiple benefits have been reported by growers for selected transgenic crops; these include more flexibility in terms of crop management (particularly important for herbicide tolerant crops), decreased dependency on conventional insecticides and herbicides, higher yields and cleaner and higher grade of grain/end product.

As expected, net economic returns to the grower vary by year, by crop product and by location, depending on factors such as level of infestation of the targeted pest, the epidemic level of a disease or the weed density. For the USA in 1996, economic benefits to growers from the following transgenic crops were estimated conservatively at $61 million for *Bt* cotton, $19 million for *Bt* corn, and $12 million for herbicide tolerant soybean for a collective national benefit of $92 million. Similarly, in 1997, economic benefits were estimated at $119 million for *Bt* corn, $109 million for herbicide tolerant soybean, $81 million for *Bt* cotton, and $5 million for herbicide tolerant cotton and <$1 million for *Bt* potato for a collective national benefit in the USA of $315 million. In Canada, benefits at a national level, due to the use of herbicide tolerant canola, were estimated at $5 million in 1996, and in 1997, $48 million, plus $5 million for *Bt* corn for a total of $53 million. Thus, in 1996 and 1997, selected transgenic crops in the USA and Canada resulted in economic benefits to growers, conservatively estimated at $465 million.

Future global markets and global food security

Global sales of transgenic crop products have grown rapidly during the period 1995 to 1998. Global sales from transgenic crops were estimated at $75 million in 1995; sales tripled in 1996 and again in 1997 to reach $235 million and $670 million respectively, and doubled in 1998 to reach an estimated value of between $1.2 to $1.5 billion. Thus, revenues for transgenic crops have increased by approximately 20 fold in the four-year period 1995 to 1998. The global market for transgenic crops is projected to increase to $3 billion or more in 2000, to $6 billion in 2005, and to $20 billion in 2010.

The number of countries growing transgenic crops has increased from one in 1992, to six in 1996, to nine in 1998, and is expected to reach 20 to 25 countries by the year 2000. In 1999, countries in North and Latin America already growing transgenic crops are expected to significantly expand the area of current products and also to introduce new single and multiple trait products. Similarly, China is expected to expand its transgenic crop area aggressively, with growth and diversification continuing in Australia and South Africa. Whereas public acceptance, including labeling of foods derived from genetically modified plants, will continue to be dominant issues that will impact on adoption of transgenic crops in countries of the European Union, the recent approval of several products may indicate that both the number of countries and the area of transgenic crops could grow significantly in the EU in 1999. It is expected that several countries in Eastern Europe may grow transgenic crops for the first time in 1999. As expansion of transgenic crops continues, a shift will occur from the current generation of "input" agronomic traits to the next generation of "output" quality traits, which will result in improved and specialized nutritional food and feed products that will satisfy a high-value-added market; this will significantly affect the value of the global transgenic crop market and also broaden the beneficiary profile from growers and consumers to food, feed and fiber processors.

Biotechnology-driven consolidations in the form of acquisitions, mergers and alliances continue to be a dominant feature of the biotechnology industry. In the last three years alone, corporations commercializing transgenic crops and involved with seeds, agricultural chemicals, and the life sciences have been engaged in more than 25 major acquisitions and alliances valued at $15 billion, and this consolidation is expected to continue; genomics is pivotal to the growth of the industry and is catalyzing a new generation of alliances, acquisitions and mergers.

Transgenic crops are proprietary, developed almost exclusively by the private sector in the industrial countries, with the majority of the global transgenic crop area to-date grown in countries of the North. However, it is important to note that developing countries such as China played a pioneering role by being the first country to introduce a commercialized transgenic crop in the early 1990s, and Argentina is a global leader in the accelerated adoption of transgenic crops with significant expansion imminent in Mexico and South Africa. Given that the food gap of many developing countries, including China, is expected to more than double in the next 25 years and that some developing countries like Argentina can meet some of those needs through exports, the long-term potential and importance of transgenic crops for developing countries is evident. There are three considerations that underpin the strategic importance of transgenic crops for developing countries.

Firstly, developing countries have potentially more to gain from transgenic crops than industrial countries because the area of almost all crops is far greater in developing than in industrial countries; for example, there is 145 times more rice, three times more cotton, twice more maize and as much wheat and soybean grown in the developing countries compared with the industrial world - this excludes important staples such as cassava and sweet potato that are grown almost exclusively in the developing countries and have the potential to benefit significantly from biotechnology.

Secondly, yields of almost all crops are significantly lower in developing than industrial countries, for example, there is almost a 3 fold difference in maize yields between the US and developing countries and almost a 2 fold difference in rice yields. Yields are low in developing countries for many reasons but one of the principal causes is that crops in developing countries suffer much more from biotic stresses, due to pests, weeds and diseases, for which current transgenic crops already offer improved protection. Thus, the potential gain for developing countries from improved control of biotic stresses is relatively greater than for industrial countries.

Thirdly, and most importantly, it is in the developing countries, not the industrial countries, where 800 million people suffer from malnutrition today and where transgenic crops could increase crop productivity and contribute to the alleviation of hunger and poverty which are inextricably linked. During the next decade an increase in productivity of 10-25% from transgenic crops is both feasible and realistic and this will be a critical and significant contribution to global food security, more nutritious food and feed, and to a safer environment. Transgenic crops have much to offer developing countries and should be an essential component of a global food security strategy that integrates conventional and biotechnology crop improvement applications to produce more food where the need is greatest, and where the welfare value of food is the highest; denial of the new technologies to the poor is synonymous

to condemning them to continued suffering from malnutrition which eventually may deny the poorest of the poor their right to survival.

Table 1 Global area* of transgenic crops in 1996, 1997 and 1998

	ha (million)	acres (million)
1996	1.7	4.3
1997	11.0	27.5
1998	27.8	69.5

* Excluding China.

Increase in area from 1996 to 1997 is 9.3 million ha (23.2 million acres); increase in area from 1997 to 1998 is 16.8 million ha (42.0 million acres).

Source: Clive James, 1998*

Table 2 Global area of transgenic crops in 1997 and 1998: industrial and developing countries (millions of ha)

	1997	%	1998	%	increase (ratio)
Industrial Countries	9.5	86	23.4	84	13.9 (2.5)
Developing Countries	1.5	14	4.4	16	2.9 (2.9)
Total	**12.8**	**100**	**29.8**	**100**	**16.8 (2.5)**

Source: Clive James, 1998*

Table 3 Global area of transgenic crops in 1997 and 1998: by country (millions of ha)

Country	1997	%	1998	%	increase 1997 to 1998 (ratio)
USA	8.1	74	20.5	74	12.4 (2.5)
Argentina	1.4	13	4.3	15	2.9 (3.0)
Canada	1.3	12	2.8	10	1.5 (2.1)
Australia	0.1	1	0.1	1	<0.1 (1.0)
Mexico	<0.1	<1	0.1	1	<0.1 (-.-)
Spain	0.0	0	<0.1	<1	<0.1 (-.-)
France	0.0	0	<0.1	<1	<0.1 (-.-)
South Africa	0.0	0	<0.1	<1	<0.1 (-.-)
Total	**11.0**	**100**	**27.8**	**100**	**16.8 (2.3)**

Source: Clive James, 1998*

Table 4 Global area of transgenic crops in 1997 and 1998: by crop (millions of ha)

Crop	1997	%	1998	%	increase (ratio)
Soybean	5.1	46	14.5	52	9.4 (2.9)
Corn	3.2	30	8.3	30	5.1 (2.6)
Cotton	1.4	13	2.5	9	1.1 (1.8)
Canola	1.2	11	2.4	9	1.2 (2.0)
Potato	<0.1	<1	<0.1	<1	<0.1 (-.-)
Total	**11.0**	**100**	**27.8**	**100**	**16.8 (2.5)**

Source: Clive James (1998)*

Table 5 Global area of transgenic crops in 1997 and 1998: by trait (millions of ha)

Trait	**1997**	**%**	**1998**	**%**	**increase (ratio)**
Herbicide tolerance	6.9	63	19.8	71	12.9 (2.9)
Insect resistance	4.0	36	7.7	28	3.7 (1.9)
Insect res. & Herb. tol.	<0.1	<1	0.3	1	0.2 (-.-)
Quality Traits	<0.1	<1	<0.1	<1	< 0.1 (-.-)
Global Totals	**11.0**	**100**	**27.8**	**100**	**16.8 (2.5)**

Source: Clive James (1998)*

Assessment of gene flow and potential effects of genetically engineered sunflowers on wild relatives

Snow, A.A.[1)], Rieseberg, L.H.[2)], Alexander, H.M.[3)], Cummings, Ch.[3)], Pilson, D.[4)]

[1)] Department of Evolution, Ecology, and Organismal Biology; 1735 Neil Ave., Columbus, OH 43214, USA;
[2)] Department of Biology, Indiana University, Bloomington, IN 47405, USA;
[3)] Departments of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045, USA; [4)] School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA

Abstract

In the USA, cultivated sunflower (*Helianthus annuus*) is often sympatric with wild *H. annuus*, which is an agricultural weed that grows along roadsides and in other disturbed sites. We found that crop-to-wild gene flow was common (~5-40% hybrids) when wild plants occurred within <1,000 m of the crop, and crop-specific genetic markers persisted in wild populations for many generations. Crop-to-wild gene flow with other wild sunflower species is far less likely due to infertility barriers and non-overlapping ranges. Field experiments with *H. annuus* demonstrated that F_1 crop-wild hybrids typically produced fewer viable seeds than wild plants, but this disadvantage varied among plants, regions, and growing conditions, and diminished with further backcrossing. Thus, the F_1 generation is not a strong barrier to introgression of transgenes into wild populations. Little is known about how introgressed transgenes will affect the population dynamics of wild plants, but we suspect that release from insect damage and disease pressure will sometimes enhance the survivorship, competitive ability, and lifetime seed production of wild sunflowers, perhaps causing them to become more invasive. Our current research focuses on the ecological effects of insect seed predators and other pests in an effort to anticipate effects of transgenes for insect resistance in wild populations of *H. annuus* (transgenic sunflowers have not yet been marketed in the USA).

Introduction

Crop-to-wild hybridization has the potential to influence the evolutionary ecology of related wild/weedy taxa, but little is known about the ecological effects of crop genes that enter wild populations via pollen movement (e.g., Rissler and Mellon, 1996; Snow and Morán-Palma, 1997). Examples of crops that hybridize spontaneously with wild/weedy populations include sunflower (Arias and Rieseberg, 1994), squash (Kirkpatrick and Wilson, 1988), radish (Klinger et al. 1992), rice (Langevin et al. 1990), foxtail millet (Till-Bottraud et al. 1992), sorghum (Arriola and Ellstrand, 1996), and canola (Crawley et al. 1993; Jørgensen and Andersen, 1995). In sunflower (*Helianthus annuus*), foraging bees carried crop-specific genetic markers to wild plants as far as 1,000 m away from small experimental stands of cultivated sunflower (Arias and Rieseberg, 1994). In addition, a 6.4 km isolation zone is recommended to protect commercial sunflower seed nurseries from unwanted wild sunflower pollen (e.g., Smith, 1978). Thus, pollen from cultivated sunflower is certain to spread to adjacent wild populations due to the movements of foraging bees.

In the USA, cultivated sunflower is grown within the center of diversity of its ancestral species (primarily *H. annuus*) and often hybridizes with wild *H. annuus* (Whitton et al. 1997; Linder et al. 1998). Hybridization among several native *Helianthus* species has been demonstrated (e.g., Rieseberg et al. 1990; Rieseberg et al. 1999), so it is important to determine possible effects of "escaped" transgenes that may be beneficial to native species. Although cultivated sunflower is capable of hybridizing with other *Helianthus* species, especially *H. petiolaris*, most offspring from interspecific crosses are unsuccessful or yield infertile F_1 progeny (Rieseberg et al. 1999). Therefore, our research focuses on wild *H. annuus*. In particular, we need to know whether wild populations that acquire transgenes conferring resistance to herbicides, insects, or diseases are likely to become more invasive weeds. This requires controlled experiments in which the effects of specific transgenes can be quantified by comparing wild plants from the same genetic lines, with or without the transgene.

Wild *H. annuus* is a native, annual weed that occurs in disturbed sites and is widespread throughout much of the USA, reaching its greatest abundance in the midwestern states (Heiser, 1954). Populations

are typically patchy and ephemeral, relying on the soil seed pool and long-distance dispersal for opportunities to become established in available clearings. In the absence of plowing or other types of disturbance, population size often declines due to competition from later successional species such as perennial grasses, herbs, and shrubs. In agricultural areas, however, repeated tilling allows wild sunflower populations to persist for many years, and this species is sometimes considered to be a noxious weed of corn, cultivated sunflower, and other crops (e.g., Burnside et al. 1996). Wild sunflower has the potential to become more troublesome due to the acquisition of beneficial transgenes, but we do not know how these transgenes are likely to affect its abundance and competitive ability in managed and unmanaged ecosystems.

The persistence of a long-lived seed bank makes it difficult to study the population dynamics of wild sunflower. Seed burial experiments show that ~30-40% of newly produced seeds remain dormant when exposed to optimal germination conditions in the spring (Teo-Sherrell, 1996; Snow et al. 1998). Buried seeds sometimes remain dormant in the soil for several years and germinate following local disturbances such as plowing. Teo-Sherrell (1996) showed that seeds buried at depths of 5-20 cm exhibited annual dormancy cycles, with highest germination rates in the spring and intrinsic dormancy at other times of the year. The average longevity of buried seeds is not known, but at least 3% were still germinable after 17 years of burial in a Nebraska study (Burnside et al. 1996). Due to the difficulties of studying seed bank dynamics over a short time period, our research examines other life history stages of wild sunflower, namely, seedling establishment, survival, and lifetime seed production.

Spread of transgenes via pollen and seeds

Gene flow occurs through the dispersal of both pollen and seeds. Because annual crops are harvested each year, it is likely that most hybridization with wild relatives occurs via the spread of pollen from the crop to the weed rather than *vice versa*. Wild sunflower is a self-incompatible species that requires visits from insect pollinators in order to set seed, and it easily crosses with cultivated sunflower. Like other outcrossing weeds, the extent of hybridization with a cultivated relative depends on the plants' proximity to the crop and the ratio of wild plants to cultivated plants. For example, in *Brassica rapa* (= *campestris*), rates of hybridization with oilseed rape (*B. napus*) were as high as 69% when the weed occurred as isolated individuals surrounded by rows of crop plants, and hence had little or no chance to cross intra-specifically (Landbo et al. 1996). In contrast, when the weed occurred in small populations adjacent to or within fields of oilseed rape, hybridization rates of 0-13% were detected (e.g. Landbo et al. 1996; Jørgensen et al. 1998; Scott and Wilkinson, 1998).

In sunflower, we found that the extent of pollen movement from the crop is greatest at the crop edge, diminishing to nearly zero at distances of 800-1,000 m. Research involving genetic markers showed that on wild plants within 3 m of crop plants, the frequency of F_1 crop-wild hybrid seeds averaged ~28% in one study (Arias and Rieseberg, 1994) and 42% in another (Whitton et al. 1997). Further away, frequencies of hybrid seeds were ~10-15% at 200 m, ~4-8% at 400 m, and 0-4% at 1,000 m. This leptokurtic pattern is typical of pollen dispersal by insects such as bees, which occasionally transport pollen to distances greater than 1,000 m from its source.

Once crop markers enter a wild population via spontaneous hybridization, they can spread further and faster by means of both pollen and seed dispersal. Long-distance seed dispersal is common in weedy species and is often facilitated by humans because seeds can be transported inadvertently by farm vehicles and as contaminants of hay, manure, topsoil, and seed lots. For sunflower and many other weeds, however, few attempts have been made to document rates of seed dispersal from a known source population. Ironically, studies of the combined effects of pollen and seed dispersal on gene flow may be easier to undertake in the future, when selectively neutral transgenes can be used as novel genetic markers in wild populations. In the meantime, efforts to produce useful mathematical models of the rate of transgene spread will be hampered by a lack of reliable data on seed dispersal.

Persistence of transgenes in weed populations

A. fitness of F_1 hybrids and backcrossed generations

Once a transgene has been introduced into a wild population, its frequency and long-term persistence depend on several factors, including the fitness of F_1 hybrids and whether the transgene confers a fitness

benefit that enhances the survival and/or lifetime seed production of these plants. Here we use the term "fitness" to describe the relative genetic contributions of different classes of genotypes, in this case wild-crop hybrids versus wild plants, to the next generation of plants in a natural population. We focus on two main components of fitness - survival and lifetime fecundity - as is standard in ecological studies of fitness-related traits. However, it is important to recognize that a genotype's fitness can also be influenced by other life history stages, such as seed longevity and dormancy characteristics.

The F_1 generation may constitute a partial barrier to backcrossing and subsequent introgression of crop genes, particularly in the case of hybrids between different species. To test for lower survival and fecundity of F_1 wild-crop hybrids of sunflower (both *H. annuus*), we carried out field experiments in 1996 using wild sunflowers from North Dakota, Kansas, and Texas (Snow et al. 1998). F_1 hybrids were obtained using two varieties of cultivated, nontransgenic sunflower. Seed burial experiments in the region of origin showed that wild-crop hybrid seeds had no appreciable dormancy, although it is likely that deeply buried seeds can remain viable in the soil for several years if they are not exposed to appropriate germination cues (Snow et al. 1998). F_1 hybrid seeds germinated a few days earlier than wild seeds and exhibited high germination rates (>90%).

Common garden experiments carried out in Kansas and Ohio demonstrated that F_1 crop-wild hybrids grew vigorously and did not exhibit lower survivorship than wild plants (Snow et al. 1998). The hybrids produced hundreds of viable seeds per plant but on average they were inferior to wild plants in terms of their lifetime seed production. The number of flower heads per plant is correlated with total seed production, so we used this variable as an index of seed production. Wild plants branched more than F_1 hybrids and therefore produced more flower heads and more seeds per plant. The magnitude of this advantage varied considerably due to different growing conditions and the origins of the wild seeds (North Dakota, Kansas, or Texas). For example, when plants were fertilized and watered in large outdoor pots, wild plants from Kansas and Texas produced ~3-4 times more flower heads per plant than F_1 hybrids, and wild plants from North Dakota produced twice as many flower heads as their corresponding F_1 hybrids. This advantage diminished, however, when plants were grown with competitors (Morán-Palma, 1998) and when they were grown in a weed-infested field in Kansas (Snow et al. 1998). At the latter site, wild plants produced more flower heads than some hybrid cross-types but not more than others, including the hybrids from North Dakota. These results show that the fecundity disadvantage of hybrids is quite variable and is sometimes insignificant. Under most conditions, however, we expect that hybrid plants contribute fewer seeds to the next generation than do wild plants.

The number of seeds a plant "sires" in a population by dispersing its pollen is also likely to be lower for hybrids because male reproductive success should be lower in plants that produce fewer flower heads. Also, in the case of wild plants from Kansas, we found that these plants flowered much later than F_1 hybrids, reaching their peak flowering when the hybrids nearly finished blooming (Snow et al. 1998; Snow et al., unpublished data). This difference in flowering time reduced their chances of mating with the more prolific wild plants and forced F_1 plants to cross mainly with each other. In contrast, flowering periods of wild and hybrid plants from North Dakota and Texas overlapped almost completely (Snow et al. 1998).

A further disadvantage of F_1 hybrids was that their seeds were more likely to be destroyed by insect seed predators than were those of wild plants (Cummings et al., unpublished data). In 1997 we compared the lifetime seed production of wild and F_1 crop-wild hybrids from Kansas at our Kansas field site (Cummings et al. unpublished data). Seed heads were collected at random throughout the growing season, and seeds were examined for damage from insect seed predators (300 heads per cross type). On average, 44% of the hybrid seeds were destroyed by seed predators, while only 6% of the wild seeds were killed. Reasons for this difference are unclear, but a preference for hybrid seeds may be related to the fact that their seeds tend to be larger than those of wild plants (Snow et al. 1998; Cummings et al., unpublished data). If these levels of damage are typical of other regions of sympatry between wild and cultivated sunflowers, seed predation could inhibit the spread of crop genes by reducing the fitness of F_1 hybrids.

B. Persistence of crop genes in wild populations

Despite fitness disadvantages of F_1 hybrids relative to wild plants, we expect that escaped transgenes will inevitably move into wild sunflower populations based on several lines of evidence. First, within each of the cross-types mentioned above the lifetime seed production of F_1 hybrids was variable and a portion of the hybrids produced as many seeds as wild plants. Thus, reporting average values of lifetime fecundity can obscure the fact that some of the F_1 plants were very successful relative to wild plants and were probably able to contribute comparable numbers of copies of their genes to subsequent generations. Second, we found that the fecundity of backcrossed plants (F_1 x wild) was intermediate between that of F_1 and wild plants (Figure 1; Morán-Palma, 1998). Therefore, with each generation of further backcrossing, the fitness disadvantage associated with a particular transgene is expected to decrease. Finally, two studies involving genetic markers showed that 1) crop alleles persisted in wild populations for at least five generations after a known hybridization event (Whitton et al. 1997), and 2) crop alleles occurred at relatively high frequencies (31-38%) in wild populations located in areas where cultivated sunflower had been grown for ~20-40 years (Linder et al. 1998). Thus, even with low rates of hybridization and backcrossing, we expect that long-term cultivation of transgenic sunflowers will result in repeated opportunities for gene flow into nearby populations of wild plants.

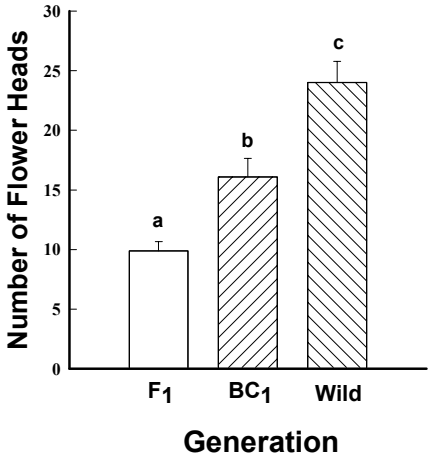

Figure 1: Performance of backcrossed (BC_1) plants relative to F_1 crop-wild hybrids and wild sunflower. The average number of flower heads per plant in each genotype category is shown (N=20-23 plants per genotype, superscripts show significant differences based on Tukey test, $P<0.05$; error bars are 1SE). Plants were grown in a greenhouse for 22 weeks; wild plants were from Texas (see Snow et al. 1998 and Morán-Palma, 1998).

The rate at which an introduced transgene spreads through wild populations can be enhanced if the transgene confers a fitness benefit, for example by protecting the plant from insect damage or disease. Most fitness-related transgenes are unlikely to be deleterious to wild plants because crop breeders will choose transgenic lines that perform well before being released commercially. In general, we expect the fitness effects of particular transgenes to be neutral or beneficial. Sunflower varieties with transgenic resistance to herbicides, insects, and various diseases are likely to be developed for marketing, although none has been deregulated yet. Some transgenes such as those conferring herbicide resistance may spread quickly because exposure to the herbicide will constitute strong selection for resistant genotypes. Other transgenic traits are less likely to confer a strong fitness advantage, as described further below, but this assumption needs to be tested empirically.

Effects of transgenes on wild populations

Finding that a specific transgene enhances the fitness of wild genotypes and becomes common in wild populations does not necessarily mean that it will result in more invasive weeds. Even if transgenic progeny produce more offspring than nontransgenic plants and the transgene eventually becomes "fixed" in the population, the number of weed populations and the number of plants within each population (= population size) may not increase. For example, the number of plant populations could be determined by the number of sites with appropriate biotic or abiotic conditions for establishment. Similarly, at a single site, greater production of seeds will not necessarily lead to a greater number of plants at that site, especially if there are a limited number of locations for seedling establishment (e.g., Bergelson, 1994). Even if seedling establishment does increase, other factors such as intraspecific competition or herbivore damage may limit the final abundance of plants at the site. For these reasons, it may be wrong to assume that greater fecundity due to the effects of transgenes will affect plant population dynamics.

Despite these caveats, however, there are reasons to expect that weedy species could become more abundant due to effects of escaped transgenes. In general, weeds have broad ecological requirements, and the size and numbers of weed populations may primarily reflect the availability of disturbed soil needed for seedling establishment. Given the prevalence of human-induced disturbances in and around agricultural fields, and thus opportunities for large populations (including dormant seeds in the soil), it seems likely that effects of transgenes on individual fitness could lead to population consequences. Greater survival and fecundity of individual plants could potentially result in larger weed populations and more effective dispersal of seeds to found new populations.

Transgenes that protect wild plants from insect herbivory and disease are of particular interest because wild plants are susceptible to many of the same pests as cultivated plants (and probably served as the original hosts prior to domestication). A recent sunflower production manual for the northern Great Plains region of the USA lists several moth, weevil, beetle, and midge larvae that frequently cause economic damage (Berglund, 1994). To reduce damage from head-feeding insects that are difficult to control, VanderHave has developed transgenic varieties with resistance to coleoptera (http://www.nbiap.vt.edu/). With regard to diseases, the most important pathogens of commercial sunflower include *Sclerotinia* wilt, rust, and downy mildew. Transgenic resistance to *Sclerotinia* has been field-tested by both VanderHave and Pioneer Hi-Bred (http://www.nbiap.vt.edu/). Eventually, we expect that biotechnology companies will develop a variety of highly effective transgenic constructs that will protect cultivated sunflowers from many economically important pests.

At present, little is known about the effects of insects or disease on the population dynamics of wild sunflower, so it is difficult to assess the ecological effects of beneficial transgenes. With regard to insect seed predators, preliminary data from wild plants in Kansas suggest that seed damage is relatively low (about 6% in 1997; Cummings et al., unpublished data), but insect damage to seed heads of wild plants in Nebraska can be substantial (D. Pilson, 1999; E. Sundvall and D. Pilson, unpublished data). Further research is needed to determine the overall benefits of transgenic, plant-produced toxins on plant fitness. Effects of *Bt* transgenes (derived from *Bacillus thuringiensis*) are uncertain because at present these toxins are only effective against specific insect taxa (e.g., lepidoptera or coleoptera), and it is not known whether other insect pests will become more abundant once a competing group of insects has been controlled. Another consideration is whether insects and pathogens that are unable to use transgenic host plants will rapidly evolve resistance to novel plant-produced toxins. Further studies are needed on the natural occurrences of insect herbivores and diseases in wild sunflower populations, and experimental studies are needed to evaluate the possible ecological impact of transgenic sunflowers on wild populations over the next few decades.

Conclusions

Transgenes from cultivated sunflower will inevitably spread to wild populations via crop-to-wild gene flow. The rate at which transgenes will disperse among populations is difficult to predict because little is known about rates of seed dispersal, especially human-mediated dispersal. However, continued cultivation of transgenic sunflowers in regions where wild sunflowers also occur will allow beneficial transgenes to accumulate in wild populations. Transgenes conferring resistance to herbicides, insects, and diseases are likely to be beneficial to wild plants, perhaps enhancing survival, competitive ability, and

lifetime seed production, but their individual and combined effects on the population dynamics of wild sunflower are unknown (we are currently investigating these questions). Wild sunflower is an agricultural weed that can be adequately controlled in most agricultural systems, but it also occurs in unmanaged areas throughout much of the midwestern USA. Although this species is not considered to be an invasive species at present, release from insect damage and disease pressure could potentially cause wild sunflower to become more problematic in both managed and unmanaged areas.

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Deep rooted strategies for the deployment of transgenic trees

Ellis, D., Gilbert, M.

Forest Biotechnology Centre, BC Research Inc., 3650 Wesbrook Mall, Vancouver, B.C., V6S 2L2, Canada

Summary

Huge differences exist between the cultivation of agronomic crops and forest trees. Forest trees are by comparison large, long-lived, take longer (typically 10+ years) to reach reproductive maturity, and have traditionally been grown in "natural" ecosystems with relatively few inputs. This is changing globally as a greater emphasis is being placed on plantation forestry and the use of high-input clonal tree farming. Due to this shift of growing fiber producing trees as crops, as well as a lack of advanced tree breeding programs, genetic engineering can have a greater over-all impact to the improvement of forest trees than to any other crop. However, because of the longer-term perennial nature of tree crops, there is a perception that the direct risks associated with the commercial release of genetically engineered trees are inherently different from those risks associated with the commercial release of genetically engineered herbaceous crops. From a risk assessment point of view this is not always the case, as the initial questions posed in risk analyses are the same - is there a risk involved with such a release, what is the impact of this risk, and how can the impact be lessened?

As with any crop, the evaluation of transgenic trees draws on examples of risk assessments that have previously been tested and then builds on this knowledge to formulate hypotheses to address and evaluate the risks. For example, the first generation of genetically engineered traits for forestry will likely be the same first generation traits that were used in agriculture, specifically insect and herbicide resistance. The primary direct risks associated with these engineered traits in trees are similar to agricultural crops, the evolution of *Bt*-resistant insect populations and increased weediness due to spread of the herbicide resistant transgene into surrounding populations. Further, similar ecological theories and strategies to lessen these risks are applicable in forest trees, such as a high expression level and the use of refugia, to manage insect biotype evolution in *Bt*-expressing trees. However, as transgenic trees move into the second generation of genetically engineered traits, such as those affecting endogenous biochemical pathways and therefore plant secondary products, some risks may not have been previously addressed in risk analyses with agricultural crops. These traits include the modification of lignin and cellulose, modifications which if altered in agricultural crops would likely be expressed in a seed specific manner and not in the main vegetative portions of the plant as in the stem of trees.

A major concern with transgenic trees are risks associated with the spread of transgenes into native populations. A lengthy history of the spread of exotics into forests suggests that weediness is most prevalent in disturbed sites and that the dynamics of natural undisturbed populations buffer against invasion. This would certainly be the case for traits such as herbicide resistance which confer no selective advantage in the absence of herbicides, yet may be different for insect resistance which would confer a selective advantage in years of severe insect infestation. Therefore, in forestry the strategy most often discussed for the containment of transgene spread is the suppression of reproductive structures.

Introduction

Due to a long breeding cycle, gains through crop improvement as seen with agronomic crops during the green revolution have not occurred in trees. Despite this, directional breeding in trees can have a huge impact on fiber and timber yields, as we have seen from the use of hybrids in eucalyptus and poplar. However, such directional breeding takes time to exploit the existing germplasm, often 10+ years between generations due not only to long generation times but also due to the time needed to select for desired phenotypic traits. Therefore, tremendous advances in tree improvement can be achieved by combining genetic engineering with advanced tree breeding programs.

Despite this, significant differences exist in the public perception of what is acceptable in agricultural crops versus forest tree crops. The major issue of course is food versus non-food crops, which would lead one to believe genetic engineering in forest trees should be much easier for the public to accept. Surprisingly, however, moral/ethical viewpoints on genetically engineered trees center on the use, esthetics, and ecology of the forest environment. For example, agricultural land is generally

acknowledged by the public to harbor an annual crop, consisting of a single species which is harvested, tilled, and planted on yearly cycles. For practical purposes, this land has always been agricultural, it is already "disturbed", and easily justifiable from an economic and sociological standpoint. People are used to seeing an annual cycle with this land and for much of the growing season every year it is flourishing in a crop. This land is therefore deemed productive, usable land. Agricultural lands are not generally public lands which are used for esthetic and recreational purposes such as hiking and picnicking. Very basically put, because people need food to eat, it is accepted that agricultural lands are dedicated for this use and relatively little other use.

In contrast, forest trees are grown in "forests" which are perceived as pristine, natural, public lands. Therefore, in the public's eye, harvesting of trees causes direct and conflicting pressures for multiple use of "our" forests. Forest regeneration after harvesting occurs on a ten-year or more cycle. This long duration causes the visible impact from harvesting trees to appear unchanging for a period which can span from childhood to adulthood in someone's life. Further, environmental concerns have linked the harvesting of all forests to a loss of biodiversity, a perception which is not only wrong but has also provided an easy environmental concept to lobby for lessening harvests in forested land. This is compounded by the notion that all forests are natural and therefore harvesting is unnatural and unacceptable, a perception strengthened by forests being non-food generating and therefore their harvest is less justified from a sociological and economic viewpoint by a majority of the public.

There is no question that some arguments against the extensive harvesting of trees from forest ecosystems have sound ecological basis and that not all ecosystems can withstand stable repeated harvesting of the trees. With the change occurring in forestry toward tree farming of selected clones, many issues stated above can be addressed by helping to offset the growing demand for timber. Here, however, is where the assessment of risk from genetically engineered trees gets confused and complicated with moral, environmental and social values. The fact that the very improvements offered by clonal forestry and genetic engineering are maximized when applied to plantation forestry is being missed by the general public. This is due in part by not distinguishing tree farming from forests in the public perception. There is opposition and distrust to trees being grown as crops and 'forests' being grown in farms. The public has a difficult time believing that tree plantations will decrease pressures on forests, and to date foresters have not proven this is the case. Therefore, there is a conflict over what practices will affect biodiversity and how the integrity of the ecosystem can be maintained.

Why are social and environmental issues relevant in a scientific discussion on biosafety issues pertaining to transgenic trees? This is because the evaluation of biosafety in fiber and timber producing trees will inevitably be influenced from a scientific, as well as from a moral basis. Recognizing this in any risk assessment strategy for fiber and timber trees is paramount to understanding the pressures that will exist when commercializing genetic engineering in forestry. Therefore, a trait-based risk assessment approach, while scientifically sound, will fall short of addressing some very fundamental biases that exist in the use of genetically engineered trees.

In this paper, we focus on the issue of transgene spread and the control of this spread from genetically engineered trees as this will alleviate a major and controversial risk associated with the commercial release of transgenic trees. This is not to say that other risks, such as the evolution of resistant insect biotypes in response to *Bacillus thuringiensis* (*Bt*) endotoxin, an increased susceptibility of lignin-modified trees to pests and diseases, or the effect on soil microorganisms from altered carbon allocation within the trees, should not be considered. Rather, that the impact and scope of these and other potential risks would be greatly diminished if the transgene was confined to the transgenic crop.

Transgene spread

As mentioned, a major concern with the use of genetic engineering in forest trees is the spread of transgenes from transgenic trees into native species and pristine forests. From a risk analysis standpoint, spread of a transgene into native populations can occur via three means; pollen, seed or vegetative propagules. If this risk is assumed to be the same for all species, then the highest probability of spread is when a sexually compatible species is within the range of viable pollen distribution. The likelihood that such a spread would happen is directly related to several variables including the distance pollen travels

from transgenic tree, the distance sexually compatible non-transformed species are from the transgenic tree, the duration transgenic pollen is viable, and the quantity of competing non-transgenic pollen.

The distance pollen travels is probably the most important and variable component. As many of the major forest trees species are wind pollinated, pollen can therefore travel great distances if it gets into the airstream. Fortunately, documented cases of extremely long-range pollen travel represent a minuscule percentage of the total pollen and therefore are probably negligible from a risk assessment standpoint. In fact, over 90% of pollen falls within a few hundred feet of the donor tree, similar to the pollen dispersion distances in radish, cotton and maize (Table; Wright 1976). Factors such as wind patterns, rain, surrounding vegetation, and topography also influence the distance pollen will travel. In addition, not all timber species are wind pollinated, for example eucalyptus which is pollinated by insects creating a whole new level of complexity in controlling pollen movement.

Table Distance 91% of the pollen traveled (ft) from the donor tree of various forest tree species (Wright 1976)

Species	Travel distance (ft) for 91% of pollen
Atlas cedar	238
Norway spruce	125-300
Slash pine	225
Douglas fir	>500

Of equal concern in the risk assessment of pollen as a vehicle for transgene spread is the duration which pollen remains viable. The distance pollen travels is of minor importance unless pollen remains viable during this travel. Pollen viability varies between species yet generally pollen remains viable for only short periods of time in nature, on the order of days to one week. In addition humid and wet environments usually hydrate pollen, greatly shortening pollen life.

Unlike pollen, seed containing a transgene will likely travel only a limited distance from the tree. Although there are exceptions, such as Chinese elm and cottonwood seed which can travel with wind currents large distances. In general, if pollen flow is contained, the risk of transgene spread through seed can be limited to a relatively close proximity of the parent. In the case of the maternal parent being a transgenic, the spread of transgenes into trees in native forests would not occur for a decade or more until seedlings reached sexual maturity. Such cases of spread of the transgene into native populations could easily be handled by monitoring or management practices.

As with the other means of transgene spread, the risk of spread through vegetative propagules is highly dependent on the species. In the case of pine and many other trees species vegetative shoots are recalcitrant to rooting. Hence a broken off branch carried away by a rodent or water has a minuscule chance of surviving. In these cases, the risk of spread through vegetative propagules would be sufficiently low such that it would be ruled out as a risk in most assessments. In the case of species where mature branches can be induced to root, such as in poplar, huge variation exists between individual genotypes in rootability and therefore, species selection may aid in controlling such spread. In addition, spread by rodents would likely not be long distance and the risk of long-range spread by streams or other permanent bodies of water can be lessened by planting away from streams, rivers or lakes. Therefore, management and cultural practices could reduce this risk to acceptable levels.

Impact of transgene spread

In a purely scientific risk assessment on the spread of a transgene into a native forest, the impact of the genetically engineered *trait* in a given tree would be considered. Such is the standard in the USA, as demonstrated with the allowance of the commercial release of papaya in Hawaii. Although this may be in a scientific assessment, as previously mentioned, this may not be adequate to satisfy a risk assessment which is forced to consider concerns which are based on ethics and morals. While such non-scientific assessments of risk can be countered with sound scientific reasoning, more may be needed to deal with the public perception of the risk.

For example, from a scientific standpoint, the risk associated with the escape of a transgene into a natural population is dependent on the trait and the species in which the trait is introduced. These variables are paramount in any risk assessment. Further, these variables and the inherent risks are then assessed in light of the environment in which they are planted. For example, glyphosate resistance would be relatively benign if introduced into a native spruce forest as spruce is not an overly weedy species needing herbicide treatment. In contrast, glyphosate resistance introduced into native aspen in the Pacific Northwest of North America, may increase weediness of the species if glyphosate were the sole controlling agent. However, under existing management schemes, integrated programs are utilized which can diminish the risk associated with the spread of such a transgene into aspen.

If risk assessment in forest ecosystems were to take a completely different track, one which analyzed the dynamics of forest ecosystems and the introduction of exotic species as a model for risk assessment of introduced genes, it could be argued that the greatest impact would be in disturbed forests (Strauss 1998). Natural forest ecosystems are composed of highly heterogenous individuals which survive in the long-term due to their ability to respond to a multitude of stresses over-time. Fitness in a forest ecosystem is measured by the ability to survive and adapt, not by the capacity for rapid growth and yield, the later qualities being important in clonal forestry. In addition, fitness and survival on an evolutionary time-scale is not a feature characteristic of single gene traits. Therefore, in absence of other factors, natural forest ecosystems will buffer against natural selection conferring long-term advantages based on single gene traits. Therefore, few transgenic traits by themselves would confer a significant advantage in natural, undisturbed forest ecosystems. Unfortunately, few such undisturbed reserves exist, however, the principals of the buffering capacity of such systems could still apply in minimally disturbed or reforested sites. As in the preceding paragraph, it can be said that from a scientific viewpoint the risk of transgene spread exists, yet the consequences of such spread will likely not impact or adversely alter natural ecosystems. Again, it could be argued that the risk is therefore negligible.

Despite the rationality of a scientific risk assessment, it remains that a large portion of the world does not view genetic engineering on a trait x genotype basis. Further, even in those countries that do, the risk of transgene spread into native forests is a concern. These and other concerns are tied to the perception that clonal forestry and genetic engineering are ecological dead ends and that the widespread planting of such stands would lead to decreased genetic diversity across the landscape. This perception extends to the notion that the risk from the escape or spread of transgenes into natural forests would result in altered ecosystems. The perception further extends to the notion that changes in ecosystems would arise from decreased genetic diversity caused by decreased adaptability, increased weediness of both weedy and previously non-weedy species, and an altered soil microflora resulting in a change in nutrient cycling. The fact that the perceived risk is there even with non-genetically engineered clonal forestry should direct our focus to the hypothetical risk of decreasing genetic diversity by the outcrossing of trees in our plantations to trees in natural forests. Therefore, decreasing the frequency or likelihood of outcrossing from genetically engineered trees should help ally fears associated with the risk of decreased genetic diversity. In addition it should also facilitate the overall use of transgenic trees as evaluations centered on the hypothetical and real risks associated with the escape of a vast array of different genes would no longer be a major issue. Finally, regulatory differences between countries in the risk evaluation of transgenic trees could be lessened.

Prevention of transgene spread

Considerable research investigating the manipulation of reproductive structures in trees to control the spread of transgenes is ongoing in numerous labs around the world. While most of these efforts have focused on genetic engineering for the manipulation of flowering, there are numerous cultural and management strategies which could be used to decrease the flow of transgenes into natural forests (Ellis and Raffa, 1997). Cultural practices would include strategies such as planting a buffer zone of sexually non-compatible trees as a physical barrier to pollen movement. Vegetative buffer zones consisting of a crop of agronomic value could also be used and could return income to the plantation owner. While this may be acceptable in small plantings to limit pollen flow, it would be impractical for larger plantations and would not completely eliminate the risk of pollen moving beyond the barrier. However, since 90+% of pollen falls within 100 meters of the tree in most cases, it could decrease pollen flow to a level that decreases risk to an acceptable level. In addition, buffer zones would effectively block the movement of

most seed and if used in conjunction with a tilled strip, could also reduce the risk of transgene spread through seed to an acceptable level.

Another approach which could be used with dioecious species such as poplar, is to only plant female transgenic clones in a plantation. This would eliminate the risk of transgene spread by pollen. Further, if isolated or planted in conjunction with a buffer zone to limit pollen flow into the plantation, seed production could be significantly reduced thereby also decreasing the risk of transgene spread through seed. Another approach is to use polyploids which are either infertile, have reduced fertility, or which produce gametes which are sexually incompatible with native species. In poplar, triploids have been identified and these have been proposed as potential targets for such an approach.

While these management approaches for limiting transgene spread are important, they do not eliminate the risk, they simply lessen it. In many cases, this may be all that is needed to reduce risk to an acceptable level. However, due to the social and political issues surrounding transgenics in forestry, the complete elimination of transgene spread has been discussed as an optimal target. Elimination of viable pollen through genetic engineering has been achieved in agronomic crops (Mariani et al. 1990) and total sterility through the ablation of flowers has been achieved in *Arabidopsis* (Nilsson et al. 1998).

The manipulation of reproductive structures in plants through genetic engineering is possible due to the identification of numerous genes involved in different aspects of floral meristem and organ differentiation. Mutations in these genes cause altered flower morphology and in some cases loss of floral organs. Of prime importance is the fact that these genes are expressed specifically in the flower and therefore their promoters can be used to target genes encoding lethal proteins such as an RNAse (Mariani et al. 1990) or a diphtheria A toxin (Nilsson et al. 1998) to ablate cells involved in the formation of reproductive structures.

The extension of this approach into forest trees has been demonstrated as genes isolated from *Arabidopsis*, an annual angiosperm, were used to manipulate reproductive structures in long-lived perennial angiosperm forest trees (Weigel and Nilsson, 1995). In gymnosperms, however, due to the vast morphological differences in reproductive structures and an estimated 300 million years separating them from angiosperms, it is not known if *Arabidopsis* genes and promoters can also be used to manipulate reproductive structures in conifers. Light is being shed on the evolution of reproductive organs as recent molecular studies have identified several homologues to *Arabidopsis* flowering genes in gymnosperms (Tandre et al. 1995; Rutledge et al. 1998; Mouradov 1998a,b) suggesting an evolution of these genes prior to the evolutionary split in these two major groups of plants. This and the fact that an *Arabidopsis* flowering gene has caused the formation of an abnormal bud reminiscent of a reproductive bud in white spruce (Ellis et al., unpublished), have yielded optimism that similar approaches could be used to control reproductive structures in gymnosperms.

One question facing foresters is whether the elimination of both pollen and seed is needed. Put another way, would the elimination of viable pollen be enough from a risk assessment standpoint? As previously mentioned, the containment of seed in a plantation generally is easier by cultural means than the containment of pollen inferring that in many cases eliminating viable pollen may be adequate. This distinction is important as constructs which functionally abort the tapetum in the anther exist and have been successfully used to disrupt pollen development in agronomic crops (Mariani et al. 1990). It can be argued, however, why go to all the trouble to eliminate only pollen? Since clonal propagules are used in forest plantations, the need for seed and hence reproductive structures is eliminated. Further, the energy used to form reproductive structures could be diverted into other productive tissues such as wood increasing growth and yield. Therefore, why not limit the formation of reproductive structures completely?

In all plants, *vegetative* meristems are induced to differentiate into *reproductive* meristems following some external (day length or temperature) or internal signal. Therefore, one target for the complete elimination of reproductive structures is to disrupt the transition between the vegetative meristem and the flowering meristem. When this transition occurs, genes in individual cells within the vegetative meristem are turned on which act as transcriptional activators to turn on a group of other genes involved in the formation of floral organs (Yanofsky, 1995). The promoters from genes which act as the master switches to induce flower development are prime candidates for use in the disruption of flower formation. Candidate genes include CONSTANS, TERMINAL FLOWER, LEAFY, APETALA 1, and CAULI-

FLOWER. In our work we are investigating the use of three of these genes, LEAFY, APETALA 1 and CAULIFLOWER for disrupting the formation of reproductive meristems in trees.

Long-term transgene expression

A question in the development of sterility by genetic engineering is whether a transgene can remain silent and non-expressed for several years and then be accurately expressed with the onset of flowering. Since very little information exists on the long-term stability of transgene expression in trees, we established a field planting of transgenic spruce and poplar expressing *uidA* (GUS) and Bt δ-endotoxin genes in 1993 to look at the stability of transgene expression over several years. Expression of GUS was intensively assayed during the growing season over a three-year period and we found very stable GUS expression in both species over this period (Pilate et al. 1997). Further, although the poplar trees have now been cut down, spruce faithfully expressed GUS after seven years in the field. Not all genes tested in trees have been faithfully expressed, as changes in transgene expression have been noted in poplars transformed with a *RolC* gene (M.R. Ahuja, personal communication). However, unlike the GUS or *Bt* endotoxin genes, expression of the *RolC* gene resulted in severe morphological and physiological changes in the plant. It is unknown if the severely altered phenotype contributes to the silencing of genes such as the *RolC* transgene.

While the above data does not answer the question of whether chimeric constructs designed to affect the formation of reproductive structures will faithfully be expressed, they do suggest that in some cases transgene expression can be very stable over long periods of time in trees. This information is in agreement with the numerous transgenic crops which have been commercialized where gene silencing has not been a major problem. Testing the expression of constructs aimed at reproductive structures in trees would normally take 5+ years as this is generally the earliest poplar or spruce can be induced to flower. However, two approaches for the induction of early flowering are being tested. One approach in spruce utilizes the combination of accelerated growth regimes and the application of GA_{4+7}. With this regime, we have been able to induce reproductive structures in two years and will begin inductions in transformed spruce in fall 1998. The other approach involves the over-expression of flowering genes as has been reported by Weigel and Nilsson (1995). In this latter case, expression specific to reproductive structures as well as the specificity of lethal constructs could be tested.

Conclusion

Considerable progress has been made with the genetic engineering of trees. Within the next decade numerous engineered traits will be commercially released in forest trees. Coupled with these releases will be the need to assess the risk imposed on natural forest ecosystems from the spread of transgenes into these ecosystems. Several strategies have been proposed to limit this spread. Of these, the majority of the research being done is focused on the use of genes expressed specifically in flowers to disrupt the formation of reproductive structures. Such an approach will not only facilitate the commercialization of transgenic trees but could also increase growth and yield of the trees by the reallocation of resources normally invested in reproduction.

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Modelling the establishment and spread of transgenes in plant populations

Giddings, G.

University of Wales, Institute of Biological Sciences, Aberystwyth, UK

Summary

The role of modelling in predicting the establishment and spread of feral transgenic and transgenic-wild hybrid plants is explored. The use of modelling as a tool to formally describe transgene dispersal and transgenic and hybrid fitness is discussed. The incorporation of such descriptions into predictive models combining population dynamics and genetics is described. It is demonstrated how the output of some of these models can lead us to useful general principles with which to guide risk assessment. This includes some which may be counter-intuitive, including the interpretation of data from monitoring programmes and the persistence of transgenes which are negatively selected. The importance of genotype by environment interaction, evolution and stochasticity are discussed, particularly in relation to predicting fitness and invasiveness.

Introduction

Escaped transgenics, or transgenic-wild hybrids, might become weeds of agriculture or natural ecosystems. Here there could be the potential for habitat modification and harm to non-target organisms, including by direct competitive exclusion. Where transgenic crops are grown close to wild relatives, e.g. forage crops and trees, rare genetic resources might be threatened by genetic assimilation or outbreeding depression (Ellstrand, 1992). Modelling can help us understand how the establishment and spread of transgenes in wild and feral populations is affected by such factors as gene flow, the population turnover rate, selective advantage and genetic drift.

Gene flow by pollen

Hybridisation between conventionally bred crops and weeds has resulted in increased aggressiveness of some weeds. Johnson grass (*Sorghum halepense*), for example, is one of the world's worst weeds (Holm et al. 1977). Its hybridisation with cultivated *Sorghum bicolor* resulted in the evolution of especially invasive weed biotypes (Arriola and Ellstrand, 1996). Plants such as forages and turf grasses, which have undergone little domestication and often have wild relatives growing nearby, are particularly at risk from hybridisation (Arriola and Ellstrand, 1996; Giddings et al. 1997a). The introduction of transgenes for pest resistance or salt tolerance, for example, might have an economic and ecological impact by increasing the aggressiveness and range of such weeds (Ellstrand and Hoffman, 1990).

Transgene transfer to wild species depends on the species and transgenic being cross compatible and flowering simultaneously. In Europe this could include forages, such as *Lolium perenne* which is cross compatible with wild *Lolium* and *Fescue* species (Raybould and Gray, 1993; Giddings et al. 1997a), and sugar beet (*Beta vulgaris*) which will cross with *Beta maritima*, the wild sea beet, and adventitious or weed type beets (Boudry et al. 1993; Bartsch and Schmidt, 1997). Genes might also be passed from oilseed rape to feral populations or to the species *Brassica rapa* and *B. juncea* (Timmons et al. 1995; Metz et al. 1997).

There are many studies of pollen dispersal (e.g. Lavigne et al. 1996; Bing et al. 1996; Giddings et al. 1997a,b; Nurminiemi et al. 1998). Some indicate that dispersal can occur over distances of hundreds or even thousands of meters (e.g. Ellstrand and Hoffman, 1990; Klinger et al. 1991; Van Raamsdonk and Schouten, 1997).

Two types of models often used for describing pollen dispersal from a source are inverse power and negative exponential models (Manasse, 1992; Giddings et al. 1997a,b; Van Raamsdonk and Schouten, 1997). Studies of wind dispersal from a *Lolium perenne* source indicated that better fits to data can be achieved by including terms to describe the effects of wind direction, in what Van Raamsdonk and Schouten (1997) describe as a Gaussian plume model (Figure 1; Giddings et al. 1997a,b). Even so many data sets showed a lack of overall fit to models in which deposition decreases smoothly with distance, and it is assumed that wind speed and turbulence also had significant effects that would be complex to model.

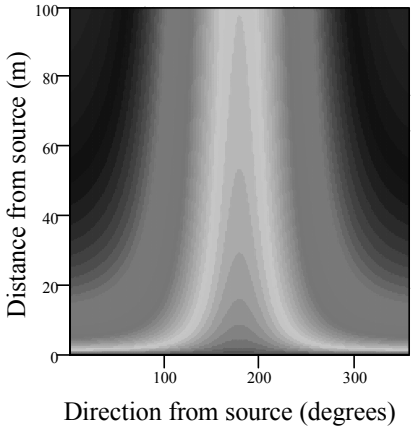

Figure 1 A Gaussian plume model of wind-mediated pollen dispersal from a *Lolium* source

Despite the problems it is interesting to calculate possible pollen deposition from the models fitted to some of the data. The simplest model for deposition (p) including wind direction was given by:

$$p = ae^{-\log D(\alpha+\beta\phi(\theta:w,s))} \qquad \text{where: } f(q:w,s) = \frac{1}{\sqrt{2ps}} e^{-\left[\frac{(x-w)^2}{2s^2}\right]}$$

D and x are the distance and direction from the source, w and s the mean and standard deviation of wind direction and a is a coefficient indicating maximum deposition. There were six data sets for which more complex models with more parameters did not give significantly improved fits.

By integrating the expression for p over D and x it is possible to calculate immigrant pollen as a percentage of the total pollen deposited on a region for any particular set of parameter values. Using a range of parameter values estimated from experiment deposition was calculated from a source population of 2 m radius (like the source on which the data is based) to a similar sized population 1,000 m downwind. Values of 7.96×10^{-3}-3.52% were obtained from the 6 data-sets. Scaling up to a source size of 320 m^2 results in a proportion of transgenic pollen between 29.74%-99.64% deposited downwind of the source and 4.87%-34.76% deposited upwind of the source.

These results alone show the difficulty involved in predicting what transgene dispersal to wild and feral populations will be. Where there is any doubt about the consequences of gene dispersal from transgenic crops measures need to be taken to minimize or prevent it. Possibilities include using intensive grazing or harvesting before flowering, or selecting for asynchronous flowering, reduced cross compatibility, or male sterility. Another effective control would be to use transgenes that kill the developing embryo of hybrid seed, a controversial technology recently patented by the US Department of Agriculture and Delta & Pine Land (US patent 5723765, 1998). Adding guard rows around transgenic crops to "mop" up pollen seems unlikely to be entirely effective at preventing pollen dispersal away from the crop.

The dispersal of seeds and asexual propagules

Transgenics may be dispersed to a new environment by seeds or asexual propagules. Voisey and colleagues (1994), for example, considered that the dispersal of clover seed is unavoidable and should be taken into account before releasing transgenic clovers into the environment. Dispersal might be by wind, water, insects, birds or other animals. There might also be accidental dispersal by humans. Feral rape

populations on motorway verges, for example, seem to be maintained by spillage from lorries transporting rape seed to seed crushing plants (Crawley and Brown, 1995). The routes by which dispersal occurs can be surprising. Oxford ragwort spread through Britain with the railway network, while soil movement during the 1980s UK construction boom facilitated the spread of asexual propagules of Japanese knotweed.

There is little quantitative data on seed and propagule dispersal but most population models assume no immigration and (in spatially explicit models) an origin from a nearby "mother-plant". Nevertheless it is possible to modify such models to take immigrant seed and propagules into account. It should be possible to get data on "natural" dispersal, for example for seeds by following the inheritance of maternally inherited traits or markers. One could also start modelling from the situation where an accidental introduction has occurred.

Modelling genetic assimilation

Simple iterative models can be used to model the establishment and spread of transgenes within a closed population, such as the anglicized version of an iterative computer model in Figure 2. The underlying assumptions are: (1) the transgenic and recipient are diploids; (2) the absence of genetic drift and (3) the presence of Mendelian segregation. Gene flow into the population is by pollen but not seed, and there is no recruitment from a seed bank, although these could be modelled by adjusting the calculation of p_m and p_f. The proportion of pollen from the crop containing one copy of the transgene is c and the proportion affecting fertilisation in the population is i. The proportion of the population replaced by recruitment from seed is R per generation (for annual plants with non-overlapping generations R=1). ω_{hom} is the selective advantage associated with being homozygous (TT) for the transgene compared with being homozygous for wild type alleles (WW), ω_{het} is the selective advantage associated with being heterozygous for the transgene (WT). Decreases in fitness can be represented using a negative selective advantage. Using two values of ω allows for the transgenic allele to be dominant or recessive. p_g and q_g are the proportions of transgenic and non-transgenic alleles in the population at generation g. Subscripts f and m denote alleles from females and males. Comments are between { and } while ← is an assignment operator (i.e., becomes equal to). The model simulates either recurrent immigration, e.g. from a forage crop, or a shorter pulse of pollen from a crop removed after one or more generations.

Function find_p **(**input: p_0, c, i, R, ω_{hom}, ω_{het}, last_generation, removed_after**)**

$p_0 \leftarrow p_0$ {most probably zero, i.e. no transgenes in the population at the start}

for $g \leftarrow 1$ to last_generation do

if g > removed_after then $i \leftarrow 0$ {crop removed so no pollen influx}

$p_m \leftarrow (1\text{-}i)\, p_{g-1} + ci$ {alleles already in the population plus incomers}

$p_f \leftarrow p_{g-1}$ {all plants from last generation flower}

$q_m \leftarrow 1\text{-}p_m \quad q_f \leftarrow 1\text{-}p_f$ {p+q=1}

{The genotypes are calculated from modified Hardy-Weinberg equations:}

$WW \leftarrow q_f q_m \quad TT \leftarrow p_f p_m(1+\omega_{hom}) \quad WT \leftarrow (q_f p_m + q_m p_f)\,(1+\omega_{het})$

{normalized to proportions by dividing by the total:}

Total ← WW + TT + WT TT ← TT/total WT ← WT/total

{The proportion of transgenic alleles in the population this generation:}

$p_g \leftarrow R(0.5\ WT + TT) + (1\text{-}R)\, p_{g-1}$

end for

end function (output: p)

Figure 2 Anglicized version of an iterative computer model for calculating genetic assimilation of a transgene into a wild or feral cross-compatible population

Output of model:

It is possible to deduce algebraic solutions to some special cases of the model. If the transgene source is removed after a year and p_0=0 then $p_1 = \frac{0.5ciR\varpi_{het}}{(ci\varpi_{het}) + (1-ci)}$.

If the transgene source is removed after a year and there are no differences in fitness between genotypes then $p_1 = p_0 - \frac{Rip_0}{2} + \frac{ciR}{2}$, or $p_1 = \frac{ciR}{2}$ if p_0=0. The maximum value of p_1=0.5 occurs only when the transgenic crop is homozygous and annual, and only transgenic pollen effects fertilisation.

Further analysis reveals that, *provided there are no differences in fitness between genotypes*, in any generation *g before* the removal of the transgenic crop the frequency of the transgene p_g can be evaluated from:

Where a_x is a coefficient determined as follows. When x is odd a_x is positive, when x is even then a_x is negative. If x=1 then a_1=g=1.

If x=2 (transgene source removed after 2 years) then $a_2 = -\sum_{N=0}^{g-1} N = -1$.

Hence $p_2 = p_0 + \frac{R^2 i^2 p_0}{4} - \frac{ci^2 R^2}{4} - Rip_0 + ciR$, or $p_2 = ciR - \frac{ci^2 R^2}{4}$ if p_0=0. The maximum value of p_1 is then 0.75.

If x=3 then $a_3 = \sum_{N=0}^{g-2} \sum_{n=0}^{N} n$. In general a_x has x-1 summations, x-2 of which are embedded.

The lower limits are equal to zero. For the embedded summations the upper limits are equal to the variable name of the limits of the preceding summation. For the first summation it is g-x+1. The variable summed is that of the final limit.

For example when x=5 $a_5 = \sum_{N=0}^{g-4} \sum_{n=0}^{N} \sum_{n2=0}^{n} \sum_{n3=0}^{n2} n3$.

Applying this to x=3, for example, gives:

$$p_3 = p_0 - \frac{R^3 i^3 p_0}{8} + \frac{ci^3 R^3}{8} + \frac{3R^2 i^2 p_0}{4} - \frac{3ci^2 R^2}{4} - \frac{3Rip}{2} + \frac{3ciR}{2}$$

Although equations for other situations can be found the recursive nature of the model means they soon become unwieldy, as we see above for even quite small values of x. For all but the most simple situations it makes more sense to use the iterative model.

Table 1 shows some results from running the model to simulate various situations. The first three simulations are of transgene dispersal into a population of an annual species. It is seen that even when pollen dispersal into the population is low transgenes can be quickly assimilated if they are associated with a high selective advantage. Conversely transgenes with a low selective advantage can spread with if there is higher gene flow. With perennials the effects of high gene flow and/or high selective advantage are tempered by lower rates of population turnover - only a small percentage of the population is replaced each year by seedling recruitment.

It is also clear that transgenes imparting a negative (or neutral) selective advantage can be maintained in a population by quite low levels of recurrent immigration. Then the expression of initially disadvantageous genes may be altered in the different genetic backgrounds of hybrid offspring, for example due to recombination between transgenes and epistatic modifiers.

Table 1 Simulations of genetic assimilation of a transgene into a cross-compatible population in various situations

I (as %)	R (as %)	$\omega_{hom}=\omega_{het}$	Removed after	p_{10} (as %)	p_{50} (as %)	p_{100} (as %)	Time when p=0.02-0.046	Time when pε 0.75
0.0001	100	1	1	0.05	93.00	97.40	12-14	28
50	100	0.05	1	31.99	57.52	74.87	<1*	101
5	100	0.1	1	6.02	51.37	81.15	1-7	82
50	1	1	not	3.30	15.65	29.04	7-15	>200
5	25	0.05	not	6.66	32.88	57.56	4-7	161
5	1	-0.2	not	0.20	0.96	1.83	>200	>200

*p>0.046 in the first year

The results in Table 1 also highlight some of the problems that could be encountered in monitoring programmes designed to detect transgene introgression into cross-compatible populations. A 10-year programme of sampling for the presence of transgenes might conclude that the first and last populations in Table 1 were at least risk from transgene introgression. If we sampled *N* plants we could be α% confident of detecting transgenes once the frequency of plants carrying transgenes $\varepsilon 1-(1-\alpha)^{1/N}$ (McArdle, 1990). For example we could be 90% confident of detecting transgenes in a sample from *N*=100 plants if the frequency of plants carrying transgenes was ε 0.023. The transgene frequency would then be between 0.023 (all plants homozygous) and 0.046 (all plants heterozygous) depending on the relative fitnesses of the different genotypes. Column 8 in Table 1 shows the minimum time by which we could be 90% certain of having detected transgenes in the population given such a sampling strategy. We can see that the population in which assimilation will ultimately occur the fastest is likely to require the greatest sampling effort over the longest time. If the transgene were to increase invasiveness then this might not be detected for many years, even though subsequent spread might then occur rapidly.

Measuring and modelling fitness

Considering that increased selective advantage can cause initially low levels of transgenes to spread through a population it will be important to consider the effect of transgenes on host fitness. Genetic linkage relationships of the transgene will also be important. If the transgene confers a selective advantage it may not be retained in the population if it is closely linked to other genes that are negatively selected. Conversely a transgene with no selective advantage, or even some disadvantage, may be retained if it is linked to genes which are selected for. It therefore makes sense to assess the effect of the transgene in a range of genetic backgrounds, including in backcross progeny of wild-transgenic hybrids if hybridisation might occur.

Potential invasiveness can be estimated from the finite rate of population change (λ), where $\lambda>1$ indicates that a population will increase in the particular experimental conditions in which λ was measured. λ can be measured by determining the ratio of seedlings in one generation to that in the preceding generation. Alternatively it can be defined as a function of the germination rate, survival to flowering and surviving offspring produced per individual (Crawley et al. 1993). Each of these are subject to genotype by environment interactions, including density dependence. This means that experimental results will be specific to the particular experimental conditions, which will therefore need to mimic the range of expected release environments as closely as possible, and preferably over several seasons. The predictive value of such data can be gauged by determining the error due to reduced sampling effort (Kareiva et al. 1996). Ideally extra sampling should lead to insignificant changes in the amount of overall variation. This will be especially important when the average multiplication rate (λ) is near to one, i.e. when it is not obvious whether the transgenic is destined to persist, become extinct, or spread.

The “natural” factors that affect the rate of population change include (1) the resource supply rate; (2) interference competition where individuals of one species reduces the fitness of another in ways not

directly related to resource capture, e.g. by allelopathy or alteration of the physical properties of the environment; (3) natural enemies such as diseases and herbivores and (4) the supply of mutualists, such as rhizobia, mycorrhizae and pollinators (Crawley, 1986). Transgenes may enhance traits affecting fitness, for example by conferring resistance to pests and pathogens (e.g. Stewart et al. 1997). As isolation from pests and pathogens appears to be an important factor influencing the success of invaders transgene induced resistance to pests and pathogens is worthy of particular attention with regard to risk assessment. Tilman's resource acquisition models could be used for predicting the effect of transgenic resistance to pests and pathogens such as insects, viruses, bacteria and fungi (e.g. Tilman, 1982; 1988; 1990; 1994).

One way such models might be used is to predict which seedlings are most likely to "win out" when more than one seed is dispersed into a gap. Suppose, for example, that two seeds are dispersed to a gap and their germination and subsequent growth is limited only by resource *R*. Seed one is of genotype *i* and can accumulate biomass at a maximum rate of 0.1 g per day per gram of existing tissue when *R* is in unlimited supply. For seed two, of genotype *j*, the rate is 0.2 g per day per gram. Both species consume 5 units of *R* for each 0.1 or 0.2 g increase in biomass respectively. Each species also loses 5% of its biomass per day, for example due to energy use and the action of herbivores. The two seeds/seedlings are the only causes of loss from the resource supply *R*, which is also replenished at a constant rate of 5 units per day. A further assumption is made that once resources are reduced below that which supports maximum growth the two species compete equally for what remains (i.e. scramble competition). If the biomass of either species drops to below 1.05 g then it dies at the next time step, except, of course, that growth is allowed from seeds which are originally 1 g. Figure 3 shows the simulated dynamics of these two genotypes.

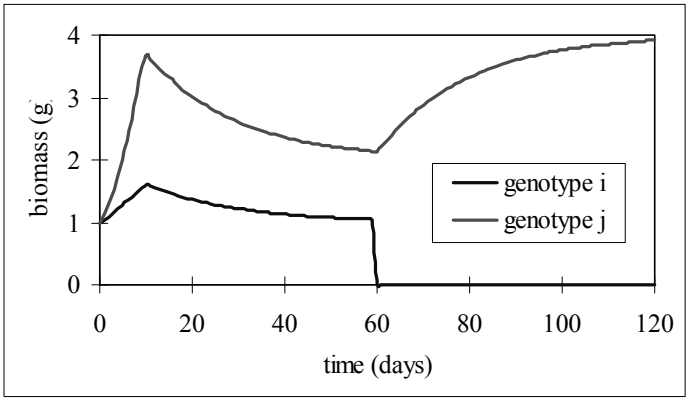

Figure 3 Simulated change in biomass of two seedlings with differential resource use

At first *R* supports both species at maximum growth rate, then both decline as they share just 5 units of *R* per day. Genotype *i* declines to less than 1.05 g and dies. Genotype *j* now has all the resources to itself and starts to grow again. Eventually it reaches an equilibrium biomass when loss of biomass equals gain, when *j* is 4 g (i.e. 5% of biomass is 0.2 g). One might take account of resistance to herbivores by lowering the loss of one genotype relative to the other (i.e. reducing either m_i or m_j), or of transgenes for increased yield, by increasing the efficiency of uptake of the transgenic, for example by increasing $f_i(R)$.

Modelling invasive spread

In the model for genetic assimilation ω_{hom} and ω_{het} represent the relative fitnesses of the different genotypes in populations whose average multiplication rate (λ) is one. As discussed above transgenes might increase the invasiveness of the recipient population. For example if λ=1.1 then the population grows by 10% per generation. The model can easily be modified to take account of transgene induced

population expansion, one possibility is demonstrated in Figure 4. If only plants with a transgene can colonise new territory then we could define 2 variables, λ_{hom} and λ_{het}. An environment carrying capacity (K) can also be included, reflecting, for example, the availability of sites suitable for recruitment. Here K represents multiples of the initial population, so K=1.5 means the population can grow to half as big again, K=2 means growth to twice the original size. Such modelling integrates population genetics and dynamics.

{ as in Figure 2}

{The genotypes are calculated from modified Hardy-Weinberg equations:}

WW ← $q_f q_m$ TT ← $p_f p_m(1+\omega_{hom})$ WT ← $(q_f p_m + q_m p_f)(1+\omega_{het})$

{Main modifications}

if (K-pop_size_now) < [TT + $(1-\lambda_{hom})p_f p_m$ + WT + $(q_f p_m + q_m p_f)(1-\lambda_{het})$]

{if there's enough space left for both genotypes to increase without competing}

{with one another then they will do so}

then TT ← TT + $(1-\lambda_{hom})p_f p_m$ WT ← WT + $(q_f p_m + q_m p_f)(1-\lambda_{het})$

pop_size_now ← pop_size_now + $(1-\lambda_{hom})p_f p_m + (q_f p_m + q_m p_f)(1-\lambda_{het})$

{if not then the remaining space is allocated according to competitive ability}

else TT ← TT + (K-pop_size_now)()

    WT ← WT + (K-pop_size_now

    pop_size_now ← K {carrying capacity of environment is reached}

{continued as in Figure 2}

{Normalized to proportions by dividing by the total:}

total ← WW + TT + WT TT ← TT/total WT ← WT/total

Figure 4 The main modification converting the genetic assimilation model to a population dynamics model

Stochasticity

When a recipient population is small stochastic processes might override deterministic ones. Similarly when there is little immigration genetic drift could cause the loss or increase of transgenes regardless of selection pressures. Then recurrent dispersal may be important in determining whether transgenes persist and spread in hybrid or feral populations.

Stochasticity can be modelled by taking random draws from a specified probability distribution function. Table 2 shows computer simulations of the simple population model $N_{t+1} = \lambda N_t$ over 500 generations for populations initially of 50 individuals (N_0=50). λ is normally distributed with a mean of 1 and standard deviation of 0.2. In agreement with the deterministic model the overall mean population size remains almost unaltered (i.e. is about 50). However, variation between individual simulations and replicates can be seen. Half of the populations go extinct, but only after an average of about 314 years, giving time for evolution to increase the mean value of λ to above 1. This simple example demonstrates how variation can be incorporated into models. The predictive value of the output can be determined by estimating whether increasing replication produces significant changes in the overall amount of variation (Kareiva et al. 1996).

Table 2 Mean population size and time to extinction of a simulated population where N_0 = 50 and λ is normally distributed with a mean of 1 and standard deviation of 0.2

Replicate number (6 simulations/ replicate)

	1	2	3	4	5	6	overall
mean population size	38.30	45.99	11.90	93.61	87.36	20.17	49.55
standard deviation	19.06	21.98	14.51	35.86	76.87	15.14	12.81
number of extinctions	3	3	5	1	4	2	18
mean time to extinction	220	251.67	230.2	480	292	408	313.64

Modelling at the level of the individual

Neighbourhood models have been specifically developed for studying the population dynamics of sessile organisms and are popular for practical applications, especially cellular automata models (e.g. Silvertown et al. 1992; Colansanti and Grime, 1993; Perry and Gonzalez-Andajur, 1993). They have been used to model clonal growth, gap colonisation, competition, succession and weed spread. Populations are represented as grids of "cells" (or sites) which can be occupied by an individual or genet, or empty. The neighbourhood of a particular cell consists of one or more concentric zones of the surrounding cells. The state of each site is determined for each generation according to the rules of neighbourhood interaction, with births, deaths, growth and dispersal being defined by probability density functions. Such models can allow for the explicit specification of environmental heterogeneity, and are useful for investigating the effects of factors such as population shape and size. Although conceptually simple in construction the use of neighbourhood models needs to be systematic and planned. As with other stochastic models the output will vary between replicate runs and requires further statistical analysis to be useful.

Discussion

If transgenes are likely to enhance the ability of a crop to establish, survive or reproduce then consideration needs to be given to the likely fitness of volunteers, ferals or transgenic-wild hybrids. Some crops may be so debilitated outside of agriculture that they present little or no risk of becoming weedy. For others increases in fitness may enhance the potential for invasiveness. If this is so then effort needs to be devoted to measuring the finite rate of population change, either directly or as a function of the components of fitness. Such measurements will need to be done in a range of conditions to match those likely in the release environment over several seasons. The predictive value of the data can be gauged by determining the error due to reduced sampling effort.

Modelling shows that even when the finite rate of population change is less than one transgenes can persist in feral or hybrid populations if there is recurrent immigration or a low rate of population turnover, as may be the case for perennials. This could give time for evolution to increase the fitness of segregant offspring, increasing the possibility of invasiveness. Thus the effect of the transgene on fitness ought to be measured in a range of genetic backgrounds, especially when hybridisation is likely. Stochasticity can also affect the fate of transgenes, especially when they are at a low frequency. Models using probability distribution functions for parameters such as λ can be used to investigate the likely effects of stochasticity. Model runs should be replicated until there is no increase in the amount of output variation with increased replication.

Modelling also shows that monitoring programmes could require considerable and long-term sampling to detect the spread of feral transgenics, or transgenic-wild hybrids, including those which might eventually become invasive. Thus if there is perceived to be any risk from hybridisation, due either to genetic assimilation, increased invasiveness or hybrids otherwise adversely affecting non target organisms, the best policy would be to use cultural or biological methods to stop pollen flow, or prevent the germination of hybrid seed. This last might be used to prevent all transgene flow from a sexually reproducing crop, although transgenes for seed or seedling death would have to be tightly linked to the other transgenic traits to minimise the chances of recombination. Furthermore the risk of hybridisation causing outbreeding depression of wild populations might then have to be assessed.

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Development of assessment methodology by the USEPA for genetically engineered pesticides

Schneider, W.R.

BioPesticides and Pollution Prevention Division, Office of Pesticide Programs, USA Environmental Protection Agency, Washington

It has been 15 years since the Office of Pesticide Programs (OPP) of the US Environmental Protection Agency (EPA) began to evaluate genetically engineered microorganisms. As a result of a law suit in May, 1983, by the Foundation on Economic Trends, the EPA undertook the review of a field test of *Pseudomonas syringae* genetically engineered to delete an ice nucleation protein gene by the University of California (Steven Lindow) and a second, similar, field test by Advanced Genetic Sciences. The law suit challenged the June, 1983, approval of the National Institutes of Health Recombinant DNA Advisory Committee for this field test and asked that EPA require an experimental use permit. It took 2 years to assemble the data and complete that first assessment in order to withstand legal challenges and to involve interested members of the public. Since that time, the EPA has refined its risk assessment methodology for genetically engineered organisms, plants as well as microorganisms.

US regulatory framework for genetically engineered pesticides

OPP regulates testing and commercialization of pesticides under the authority of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA) as amended by the Food Quality Protection Act (FQPA) of August 3, 1996. The law describes a pesticide, in part, as any substance intended for preventing, destroying, repelling, or mitigating any pest. EPA must ensure that a registered pesticide will not generally cause unreasonable adverse effects on man and the environment.

All biological control organisms, e.g. nematodes, parasitic wasps, spiders, mites, etc., are exempted from regulation on the basis that another federal agency is adequately regulating them, except that EPA continues to regulate microbial pesticides, described as eucaryotic and procaryotic microorganisms such as viruses, bacteria, fungi, and protozoa (40 CFR 152.20). In 1994, a policy statement was published describing how we regulate pesticidal transgenic plants. Although EPA had exempted the plants themselves from regulation, in this case EPA would be regulating the "pesticidal substance that is produced in a living plant and the genetic material necessary for the production of the substance, where the substance is intended for use in the living plant". Registered pesticides currently include 57 microbial and 6 plant pesticide active ingredients (Table 1).

Table 1 Registered microbial pesticides 9/4/1998

Tolerance	Active ingredient	EPA code	Target pest
BACTERIA			
180.1076	*Bacillus popilliae* & *B. lentimorbus*	54501	Japanese beetle larvae
180.1011	*Bacillus thuringiensis* subsp. *kurstaki*	6402	Lepidopteran larvae
	Agrobacterium radiobacter K84	114201	*A. tumefaciens*
180.1011	*B. thuringiensis* subsp. *israelensis*	6401	Dipteran larvae
180.1011	*B. thuringiensis* Berliner	6400	Lepidopteran larvae
180.1011	*B. thuringiensis* subsp. *tenebrionis*	6405	Coleopteran larvae
180.1114	*P. fluorescens* A506	6438	Ice-crystallizing Pseudomonas species
180.1114	*P. fluorescens* 1629RS	6439	Ice-crystallizing Pseudomonas species
180.1114	*P. syringae* 742RS	6411	Ice-crystallizing Pseudomonas species

Tolerance	Active ingredient	EPA code	Target pest
180.1011	*B. thuringiensis* subsp. *kurstaki* EG2348	6424	Lepidopteran larvae
180.1011	*B. thuringiensis* subsp. *kurstaki* EG2424	6422	Lepidopteran larvae
180.1011	*B. thuringiensis* subsp. *kurstaki* EG2371	6423	Lepidopteran larvae
	B. sphaericus	119801	Dipteran larvae
180.1111	*B. subtilis* GBO3	129068	Damping off disease
180.1011	*B. thuringiensis* subsp. *aizawai* GC-91	6426	Lepidopteran larvae
180.1011	*B. thuringiensis* subsp. *aizawai*	6403	Lepidopteran larvae
180.1115	*Burkholderia cepacia* type Wisconsin M36	6419	Damping off disease & nematodes
180.1120	*Streptomyces griseoviridis* K61	129069	Damping off disease
180.1011	*B. thuringiensis* subsp. *kurstaki* BMP123	6407	Lepidopteran larvae
180.1128	*B. subtilis* MBI 600	129082	Damping off disease
180.1011	*B. thuringiensis* subsp. *kurstaki* EG7673 Lepidopteran toxin	6448	Lepidopteran larvae
180.1145	*P. syringae* ESC 10	6441	Post harvest decay-causing fruit pathogens
180.1145	*P. syringae* ESC 11	6451	Post harvest decay-causing fruit pathogens
180.1011	*B. thuringiensis* subsp. *kurstaki* EG7841	6453	Lepidopteran larvae
180.1011	*Burkholderia cepacia* type Wisconsin IsoJ82	6464	Damping off disease & nematodes
180.1011	*B. thuringiensis kurstaki* M200	6452	Lepidopteran larvae
180.1011	*B. thuringiensis* subsp. *kurstaki* EG7673 Coleopteran toxin	6447	Coleopteran larvae
180.1181	*Bacillus cereus* strain BP01	119802	Plant regulator
YEAST			
180.1144	*Candida oleophila* I-182	21008	Post harvest decay-causing fruit pathogens
FUNGI			
180.1057	*Phytophthora palmivora* MWV	111301	Citrus strangler vine
180.1075	*Colletotrichum gloeosporioides* f.sp. *aeschynomene* ATCC 20358	226300	Northern joint vetch
	Trichoderma harzianum ATCC 20476	128903	Tree wound decay
	Trichoderma polysporum ATCC 20475	128902	Wood rot
180.1100	*Gliocladium virens* G-21	129000	Pythium, Rhizoctonia
180.1102	*Trichoderma harzianum* Rifai KRL-AG2	119202	Damping off disease
180.1113	*Lagenidium giganteum*	129084	Mosquito larvae
180.1116	*Metarhizium anisopliae* ESF1	129056	Cockroach & flies
180.1123	*Puccinia canaliculata* (Schweinitz) *langerheim* ATCC 40199	129085	Yellow nutsedge
180.1131	*Ampelomyces quisqualis* M10	21007	Powdery mildew
180.1146	*Beauveria bassiana* GHA	128924	Grasshoppers, crickets, locusts & whitefly
	Beauveria bassiana ATCC 74040	128818	Whitefly, boll weevil
180.1198	*Gliocladium catenulatum* strain J1446	21009	fungi
	Paeciliomyces fumoroseus Apopka strain 97	115002	Insecticide non-food
PROTOZOA			
180.1041	*Nosema locustae*	117001	Grasshoppers
VIRUSES			
180.1027	*Heliothis Nucleopolyhedrosis* virus (NPV)	107300	Cotton bollworm,
	Douglas budworm fir tussock moth NPV	107302	Douglas fir tussock moth
	Gypsy moth NPV	107303	Gypsy moth larvae
180.1118	Beet armyworm NPV	129078	Beet armyworm larvae

Tolerance	Active ingredient	EPA code	Target pest
180.1125	*Autographa californica* NPV	128885	Alfalfa looper
180.1149	*Anagrapha falcifera* NPV	127885	Lepidopteran species
180.1148	*Cydia pomonella* Granulosis virus	129090	Codling moth larvae

Under the authority of FIFRA, OPP registers both active ingredients and formulated end-use products for commercial use and grants Experimental Use Permits (EUP) for field testing designed to produce data for registration. An EUP is required for pesticides tested on more than ten acres of land or more than one surface-acre of water. If the experimental use is for food or feed uses, or on water containing animals or plants that may be used for food or feed (40 CFR 172.3) a food tolerance (40 CFR 180) and an EUP are both needed, unless the crops are destroyed following the experiment. The legal description of an EUP presented a problem because, before a plant pesticide is registered for commercial use, the company needs to plant a relatively large number of acres to produce seed to sell. An EUP cannot be issued for this because FIFRA states that EUPs are only allowed for the purpose of producing data for registration. This is why EPA issues a "conditional" registration for seed increase while the full registration for commercial use is pending.

An EUP is not needed for most small-scale field testing of conventional chemical pesticides due to low exposure. However, EPA reconsidered this rule for some microbial pesticides, because they have the potential for multiplying in the environment. Naturally occurring microbial pesticides may already be found in the environment and, generally, would not present more risk than the more toxic conventional pesticides in small-scale field tests. However, a genetically engineered microbial pesticide has the potential to create a much greater and/or novel exposure for pesticidal toxins or traits. Therefore, since 1984, EPA has required that a Notification be submitted prior to any environmental release of certain genetically engineered microbial pesticides. These are defined in 40 CFR 172, subpart C, as "Microbial Pesticides whose pesticidal properties have been imparted or enhanced by the addition of genetic material that has been deliberately modified". This is intended to exclude conventional mutagenesis or incidental genetic modifications such as tagging with a marker gene.

Risk management, microbial pesticides

Risk management in OPP includes an initial risk assessment to identify any unreasonable adverse effects and uses mitigation measures to reduce those risks to an acceptable level. OPP has developed risk assessment methods to address the unique properties of microorganisms. These assessment methods are based on the pesticidal chemical risk assessment paradigm, risk = (hazard x exposure). In this approach, OPP assesses risk by evaluating the potential hazard of the pesticide (e.g. the effects, especially toxicity and/or pathogenicity, on a non-target species, including man) and determining the exposure (e.g. the actual amount of pesticide that might reach the non-target species at risk). A list of data required to support EUPs or registration of microbial pesticides is in 40 CFR 158.740. OPP recognizes that it is difficult to set forth absolute data requirements for such a heterogenous group of microorganisms and often will allow modifications on a case-by-case basis. Waivers may be granted for specific data requirements if warranted. In addition, OPP may ask for additional information if necessary to evaluate the product. Because it is very difficult to get a quantitative exposure assessment for environmental microorganisms, OPP asks for data on the effects, anticipating that there will be no significant effects seen. A microbial exposure assessment may have to address: scale of use, use patterns, application rates, persistence, degradation, mobility, population dynamics, infectivity, and gene transfer. Exposure data is required for microbial pesticides only if unreasonable adverse effects are indicated by the toxicology studies for non-target organisms including man.

Microbial pesticides risk mitigation for registration and EUPs may use a number of tools. These include removing or limiting toxic components, changing the manufacturing process, changing the formulation, modifying use rates, or labeling for application restrictions or the required use of protective personal equipment.

The assessment of small-scale field tests (genetically engineered microorganisms) is more specifically directed at unique risks. OPP tries to identify the risks posed by the genetic modifications and compares

those risks to risks posed by naturally occurring microbial pesticides which is a standard for acceptable risk. OPP generally relies on containment to mitigate any unreasonable or insufficiently defined risk. Thus, the first field trials of a totally new construct often require relatively high levels of containment which may be reduced for later trials as more data is collected. To aid in identifying the specific risks posed by a new construct, OPP has published informational requirements in 40 CFR 172 to be addressed in the Notification (Table 2).

Table 2 Information required for Notifications (40 CFR 172 Subpart C)

(a) The identity of the microorganism which constitutes the microbial pesticide including:
Summary of data supporting the taxonomic designation and its interpretation.
Means and limit of detection using sensitive and specific methods (e.g., note the use of any markers that are used to distinguish the introduced population from native microorganisms). Introduction into the microbial pesticide of a unique genetic marker is encouraged.
(b) Description of the natural habitat of the parental strain of the microbial pesticide including information on:
Physical and chemical features important to growth and survival of the parental strain.
Biological features of the parental strain that would have an impact on the microbial pesticide (e.g., presence of phages that infect the microorganism).
Competitors.
(c) Information on the host range of the microbial pesticide, if any, with an assessment of infectivity and pathogenicity to nontarget organisms.
(d) Information on survival and the ability of the microbial pesticide to increase in numbers (biomass) in the environment (e.g., in the environment into which the microbial pesticide will be introduced, and in substantially different environments that may be in the immediate vicinity). These data may be derived from the scientific literature or from tests conducted in a laboratory or other containment facility.
(e) The identity of possible transmission vectors (e.g., insects).
(f) Data on relative environmental competitiveness compared to the parental strain of the microbial pesticide.
(g) Description of the methods used to genetically modify the microbial pesticide.
(h) The identity and location of the gene segments that have been rearranged or inserted/deleted (host source, nature, and, for example, base sequence data, or restriction enzyme map of the genes).
(i) Information on the control region of the genes, and a description of the new traits or characteristics that are expressed.
(j) Data on potential for genetic transfer and exchange with other organisms and on genetic stability of any inserted sequences in the microbial pesticide.
(k) A description of the proposed testing program including:
The purpose or objectives of the proposed testing.
Designation of the pest organisms involved (common and scientific names).
The States in which the proposed program will be conducted.
The exact location of the test sites (including proximity to residences and human activities, surface water, etc.).
The crops, fauna, flora, geographical description of sites, modes, dosage rates, frequency, and situation of application on or in which the pesticide is to be used.
The total amount of pesticide product proposed for use in the testing.
The method of application.
A comparison of the natural habitat of the microbial pesticide with the proposed test site.
The number of acres, structural sites, or animals/plants by State, to be treated or included in the area of experimental use.
Procedures to be used to protect the test area from intrusion by unauthorized individuals.
The proposed dates or periods during which the testing program is to be conducted, and the manner in which supervision of the program will be accomplished.
Description of procedures for monitoring the microbial pesticide within and adjacent to the test site during the test.
The method of sanitation or disposal of plants, animals, soils, farm tools, machinery etc., that will be exposed to the microbial pesticide during or after the test.
Means of evaluating potential adverse effects and methods of controlling the microbial pesticide if detected beyond the test area.
(l) A statement of composition for the formulation to be tested, giving:

The name and percentage by weight (or other suitable units) of each ingredient, active and inert.

Production methods.

Extraneous microorganisms present as contaminants.

Amount and potency of any toxin present.

Where applicable, the number of viable microorganisms per unit weight or volume of the product or other appropriate system for designating the quantity of active ingredient.

(m) Any additional factual information regarding the potential for unreasonable adverse effects on the environment.

Certain Notifications are particularly good to illustrate these points. The first Notification, in 1984, was a *Pseudomonas syringae* with a deletion of a gene which expressed an ice nucleation gene to allow competitive replacement of ice enucleating bacteria. For this first field release, a full analysis of the organism was required. For example, this included an extensive evaluation of the potential for plant pathogenicity. This kind of modification (a deletion) would no longer require a Notification. However, if OPP evaluated it today, the assessment would examine the possible effects caused by the genetic modification and not properties that were inherent in the parental strain such as plant pathogenicity because the naturally-occurring organism presents no unreasonable risk for small-scale field testing.

A later Notification was for a poorly described class of bacteria, *Clavibacter xyli cynodontis* engineered to express a *Bacillus thuringiensis* delta endotoxin gene (Cxc/Bt). Little literature information was available on the biology or taxonomy of this organism. In order to allow field testing, stringent containment requirements were imposed. A Scientific Advisory Panel of outside experts recommended containment requirements consisting of an extensive plant free barren zone, a dike, and decontamination. The dike was to be of sufficient height to prevent runoff from flooding and/or rainfall. In addition, a wire fence was used to prevent incursion on the plot by large animals. Field equipment was to be disinfested by 10% household bleach. Monitoring included rice "trap" plants to be sampled for the presence of the recombinant bacterium. If the bacterium was detected, more extensive sampling was to be performed to determine the extent of movement. In addition, flying insects were to be sampled to see if they had transported Cxc/Bt. If the recombinant bacterium were detected in outer "trap" plants, or the insects, the experiment would be terminated. After several seasons of testing, sufficient data was gathered to conclude that the Cxc/Bt was a very poor colonizer and would not survive outside the plants. Therefore the containment provisions were reduced to two buffer rows, disinfection of hand tools and the crops were to be plowed under at the conclusion of the experiment.

In 1994, OPP received the first of a series of Notifications from American Cyanamid and, later, Dupont Corporation, to field test baculoviruses, *Autographa californica* MNPV, genetically engineered to express insect-specific toxins from scorpions (AAIT). The only significant potential adverse effect identified by the OPP assessment was that the modification could increase the host range. Initially there was little direct data to allow an assessment. Therefore OPP specified relative high containment for the first tests: buffer rows and a buffer zone, mesh soil cover in order to enable collection of infected insects to be assayed, disinfection of equipment, liming the soil following the tests and sampling of the soil nine months afterwards. Subsequent years of testing has shown that the modification does not affect the host range and the wild type baculovirus can outcompete the engineered constructs because relatively few progeny are formed when the infected insects are killed by the toxin. Therefore OPP has decided that no containment is needed for these tests and these companies may use these constructs in small-scale field tests without submitting any more Notifications.

In 1997, EPA received a Notification for field testing of fluorescent Pseudomonads that were genetically engineered to express an antimicrobial toxin from other fluorescent Pseudomonads to control wheat diseases (take-all, Rhizoctonia root rot, and Pythium root rot). For example, in one construct, the genes for phenazine-1-carboxylate from *Pseudomonas fluorescens* 2-79, isolated from wheat, were transferred into the chromosome of *Pseudomonas fluorescens* Q8R1-96, which is a better wheat root colonizer than strain 2-79. OPP's assessment concluded that since all fluorescent pseudomonads in soil may exchange genes naturally, the new genetic constructs do not significantly differ from those that might be found in nature. Therefore, all small-scale field testing was approved on the condition that the donor and recipient isolates used are non-phytopathogenic fluorescent *Pseudomonas* species, isolated from the wheat rhizo-

sphere. The tests must include a containment border such as a 20 foot wide unplanted area, fallow-field, or sod-berm, and must be direct-seeded (without tilling).

Risk management, plant pesticides

The first regulatory review of a plant pesticide field release by EPA was in 1986 of a transgenic tobacco plant producing a delta-endotoxin from *Bacillus thuringiensis* subsp. *berliner*. The test was approved with containment provisions which included daily monitoring, fencing, chemical growth regulator treatment to inhibit lateral shoot growth, and topping of the plants to remove flowers to prevent pollen formation.

Since that time, EPA has registered seven plant pesticides for commercial use. The registrations are for various truncated delta-endotoxins and their associated genetic material for use in potato, cotton, and corn (Table 3).

Table 3 Non-viable microbial pesticides

180.1107	*B. thuringiensis* subsp. *kurstaki* delta-endotoxin in killed *P. fluorescens*	6409	Lepidopteran larvae
180.1108	*B. thuringiensis* subsp. *san diego* delta-endotoxin in killed *P. fluorescens*	6410	Coleopteran larvae
180.1154	*Bt* CryIA(c) & Cry I(c) delta-endotoxin in killed *P. fluorescens*	6457	Lepidopteran larvae
180.1154	*Bt K* CryIC in killed pseudomonas	6462	Lepidopteran larvae
180.1163	Killed fermentation solids & solubles of *Myrothecium verrucaria*	119204	Nematodes
180.1147	*Bt* Cry3a delta-endotoxin and the genetic material necessary for its production in potato	6432	Colorado potato beetle
180.1152	*Bt* Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn	6430	Lepidopteran larvae
180.1173	*Bt* Cry1Ac delta-endotoxin and the genetic material necessary for its production in cotton	6445	Lepidopteran larvae
180.1173	Cry1Ab delta-endotoxin and the genetic material necessary for its production in corn	6458	Lepidopteran larvae
180.1155	*Bt K* delta-endotoxin (Cry1Ab) and the genetic material necessary for its production in corn	6444	Lepidopteran larvae
180.1155	*Bt K* Cry 1Ac delta-endotoxin and the genetic material necessary for its production in corn	6463	Lepidopteran larvae
180.1192	*Bt tol*. Cry 9C delta-endotoxin and the genetic material necessary for its production in corn	6466	Lepidopteran larvae

As part of the assessments EPA considered the potential for gene transfer to wild relatives. There are no naturally occurring relatives of corn in the USA, however, there are two wild species of potatoes that might be capable of hybridizing with the transgenic potatoes. However, the wild species differ in chromosome numbers and also grow in mountainous areas remote from cultivated species which would prevent any hybridization. Cotton also has some relatives that occur in several areas in the USA. There is a wild cotton relative in southern Arizona mountains but it does not grow near domestic cotton and, because of chromosome number differences, hybrids would be triploid and sterile. However, a wild relative to cotton in Hawaii is not geographically or temporally isolated (it may bloom at the same time as domestic cotton) and is genetically compatible. In addition, feral cotton grows wild in southern Florida. Therefore, EPA has prohibited commercial sale or use in Hawaii and southern Florida and has required containment provisions for testing in these areas. This illustrates how pesticide labeling can mitigate a risk identified for commercial use of these plants.

The pesticidal gene products for all plant pesticides registered by EPA have been one of several protein delta-endotoxins derived from *Bacillus thuringiensis*. These are Cry1Ab, Cry1Ac, Cry9C (for lepidopteran pests), and Cry3A (for coleopterians, specifically the Colorado potato beetle). Each has been tested for effects in both human health toxicology tests and in non-target species assays. In some

cases, where maximum levels of toxin were needed for a feeding assay, the toxin was produced in a microbial culture providing that an analysis showed the toxin was sufficiently similar to that produced in the plant. Information has also been collected on the levels of toxin expressed in the different parts of the plant for each of the registered plant pesticides and various studies submitted to OPP have shown that the half life of delta-endotoxins in soil is from four to seven days.

As was predicted by the long history of safe use of *Bacillus thuringiensis* as a microbial pesticide, no toxic effects were seen in the mammalian studies. However, EPA does not yet have definitive data to show that Cry9C is not a food allergen. In the absence of a suitable animal model to predict food allergenicity, OPP is using the screening model recommended by the April 18-19, 1994 conference in Annapolis, Maryland, "Scientific Issues Related to Potential Allergenicity in Transgenic Food Crops". The participants recommended that new proteins be evaluated by determining their similarity to characteristics of known food allergens. Specifically, does it have a similar protein sequence, is it resistant to enzymatic and acid degradation, is it heat stable, is it found in high amounts in edible plant parts, and is it of the appropriate molecular size (FDA Docket 94 N-0053, document TR-1). EPA does not believe Cry9C is a food allergen because it shows no sequence homology with known food allergens and is not expressed in great amounts, however, it was resistant to degradation by heat, acid and proteases. Thus, EPA took a very conservative approach and approved Cry9C for use only in field corn that would have no direct human dietary exposure.

Extensive tests were performed on the standard EPA non-target indicator species and on some additional species such as earthworms and collembola. No significant adverse effects were seen to birds, honeybees, non-target insects, fish, aquatic invertebrates, or earthworms. In some cases, the study requirements were waived because there would be no exposure. For example, it was demonstrated by a study using a very susceptible insect, *Manduca sexta*, the tobacco hornworm, that corn pollen had no detectible levels of Cry1Ac, so the requirement to submit a honeybee larvae study was waived. There was one positive Collembola study submitted for a Cry1Ab product although other similar studies were negative. A worst-case risk assessment calculation showed that there would be a maximum of 4.2×10^{-4} mg toxin/kg soil for a few days following tilling of the corn into the soil. Since the no effect environmental concentration is 8.8×10^{-2} mg toxin/kg soil, there is a safety factor of approximately 200. Furthermore, it is not certain that adverse impacts on collembola in agricultural fields would result in adverse environmental effects. The concern was that, if collembola were killed, plant detritus could accumulate in agricultural fields, however, this effect has not been seen in fields treated with pesticides that undoubtedly kill collembola.

This section identifies the data and information to be included in each Notification. When specific information is not submitted, an explanation of why it is not practical or necessary to provide the information is to be provided.

Table 4 Toxicology and ecological effects data submitted in support of plant pesticide registrations

Cry1Ab	Cry1Ac	Cry3A	Cry9C
acute oral, mice, MT no effect >4000 mg/kg	acute oral, mice, MT no effect >4200 mg/kg	acute oral, mice, MT no effect >5220 mg/kg	acute oral, mice, MT no effect >3760 mg/kg
acute oral, mice, MT noeffect >3280 mg/kg	acute oral, mice, MT no effect >5000 mg/kg	degraded by gastric fluid but not intestinal fluid	not degraded by gastric fluid or heat (90°C-10 min), MT
acute oral, mice, PT no effect >5050 mg/kg	degraded by pepsin	acute oral, quail, PT in diet no effect >50,000 ppm	no homology found with allergenic protein sequences in SWISS database.
degraded by pepsin, MT & PT	degraded by gastric fluid	honeybee larvae, PT no effect >100 ppm	acute oral, quail, MT no effect >58 ug
degraded by gastric fluid but not intestinal fluid, MT	acute oral, quail, PT in diet, no effect >200,000 ppm	earthworm, PT no effect >100 mg/kg soil	Daphnia, PT in pollen no effect

Cry1Ab	Cry1Ac	Cry3A	Cry9C
acute oral, quail, PT in corn meal, no effect >100,000 ppm	acute oral, quail, PT in diet, no effect >10,000 ppm		honeybee, PT in pollen no effect
acute oral, quail, PT no effect >2000mg/kg	*Manduca sexta*, PT in pollen no effect		collembola, PT no effect >180mg/kg soil
honeybee adult, MT no effect >20 ppm	parasitic wasp, MT no effect >10,000 x levels found in pollen & nectar		collembola, MT no effect >20 gm/kg soil
honeybee larvae, MT no effect >20 ppm	collembola, PT no effect >8.0 g/kg		earthworm, PT no effect >1.84 mg/kg soil
honeybee larvae, PT in pollen, no effect	collembola, MT no effect >0.1 mg/kg		ladybird beetle, PT no effect >0.36 ug/l diet
ladybird beetle, MT no effect >20 ppm	earthworm, PT no effect >2 g/kg		
ladybird beetle, PT in pollen, no effect	earthworm, MT no effect >0.1 mg/kg		
green lacewing larvae, MT no effect >20 ppm			
parasitic wasp, MT no effect >20 ppm			
daphnia, MT no effect >150 mg/l			
collembola, MT no effect >200 ppm			
collembola, PT LD_{50} 240 mg/kg/soil NOEL 125 mg/kg/soil			
catfish, PT in corn meal no effect at 100% of diet			
earthworm, MT non toxic			
earthworm, MT non toxic >200 ppm			

MT = Purified toxin produced by a bacterium. The toxin was shown to be equivalent to the plant-produced toxin; PT = Toxin produced in the plant tissue.

Field release of a RecA-deficient Sinorhizobium meliloti strain – test of a novel safety concept

Keller, M.[1)], Dresing, U.[1)], Homann, I.[1)], Dammann-Kalinowski, T.[1)], Selbitschka, W.[1)], Miethling, R.[2)], Tebbe, C.[2)], Pühler, A. [1)]

[1)] Universität Bielefeld, Biologie VI, Postfach 100131, 33501 Bielefeld, Germany,
[2)] FAL, Institut für Agrarökologie, Bundesallee 50, 38116 Braunschweig, Germany

Abstract

To test the suitability of the *rec*A mutation as a novel biological containment system two isogenic RecA$^-$ and RecA$^+$ *S. meliloti* strains were constructed which carry the firefly luciferase gene as an identification marker (Selbitschka et al. 1992, 1995). Laboratory-based experiments had shown that the RecA$^-$ *S. meliloti* strain exhibited a reduced survival in broth culture and in microcosms and was less competitive for alfalfa nodulation compared to the isogenic RecA$^+$ strain (Selbitschka et al. 1992, 1995; Dammann-Kalinowski et al. 1996). Both genetically tagged *S. meliloti* strains were released in field experiments at a cell density of 10^6 cells g^{-1} soil. In a first release lysimeters were used, whereas in a second experiment the strains were released in field plots. In both experimental settings the cell density of the RecA$^+$ and RecA$^-$ strain reached a level of approximately 10^4 cells g^{-1} soil with no significant difference between both strains. Surprisingly, during a three-month heat period after the release into the field plots a ten times more pronounced drop of the viable count of the RecA$^-$ strain compared to the RecA$^+$ strain was detected. Microcosm experiments showed that the observed difference in viable count of both strains was due to a heat-enhanced mortality of the RecA$^-$ strain (Dresing et al. 1998). Analyses of the nodulating *S. meliloti* population isolated from field plots revealed that the RecA$^-$ strain was found only in soils from plots inoculated with this strain. In contrast, the RecA$^+$ strain was isolated also from non-inoculated plots. This result indicates that the RecA$^-$ strain is reduced in horizontal dispersal. In summary, the *rec*A mutation represents an interesting biological containment system.

Introduction

The soil bacterium *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) forms nitrogen fixing root nodules in symbiosis with the pasture crop alfalfa (*Medicago sativa*). The atmospheric nitrogen fixed by the microsymbiont is supplied to the host plant which is then independent of exogenously supplied N fertilizer. Consequently, in agriculture rhizobia have been used as inoculants for the past 100 years in order to increase the yield of leguminous crops. The molecular analysis of the symbiotic process has resulted in a detailed knowledge on nodule development and symbiotic nitrogen fixation, but only little is known of the behaviour of the microsymbiont in its natural environment. The use of genetic markers facilitated the study of how these bacteria behave in soil. In the case of *Sinorhizobium meliloti*, we used the luciferase gene of the North American firefly (*Photinus pyralis*) as a genetic marker (De Wet et al. 1987). This gene directs the synthesis of the enzyme luciferase which is responsible for light emission provided the substrate luciferin is added.

Since there is concern that unintended ecological impacts arising from the long-term persistence and spread of genetically modified microorganisms (GMMs) might be associated with the release, several containment strategies to minimize these potential risks have been developed (Gerdes et al. 1986; Molin et al. 1987, 1993; Knudsen and Karlstrom, 1991; Contreras et al. 1991). We proposed to use the RecA-deficiency of *S. meliloti* as a novel biosafety concept in field release experiments. For this purpose luciferase marked *S. meliloti* strains were constructed which differed in their RecA phenotype (Selbitschka et al. 1992, 1995; Dammann-Kalinowski et al. 1996). These *S. meliloti* strains were characterized intensively first in laboratory-based experiments and in the greenhouse before they were used in field experiments. In this paper we report on long-term monitoring, heat sensitivity and horizontal spread of both GMMs.

Results and discussion

Properties of the luciferase marked RecA$^+$ / RecA$^-$ *Sinorhizobium meliloti* strains L1 and L33

S. meliloti wildtype strain 2011 was used as the parent for the construction of the two bioluminescent *S. meliloti* strains L1 (RecA$^-$) and L33 (RecA$^+$) (Figure 1). The intronless *luc* gene of the firefly *Photinus pyralis* was put under the control of the constitutive *nptII* promoter of transposon Tn*5* and the resulting *luc* cassette was inserted into the chromosome of strain 2011 (Selbitschka et al. 1992, 1995). In the case of the strain L1 the cassette was inserted directly into the *rec*A gene resulting in a RecA deficient phenotype. The isogenic *S. meliloti* strain L33 carried the *luc* cassette downstream the *rec*A coding region and was not affected in its RecA phenotype. Both luciferase marked strains can be easily distinguished from each other at the molecular level by the use of specific PCR primers. One primer hybridizes to the *rec*A gene, the other primer to the *luc* gene resulting in the specificity for the detection of the *luc* marked strains (Figure 1). In the case of strain L1 a PCR product of 1011 bp is obtained, in the case of strain L33 the PCR product has a size of only 415 bp.

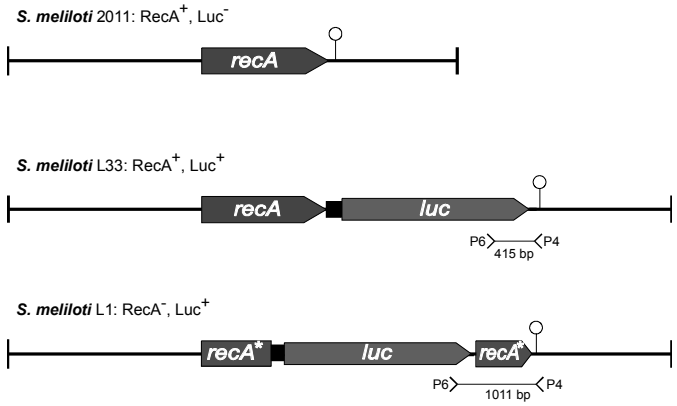

Figure 1 The *recA* gene regions of the *S. meliloti* strains used for release experiments.The *recA* gene regions of the *S. meliloti* strains 2011 (RecA$^+$, Luc$^-$), L33 (RecA$^+$, Luc$^+$), and L1 (RecA$^-$, Luc$^+$) are presented. Using the primers P4 and P6 the strains L33 and L1 can easily be distinguished by employing the PCR technique. - Abbreviations: *recA*: *recA* gene; *recA**: incomplete *recA* gene; *luc*: luciferase gene; small black box: constitutive *nptII* promoter upstream of the *luc* gene; needle: transcription termination signal of recA

Analyses of the genetic stability of the luciferase marker gene in the *S. meliloti* strains L1 and L33 showed that it was stably inherited in both strains during growth in liquid culture as well as during root nodule passages (Dammann-Kalinowski et al. 1996).

Using the RecA$^-$ *S. meliloti* strain L1 and the RecA$^+$ *S. meliloti* strain L33 we revealed that the *rec*A mutation has a significant impact on several properties of the mutated strain (Table 1). Strain L1 exhibited a dramatically reduced homologous recombination frequency (Selbitschka et al. 1995). In addition, in broth culture the RecA$^-$ strain L1 showed an enhanced doubling time of at least 10% compared to its wildtype parent strain 2011 or to strain L33. Moreover, growth competition experiments demonstrated that the proportion of strain L1 in a mixed broth culture with its wild type 2011 decreased within 60 generations of log-phase growth from an initial ratio of 1:1 to a ratio of $1:10^{-4}$ (Selbitschka et al. 1995). Experiments performed in microcosms demonstrated that strain L1 was also affected in its growth competition in sterile soil (Selbitschka et al. 1995). However, with respect to symbiotic nitrogen fixation strain L1 showed wild type properties (Selbitschka et al. 1995; Dammann-Kalinowski et al. 1996). The RecA$^+$ strain L33 was indistinguishable from its parent 2011 in the ability to perform homologous recombination, in doubling time and in growth competition properties as well as in symbiotic nitrogen fixation.

Table 1 Properties of the luciferase marked RecA$^+$ / RecA$^-$ *S. meliloti* strains L1 and L33

Properties	***S. meliloti* RecA$^+$**	***S. meliloti* RecA$^-$**	**References**
	Strain L33	**Strain L1**	
Recombination	wt	reduced	Selbitschka et al. 1992, 1995
UV sensitivity	wt	enhanced	Selbitschka et al. 1992, 1995
Doubling time	wt	enhanced	Selbitschka et al. 1992, 1995
Nitrogen fixation	wt	Wt	Selbitschka et al. 1992, 1995
Growth competition	wt	reduced	Selbitschka et al. 1995, Niemann et al. 1997
Nodulation efficiency	wt	reduced	Dammann-Kalinowski et al. 1996, Niemann et al. 1997
Horizontal dispersal	wt	reduced	this paper
Heat sensitivity	wt	enhanced	Dresing et al. 1998

From the properties of *S. meliloti* strain L1 we concluded that the RecA-deficiency may represent a biosafety concept for the field release of genetically engineered *S. meliloti* strains. In order to test this hypothesis a field release experiment was performed at the FAL (Federal Agricultural Research Centre) in Braunschweig (Germany) which made use of lysimeters (Keller et al. 1994) as well as field plots (Figure 2).

Long-term monitoring of the luciferase marked RecA$^+$ / RecA$^-$ *S. meliloti* strains L1 and L33 in a field release experiment

In September 1994 the two bioluminescent *S. meliloti* strains L1 and L33 and the wildtype strain 2011 were released in a peat-soil mixture onto the upper layer of field lysimeters (diameter 30 cm), with a fourfold repetition for each strain. 10^6 cells per gram soil as calculated for the upper 30 cm were released. A second release with the same *S. meliloti* strains was performed in April 1995. Using a specific spraying machine the bacteria were spread with low pressure onto plots of 3 m x 3 m, again resulting in a start titer of 10^6 cells per gram soil for the upper 30 cm (plough horizon). This time a fivefold repetition was applied (Figure 2). On lysimeters as well as on field plots alfalfa was grown in order to allow the released *S. meliloti* strains to colonize the rhizosphere and to form nitrogen fixing root nodules.

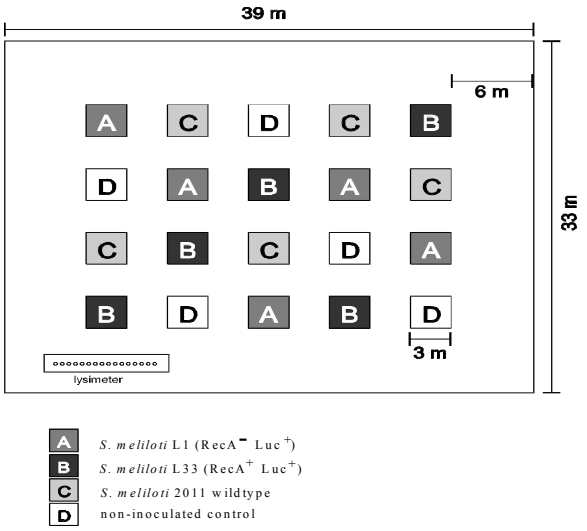

Figure 2 Field plot design at the release site in Braunschweig: The arrangement of the field plots is shown. Each of the four variants are repeated fivefold. The plots were arranged in a blockwise randomized manner. In addition, the location of the lysimeters is presented. Here a fourfold replication was employed.

The cell density of the released strains dropped during the first weeks after the release in both experimental settings for two orders of magnitude, from 10^6 cfu g^{-1} soil to 10^4 cfu g^{-1} soil. In the subsequent 30 months the cell density of both strains showed no significant difference in their survival with one exception during summer 1995 (Figure 3). Evidently, the RecA deficient strain L1 was able to establish in soil in a similar manner like the RecA proficient strain L33. This result is most likely attributable to the following facts. First, the strains were released at a very high cell density, and second, an indigenous *S. meliloti* population on the field plots was hardly detectable just before the field release. Thus, under these conditions the establishment of the RecA deficient strain L1 seems to be facilitated. However, it is worth noting that the $RecA^-$ strain L1 has the ability to compete with the indigenous population of soil microorganisms.

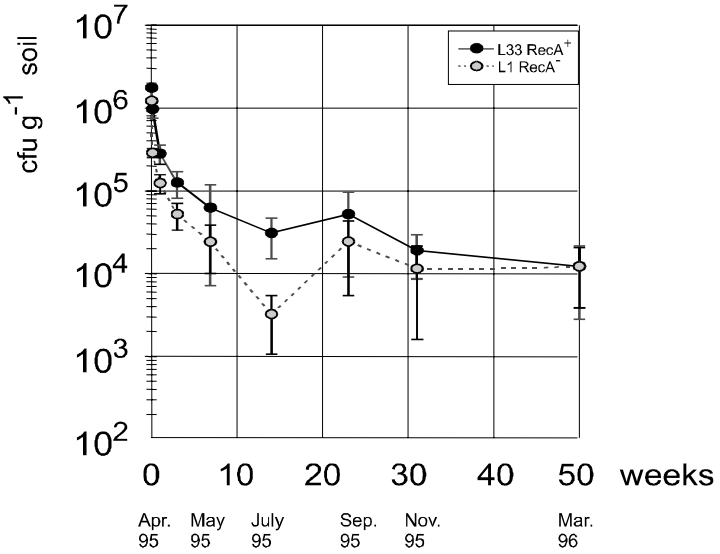

Figure 3 Titer development in the field plots of two isogenic *S. meliloti* strains differing in the RecA phenotype. Titer development in the field plots of two isogenic *S. meliloti* strains L33 ($RecA^+$, Luc^+) and L1 ($RecA^-$, Luc^+) is presented for the first 50 weeks after the release. Soil samples from the field plots were collected and used for bacterial cell extractions. The released *S. meliloti* cells were identified by their bioluminescent phenotype.

The RecA-deficient *S. meliloti* strain L1 exhibited an enhanced heat sensitivity and an impaired horizontal dispersal

During summer 1995 a heat period of several weeks was recorded which affected the viable count of the released RecA-deficient *S. meliloti* strain L1 more dramatically than that of the RecA proficient strain L33 (Figure 3). The cell density of the $RecA^-$ *S. meliloti* strain L1 and the $RecA^+$ strain L33 dropped to 4 x 10^3 and 3 x 10^4 cfu g^{-1} soil, respectively. After the heat period both strains recovered and in the subsequent spring reached the same cell density of about 10^4 cfu g^{-1} soil.

This heat sensitivity of the RecA-deficient *S. meliloti* strain L1 was not only detected in field experiments but also in greenhouse experiments where peak soil temperatures of up to 43°C were recorded. This phenomenon was further investigated and confirmed in various microcosm experiments. It was shown by a live/dead staining procedure that the RecA-deficient strain showed a higher mortality when exposed to short-term and long-term heat stress compared to the RecA proficient strain L33 (Dresing et al. 1998).

During the field release it was found that most probably due to wind transportation the released strains were slightly dispersed outside the respective field plots. Therefore, we performed analyses on the

presence of the released strains on various plots. Alfalfa was used as a sensitive trap plant to extract the *S. meliloti* population from soil samples of the field plots. The nodulating population was further analysed revealing that on L1 inoculated field plots the *S. meliloti* strain L1 dominated, whereas on L33 field plots *S. meliloti* strain L33 was dominating. On non-inoculated field plots the indigenous population dominated in a range of 45-70% (Figure 4). The released wildtype strain *S. meliloti* 2011 was found in a proportion of about 7-20%, whereas the luciferase marked $RecA^+$ strain L33 was detected in the range of 10-40%. Interestingly, the RecA-deficient strain L1 was not detected at all in the non-inoculated plots. This indicates that the RecA-deficient strain is reduced in its horizontal spread.

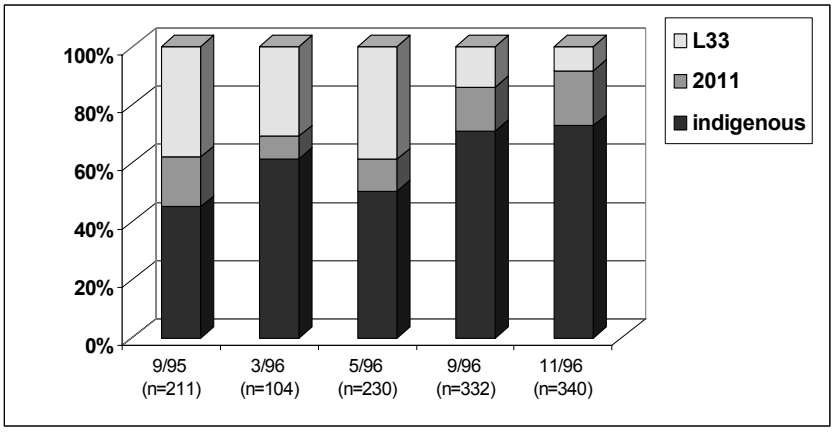

Figure 4 Composition of the nodulating *S. meliloti* population in non-inoculated field plots.
The nodulating *S. meliloti* population in non-inoculated field plots was determined by using alfalfa as a trap plant. The measurement was repeated several times after the start of the relase experiment. The bioluminescent strains L33 ($RecA^+$, Luc^+) and L1 ($RecA^-$, Luc^+) were distinguished by PCR using the primers P4 and P6. The wildtype strain was distinguished from the indigenous strains by ERIC-PCR.

Conclusions

Laboratory-based and greenhouse experiments revealed an enhanced heat sensitivity, an enhanced doubling time and a reduced nodulation efficiency of a RecA-deficient *S. meliloti* strain. In field experiments it was shown that the $RecA^-$ strain was impaired in its horizontal spread, but not significantly in its persistence in soil. In summary we conclude that the employment of the *rec*A mutation provides a useful biosafety system in respect to horizontal spread which can be applied at least for the release of the agriculturally important soil bacterium *S. meliloti*.

Acknowledgements

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Fructans as prebiotic food additives

Heyer, A.G.

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany

Abstract

Fructans are gaining increasing interest as additives for "healthy food". They show anti-tumoral effects and are interesting low calorie fibers.

Fructans are fructose polymers that are synthesized from sucrose. Enzymes of bacterial and plant origin have been characterized that are capable of fructan synthesis.

We have expressed two fructosyltransferase genes of artichoke in potato and observe a fructan accumulation of up to 1% of the fresh weight in the transgenic plants.

Considering food safety, the transgenic plants can be compared to plants expressing similar enzymatic activities, e.g. invertases.

Expression of invertase from baker's yeast as apoplastic enzyme in potato tubers leads to a decrease in tuber number per plant and increase in the size of individual tubers. In the tubers, the glycoalcaloid content is strongly reduced. It is proposed that this change could be due to a reduced UDP-glucose content.

Changes in transgenic plants in a metabolic pathway that seems not obviously related to the target pathway of the genetic modification indicate that safety assessment of transgenic food should not rely on the analysis of a modified pathway and its intermediates, but on those compounds that are relevant to the safety of a given foodstuff.

Introduction

Fructans are gaining increasing interest as additives for "healthy food". They are regarded as "prebiotic" because of anti-tumoral effects that seem to be associated with a stimulation of Bifidobacteria in the human intestine. This stimulation causes an increased propionate and butyrate fermentation and lowers the faecal concentration of tumor promoting substances like ammonia and ß-Glucuronidase. In a mutant mice strain that spontaneously develops colon tumors because of a genetic defect in the APC gene, a fructo-oligosaccharide containing diet significantly reduces the number of colon tumors, whereas starch and wheat bran have no effect on total tumor number, as reported by Pierre et al. (1997).

Besides, fructans are interesting low calorie fibers, because the ß-linkage of the fructose moieties cannot be cleaved by human enzymes. Bacterial fermentation and resorption of fermentation products yields an energy value of 1 kcal/g, which is about 30% of that for the free hexoses. The texture of the fiber gives a fat-like mouth feeling and therefore fructans are excellent bulking agents for low calorie foods. For example, a combination of Maltitol as sweetener with inulin as bulking agent in the production of low-fat, sugar-free chocolate leads to a reduction of the caloric value by 23% (Rapaille et al. 1995).

Fructans are fructose polymers that are synthesized from sucrose as substrate by transfer of the fructose moiety from sucrose to a growing chain. The glycosidic C-2 hydroxyl group can be transferred either to the C-6 position. In this case, a Levan type fructan is synthesized. Or it can be transferred to the C-1 position, leading to an inulin type fructan. In both cases the fructan chain contains a terminal glucose and is therefore a non-reducing sugar. Low molecular weight fructans, which are nowadays used as food additives, are naturally found in plants or can be produced by help of fungal enzymes. Fructans synthesized by bacteria are typically of high molecular weight. In most cases bacterial fructans are of the Levan type; only one high molecular weight inulin has been described.

There are also some reports on fungal high molecular weight inulin synthesized by certain strains of *Aspergillus* or *Penicillium*. But to date, no fungal enzymes capable of fructan synthesis are described in detail and no genes have been cloned.

Fructan synthesis in plants is brought about by the action of at least two enzymes, one of them, the sucrose dependent sucrose:sucrose-fructosyltransferase (SST) producing the trisaccharide Kestose, the

other, fructan dependent fructan:fructan-fructosyltransferase (FFT) being a transfructosylase that uses fructans as donor and acceptor of fructosyl residues.

Results

We chose artichoke as the source for the fructosyl transferase genes, because artichoke produces the largest inulin known among the plant kingdom. We believe that molecule size could influence fructan yield in transgenic crops, because fructans are water-soluble carbohydrates and the contribution of longer chains to osmotic potential would be lower than for short chains. The maximal chain length of artichoke inulin is 200 fructose units; the mean DP is about 65 molecules.

Transformation of potato with the SST and FFT genes was performed in two steps. At first, we transformed potato with a construct that contains the SST under control of the CaMV 35S promoter. Transgenic plants produce the trisaccharide Kestose and also Nystose, which is the next higher homologue, in substantial amounts. The Oligofructans are located in the vacuole and would be subject to degradation by invertases. Fortunately invertase activity is low during loading of the tubers with photosynthates. Under conditions of cold storage, only longer chains would be resistant to invertase activity.

Transformation of the SST-expressing potato with an FFT-construct leads to the accumulation of inulin in tubers. The inulin resembles the artichoke inulin in size, and the yield reaches up to 1% of the fresh weight which is high considering the low concentration of sucrose in potato.

A closer look at the carbohydrate composition of the potato tubers reveals that fructan synthesis might take place at the expense of starch, but as starch content is about 20-fold higher, the reduction is only minor, at least in greenhouse experiments.

We are now performing field tests to analyze biomass production in more detail.

What are the biosafety aspects of genetically modified potato plants that produce inulin?

As inulin is regarded as “healthy food” we believe that these potatoes are of a high nutritional value. But still we have to raise the question of an appropriate risk assessment strategy for this foodstuff.

The reaction type catalyzed by a fructosyltransferase is the ß-fructofuranosidase reaction, very much alike the reaction catalyzed by plant invertases. The enzyme catalyzes the reaction of a nucleophilic oxygen atom with a carbonyl thereby exchanging the glycosidic bond of a sucrose molecule against a hydroxyl group of a sugar in case of the transferase or water in case of an invertase. Indeed fructosyltransferases share a fairly high homology to invertases, showing an identity of about 55-60% on amino-acid level.

In the context of a project addressing photoassimilate partitioning we have created potato plants expressing the invertase of baker’s yeast with a targeting sequence for cell wall localization. The effect was quite profound: Expression of the invertase leads to an increase in the size of individual tubers and a reduction of tuber number per plant. The sucrose content of the tubers is drastically reduced in the transgenic plants, while the glucose content is notably elevated. Interestingly fructose is not changed, but the rise in glucose accounts for an overall twofold increase in soluble sugars. In the invertase expressing transgenic plants, the glycoalkaloid content is significantly reduced. The calculated glycoalkaloid content for a standard 100 g tuber from the Line 33, which has the strongest invertase activity, shows a nearly 50% reduction in α-solanine and α-chaconine. Glycoalkaloid content is depending on tuber size, because the alkaloids are mainly located in the outermost cell layers directly underneath the peel. As the volume-to-surface ratio increases with increasing tuber size, bigger tubers contain less alkaloids. The reduction in glycoalkaloid content of invertase tubers holds true for all sizes, but it is more pronounced for smaller tubers. The fact that in the wild-type a 50 g tuber contains more than twice as much glycoalkaloids than a 200 g tuber raises the question of the significance of the reduction in the transgenic line. Moreover, glycoalkaloid content is also differing among varieties - and in a given variety it is depending on environmental factors. For example, total glycoalkaloid content in the “Hela” variety is about 50% that of the variety “Sieglinde”, at least when grown in Italy. From a German field test, the glycoalkaloid content of “Sieglinde” was 1.7-fold higher than that of “Hela”. More important than the observed reduction in glycoalkaloids is the question: Why does expression of an invertase lead to a reduced

glycoalcaloid content? Is this a pleiotropic effect? Does this prove that the consequences of genetic modification are unpredictable? We believe it does not.

In potato tubers, imported sucrose is primarily metabolized by sucrose synthase, yielding UDP-glucose and fructose. Only at very early stages of stolon development, invertases play a significant role in sucrose metabolism. Both pathways ultimately lead to glucose-6-phosphate that is imported into the amyloplast. In case of the invertase-pathway, glucose-6-phosphate is produced directly from glucose in a reaction catalyzed by glucokinase. In such a case, the UDP-glucose pathway is not involved.

If this affects UDP-glucose concentration, glycoalkaloid synthesis would also be involved, as UDP-glucose is the substrate for the synthesis of the sugar moiety of the molecule. It is therefore not astonishing that glycoalkaloid content is lowered in invertase expressing potato tubers. We are currently testing whether this model applies to the invertase potato and also to the fructan producing plants.

Reduction of glycoalkaloid content is not at all a problem in respect to food safety. But still the fact that a change occurs in the transgenic plants in a pathway that seems not obviously related to the target pathway for the modification is an important aspect of risk assessment for the genetically modified foodstuff.

Conclusion

Transgenic potato plants expressing fructosyltransferase genes contain fructans that add to the content of soluble sugars. An influence of the new enzymatic activity on the concentration of soluble sugars like glucose, fructose or sucrose cannot be excluded.

For invertase expressing potato tubers that show a strong reduction in sucrose content, a reduction in the content of glycoalkaloids is observed. This change seems not obviously related to the genetic modification. Still there is a model that can explain the effect on the basis of a reduced UDP-glucose content of the tubers.

A risk assessment strategy that was designed specifically for the genetic modification and the metabolic pathway that is the target for the modification could fail to detect a change in the glycoalkaloid content in case of invertase expression. Yet this is the most important aspect of food safety in potato.

Therefore a more appropriate way to assess the safety of a transgenic food would be analyzing all compounds that are relevant to food safety of a given foodstuff. In case of potato such a list would certainly contain the glycoalkaloids.

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Engineering plant viruses to produce peptides or proteins of medical, veterinary or industrial importance

Wilson, T.M.A., Oparka, K., Chapman, S., Lacomme, Ch., Smolenska, L., Ingram, A., Santa Cruz, S.

Scottish Crop Research Institute, Invergowrie, Dundee, DD2 5DA, UK

Abstract

More than one thousand plant viruses have been described in greater or lesser detail and over 75% of these contain single-stranded RNA as their sole genetic material. Many of these viruses multiply to extremely high copy numbers in appropriate host plant cells, reaching titres of up to one million particles per cell. Over the past 14 years, several of these viruses have been developed as high-level transient expression systems for foreign proteins and, more recently, as particulate vaccines by fusing candidate antigenic peptides to one or more suitable sites on the viral coat protein. A wide variety of ingenious molecular genetic strategies have been developed to achieve the desired product. In terms of biological safety, as well as genetic and environmental containment, several intrinsic or introduced features of these viral vectors have been shown to confer desirable properties. In at least one case, large-scale open field releases and commercial production has occurred over the past several years in the USA. In addition to their obvious commercial attractions, foreign reporter gene expression systems using plant viruses also reveal increasing details of the molecular and cell biology of plant developmental, defence and gene regulatory processes. Among the many advantages of using transient expression vectors based on plant RNA viruses are the speed and efficiency with which candidate proteins, peptides or genomic fragments can be expressed and assayed for their biological function within the plant or following extraction, if required. Moreover, there is no interaction with the heritable material or propagules of the crop. Among an apparently limitless array of applications for which plant bioreactors would seem appropriate are: vaccines, prophylactics, therapeutics, bioremediation molecules (or even crops), crop protectants, food additives or neutraceuticals, industrial enzymes, or biocatalysts either in soluble or particulate form.

Introduction

Many useful and novel phenotypes can be introduced into model or crop plant species by transgenesis using *Agrobacterium*-mediated transformation or by direct DNA-transfer methods. In general, the levels of functional (or dysfunctional) protein expressed, or the amount of untranslatable RNA produced, range from less than 1% of total cellular protein to virtually undetectable levels. Nevertheless, transgenic lines are selected which display the desired phenotype, whether that be conferring resistance to a pest or pathogen, a herbicide or some other alteration to plant cell metabolism.

Crop plants offer extremely cost-effective, efficient and high levels of energy conversion into biomass; however, to extract a transgene product expressed at vanishingly low levels from such a large volume provides new and challenging technical problems during commercial scale-up. For these reasons, as well as for additional beneficial features such as biological containment, restricted gene flow, speed, precision and efficiency, a number of plant viruses have been developed to express much higher levels of valuable foreign proteins in transfected crop plants. This paper will describe and review some recent developments using plant RNA viruses as high level, transient expression systems for proteins and peptides with medical, industrial and agro-environmental use.

Since the early 1980's, plant viruses have been proposed and developed as possible vectors for the expression of foreign peptides or proteins. Much research has been motivated by the potential economic advantages available through cheap, high-level expression of valuable proteins in systems capable of producing high yields of biomass at extremely low input costs, for example by using field crops as hosts for these genetically engineered viruses.

Early efforts to develop virus-based vectors for plant transformation focused on DNA viruses, rather than RNA viruses. Two reasons for this were that DNA genomes could be manipulated directly *in vitro,* whereas RNA genomes required a cDNA that could subsequently be transcribed to generate infectious RNA; and there was a widespread belief that the error rate of RNA-dependent RNA polymerases, which lack a proof-reading mechanism, would render RNA virus-based vectors genetically unstable and so of no value in biotechnology (Van Vloten-Doting et al. 1985). For several other reasons, it was generally

concluded that the single-stranded DNA-containing geminiviruses would be the most appropriate candidates to be developed as episomal foreign gene expression systems in plants. The main reason was that geminiviruses replicate only through a DNA intermediate within the nucleus of the host cell. Consequently, they benefit from DNA-dependent DNA polymerase proof-reading mechanisms. However, the capacity of the geminivirus particle to package DNA imposed a severe constraint on the size of recombinant genomes that could be encapsidated which limited their utility as gene delivery systems (Timmermans et al. 1994; Scholthof et al. 1996).

Despite these early concerns, studies from 1990 onwards have demonstrated that plant RNA viruses are well-suited to serve as transient expression vectors for foreign genes (Scholthof et al. 1993; Joshi et al. 1990; Takamatsu et al. 1987; Chapman et al. 1992; Kumagai et al. 1993; Dolja et al. 1992). Thus a wide variety of RNA-containing plant viruses, displaying various genome organisations, expression strategies and particle architectures have been used as vehicles to express extremely high levels of foreign peptides and/or proteins in infected host plants. Although most foreign gene inserts are not retained by RNA viruses during sequential passaging through susceptible hosts, this should be seen as an advantage. The revertant, "wild-type", virus is better adapted and therefore ultimately likely to prevail in the overall population, so reducing the risk of escape of a recombinant viral genome into the environment. For practical purposes, it is sufficient only that the foreign gene be retained and expressed transiently in a single generation of inoculated plants. The inability of a recombinant virus carrying a foreign sequence to move to successive generations of plants or to adjacent plants is beneficial.

All viruses, including the approximately 1,000 or more known to infect plants, exploit their host plant cells as a source of energy, preformed constituents, and biosynthetic machinery in order to achieve high-levels of replication and accumulation of progeny virus via an infection cycle involving particle disassembly, translation, genome replication, and encapsidation of progeny virions. Most plant viruses encode protein(s) which promote local and systemic movement within the infected plant. Some viruses also encode proteins which assist in the long-distance transmission of infection between plants via invertebrate or fungal vectors. The fact that relatively few plant viruses are pollen- or seed-transmitted is a further advantage of using plant virus expression vectors, with respect to environmental containment of foreign genes. The majority of plant viruses (76%) have single-stranded RNA genomes between 3.5-10 kb. Thus the control elements which govern replication and expression of virus genes are, of necessity, highly efficient, often small and frequently multifunctional.

Stable plant transformation and regeneration methods are laborious, unpredictable and remain limited to relatively few plant species which exclude many of the most important monocotyledonous crops, legumes and woody perennial species. In contrast, one or more plant viruses, capable of acting as a foreign gene expression vector, could be designed for every major crop plant species.

To develop more efficient systems than transgenesis (Benfey and Chua, 1990; Sleat and Wilson, 1992) for high-level synthesis of specific foreign proteins in plants, attention has recently focused on high-copy-number RNA viruses as vectors for foreign gene expression. Plant viruses offer numerous advantages as vehicles for transient expression of foreign genes, including their characteristically rapid multiplication to high-levels with concomitant high-level gene expression. Maximum levels of foreign gene expression from a viral genome can occur within one or two weeks of inoculation. Another practical advantage is that many plant viruses can be transmitted mechanically and so can be used for simple, commercial-scale inoculation over large areas of a crop. Also, once a suitable virus expression vector is constructed, expression levels of the foreign gene can be screened rapidly in a variety of different host plant species to choose the most suitable crop which is least affected by virus infection and which gives the highest yield of foreign protein. This paper reviews recent and future strategies for the utilisation of plant viruses as vectors for transient, rapid, high-level expression of foreign genes in cheap, safe and readily available crop plants.

Development of plant virus-based expression vectors

The choice of virus to develop a viral-based expression vector depends on several features including:

i. facile genetic manipulation of the viral genome via a full-length infectious clone,
ii. wide host range,
iii. efficient mechanical infection,

iv. optimised viral functions [high-level replication, efficient promoter(s) for foreign gene expression, efficiently translated mRNA, local and systemic movement],
v. hypovirulent virus strains (i.e. cause mild symptoms),
vi. genetic stability, and
vii. no size constraint on recombinant genome packaging.

For any given virus, the complete genome organisation, a full knowledge of essential or non-essential viral genes, and requirements for *cis*-active sequences will determine the possible strategies that could be used for foreign gene expression (Table 1).

Table 1 Advantages of plant viruses as foreign protein expression vectors and carriers

Many plant viruses multiply rapidly to very high levels (up to a few grams per kg fresh weight)
Plants are cheap factories (need only water, minerals and sunlight to accumulate high biomass)
Plants are safe source for protein (no human or animal pathogens - cf. blood products or microbial cultures)
Plant virus particles are very stable and can be stored at ambient temperatures, often for decades
Edible plants offer direct routes for application
Plant viruses provide highly ordered, multivalent carriers for vaccine peptides (60-2,000 copies per virion promise greater immunogenicity)
Plant virus-based expression vectors can be assembled and candidate therapeutics, vaccines etc., tested in days-to-weeks
High expression levels and novel IP facilitate profitability
Lack of heritability and no virus movement by pollen, seed or invertebrates aids containment (cf transgenes)
Plant-based non-food production systems raise fewer ethical and public outrage issues than use of transgenic mammals.

Because of the ease of manipulation of their genomes, double-stranded DNA plant viruses were first used as gene replacement vectors (Brisson et al. 1984). However, numerous techniques have been developed to permit reverse transcription of viral RNA into cDNA that can be inserted into plasmid vectors for subsequent *in vitro* manipulation. The generation of full-length infectious viral RNA from *in vitro* transcription systems has become routine since 1984 (Ahlquist et al. 1984) and provides unlimited possibilities for designing and using single-strand RNA (ssRNA)-based viruses as gene expression vectors.

Gene replacement vectors

This strategy was the first used for foreign gene expression. The approach is based on replacement of a non-essential viral gene by a foreign gene in order to avoid the possible negative effects of increased genome size. For example gene II (470 bp) of CaMV, which encodes a protein responsible for aphid transmission of the virus, was replaced with foreign DNA without abolishing infectivity on plants (Brisson et al. 1984; De Zoeten et al. 1989). This vector was used successfully to express bacterial dihydrofolate reductase (DHFR) (Brisson et al. 1984) and human α-D-interferon (α-D-IFN) (De Zoeten et al. 1989). In the case of α-D-IFN, yields up to 2 µg per gram fresh leaf weight were obtained.

Other groups have attempted to use ssDNA geminiviruses, tombusviruses and several different rod-shaped RNA viruses to express reporter genes, mostly by replacement of some or all of the coat protein gene. With some rare exceptions [e.g. coat protein (CP) replacement in tomato golden mosaic geminivirus (TGMV) (Hayes et al. 1989), African cassava mosaic geminivirus (ACMV) (Ward et al. 1988), tomato bushy stunt virus (TBSV) (Scholthof et al. 1993), and replacement of a portion of RNA3 and upstream inhibitory sequences of beet necrotic yellow vein furovirus (BNYVV) (Jupin et al. 1992)], only low levels of foreign gene expression were observed. Foreign gene expression by the gene replacement strategy can be affected by alterations in replication [e.g. replacement of the CP in barley stripe mosaic virus (BSMV) (Joshi et al. 1990)]; in local and/or systemic movement [e.g. replacement of the CP in tobacco mosaic virus (TMV) (Takamatsu et al. 1987), potato virus X (PVX) (Chapman et al.

1992) and brome mosaic virus (BMV) (French et al. 1986)]; in encapsidation [size modification of CP in TGMV (Hayes et al. 1989) and ACMV (Ward et al. 1988)]; in virus accumulation [CP replacement in TBSV (Scholthof et al. 1993)]; and by recombination events inherent in some virus genomes [TBSV (Scholthof et al. 1996), CaMV (Gronenbom and Matzeit, 1989)].

Gene insertion vectors

Stringent biological selection means that all viral genes contribute to the overall fitness of the virus, so gene replacement often compromises virus yield. To avoid such deleterious effects, foreign gene insertions have been made and tested.

For some viruses, foreign genes can be inserted under the transcriptional control of a viral subgenomic promoter sequence. However, duplicating a native viral subgenomic RNA promoter for foreign gene expression can lead to high frequencies of homologous RNA recombination between the duplicated sequences, thus resulting in rapid deletion of the inserted foreign gene (Dawson et al. 1989) and reversion to wild-type. One solution is to use a subgenomic RNA promoter from a closely related virus, to provide a more genetically stable vector for foreign gene expression (Donson et al. 1991). Using this approach, Biosource Technologies Inc., created a TMV-based vector to express a sequence encoding α-trichosanthin (a ribosome inactivating protein) under the control of the coat protein subgenomic promoter of U1 strain TMV, while TMV coat protein synthesis was directed by a promoter sequence from another tobamovirus, odontoglossum ringspot virus (ORSV). The two promoters shared low nucleotide sequence identity (45%). No alteration of virus movement was observed and biologically-active α-trichosanthin accumulated at up to 2% of total soluble leaf protein (Kumagai et al. 1993). Using this strategy, Biosource Technologies have also shown that it is possible to manipulate the carotenoid biosynthetic pathway in TMV vector-infected plants, either by over-expression of a phytoene synthase gene, causing a bright orange phenotype, or by inhibition (virus-based expression of the corresponding antisense RNA) of the endogenous gene encoding a phytoene desaturase, leading this time to a "white" phenotype (Kumagai et al. 1995).

Other examples of vectors based on rod-shaped viruses have been described including tobacco etch virus (TEV) and PVX. In the case of TEV, whose expression requires regulatory *cis*-elements for proteolytic polyprotein processing, an insertion-fusion strategy [insertion of the *uid*A gene from *Escherichia coli*, encoding *ß*-glucuronidase, between the N-terminal 35 kDa proteinase and the helper component-proteinase (HC-Pro)] gave high-level expression of the inserted gene (Dolja et al. 1992).

Duplication of the homologous coat protein subgenomic promoter has proved to be an efficient way to express foreign genes from PVX without rapid recombination/deletion. The gene insertion strategy has been used successfully with PVX to study virus infection (Baulcombe et al. 1995), and for structure-function analyses of a *myb*-like transcription factor from potato (Sablowski et al. 1995), the avirulence gene *avr9* (a fungal elicitor of disease resistance) from *Cladosporium fulvum* (Hammond-Kosack et al. 1995) and the *fen* gene (a homologue of the *pto* resistance gene) from tomato (Rommens et al. 1995) amongst other examples.

Complementation systems

To overcome possible effects of the gene replacement or gene insertion strategies on viral fitness, plant virus complementation systems have been designed in which the foreign gene is inserted into a defective virus component that is rescued *in trans* by transgenic host expressed viral genes, or by co-infection with a helper virus.

Transgenic plant complementation of viral functions has been used for the 30 kDa movement protein of TMV (Deom et al. 1987), or gene VI for long-distance movement of CaMV (Schoelz et al. 1991). Recent work on functional complementation by co-inoculation with a helper virus was also developed with CaMV. Rescue was performed either by wild-type CaMV, or by a second defective virus bearing another deletion, to avoid any selective advantage in favour of a wild-type helper virus. This strategy, however, is very inefficient, because the defective CaMV genomes readily recombine *in vivo*, restoring an infectious wild-type genotype (Mushegian and Shepherd, 1995). To reduce the frequency of virus recombination, a pair of CaMV mutants with long deletions was constructed. In one genome, the deletion spanned most of the essential gene I and non-essential gene II, while in the other, most of gene II and the essential gene III

were deleted (Hirochika and Hayashi, 1991). This overlap should prevent recombination events and result in forced genetic complementation, and co-existence of two defective CaMV mutants. The complementation approach could also be exploited for a gene replacement strategy despite the genetic instability inherent in caulimoviruses (Vaden and Melcher, 1990).

Another cross-complementation scheme has been described with monopartite geminiviruses based on wheat dwarf virus (WDV). A WDV shuttle vector was created in which the *npt*II reporter gene and a prokaryotic origin of DNA replication replaced the virus CP gene. Kanamycin resistance was expressed in plant protoplasts where the replication defective mutant was rescued by a co-introduced vector providing the C2 replication protein *in trans* (Timmermans et al. 1992).

Other complementation systems, for the expression of foreign genes, have involved the use of subviral agents "parasitic RNAs" which depend on their helper virus for essential functions. Satellite RNAs [e.g. replacement of a 20-kDa non-structural protein encoded by satellite RNA of bamboo mosaic potexvirus (Lin et al. 1996)] or defective interfering (DI) RNA replicons (Scholthof et al. 1996) may be employed as helper-dependent foreign gene vectors. These approaches require considerable further investigation into the effects of sequence modifications on foreign gene expression, virus fitness, genetic stability, host plant symptomatology and virus transmissability.

Epitope presentation systems

This expression strategy overcomes the inconvenience of duplicated promoter sequences in the viral genome which can lead to RNA recombination (Gronenbom and Matzeit, 1989). An in-frame translational fusion to a highly expressed viral gene (e.g. the CP gene) produces large amounts of the foreign protein or peptide sequence. Fusions can be N- or C- terminal, or inserted into the CP sequence, usually at a site located on the outer surface of the final virion, such that the additional sequence does not prevent particle assembly. This strategy is attractive since the unique physico-chemical characteristics of plant virus particles should allow easy purification of modified particles. Some CP fusions were deleterious, however, as exemplified by fusion of a pentapeptide corresponding to Leu-enkephalin to the C-terminus of TMV CP which inhibited assembly of virus particles and abolished long distance movement (Takamatsu et al. 1990). Nevertheless, other foreign peptide sequences have been fused successfully to the CPs of TMV, TBSV and cowpea mosaic virus (CPMV) whilst still allowing particle assembly from the tagged subunits (Takamatsu et al. 1990; Hamamoto et al. 1993; Joelson et al. 1997; Porta et al. 1994).

Recent developments have defined a more "targeted approach" to construct internal or terminal CP fusions to display peptide epitopes. This approach benefits from having extensive knowledge of the carrier virus structure at the atomic level, which is currently available for only a few plant viruses. Using TMV, a 12-amino acid malarial epitope was inserted into a surface loop in the CP (Turpen et al. 1995) between successive α-helical segments (around amino acid position 60). This mutant was recovered in yields up to 0.4 mg/g fresh systemic tissue. No data concerning the immunogenic character of this fusion are available.

Epitope presentation has also been achieved successfully with CPMV. CPMV is an icosahedral virus with a bipartite positive-sense RNA genome. The virus capsid comprises 60 copies each of a large and a small CP subunit. A detailed knowledge of the three-dimensional structure of the CPMV particle allowed a site of insertion to be selected such that the heterologous sequence would not interfere with virus assembly. Inserts of approximately 15-30 amino acids in the βB-βC loop, in the smaller CP subunits (S-CP), are clustered around the twelve five-fold axes of symmetry on the outer surface of virus particles (Lomonossoff and Johnson, 1995). Once it had been determined that all the S-CP amino acid sequence had to be retained, and an optimal insertion site existed, this approach led to the production of genetically and structurally stable CPMV particles containing epitopes from foot-and-mouth disease virus (FMDV), human rhinovirus 14 (HRV-14) and human immunodeficiency virus type 1 (HIV-1) (Porta et al. 1994) among many others. Purified particles containing epitopes of HRV-14 and HIV-1 yielded 1 mg virus/gram leaf tissue and raised antibodies, when injected into rabbits or mice. The latter were capable of neutralising an HIV-1 infection of laboratory T-cells (Lomonossoff and Johnson, 1995). Recently, protective immunity against mink enteritis virus (MEV) has been achieved using a 17-amino acid epitope from the VP2 MEV capsid protein inserted into the CPMV S-CP (Dalsgaard et al. 1997). However, one

limitation of this approach is a lack of CPMV assembly and intraplant movement with most peptides greater than about 25 amino acids (most epitopes tested range from 14-24 residues) and problems in recovering virus particles from plant debris in some cases, where large paracrystalline inclusions form.

To overcome this limitation, Yusibov et al. (1997) used a TMV gene insertion vector, with duplicated CP promoters, to express the CP of alfalfa mosaic virus (AMV) carrying N-terminal fusions of foreign epitopes. The choice of AMV CP as a carrier molecule was dictated by the fact that the AMV genome is known to accommodate larger sequences by forming particles of different sizes and shapes, moreover the N-terminus of AMV CP is located on the outer surface of virus particles and fusions do not appear to interfere with virus assembly. The autonomous expression of AMV CP-peptide fusions from a TMV gene insertion vector required an origin-of-assembly RNA sequence (OAS) for AMV CP which was provided by incorporating the 3'-untranslated region of AMV RNA4 (the CP mRNA). The authors were thus able to express and purify two antigenic peptides (fused to AMV CP) of 47 and 40 amino acids corresponding, respectively, to the V3 loop of HIV-1 and a B-cell epitope from Rabies virus glycoprotein. Both AMV-chimaeras elicited specific virus-neutralising antibodies in immunised mice (Yusibov et al. 1997).

Vectors have also been designed to synthesize both fused and unfused forms of the TMV CP from the same viral mRNA, thus enabling virus assembly to occur normally but using predominantly unmodified CP. Incorporation of a nucleotide sequence which promotes approximately 5% "leaky" readthrough of the amber stop codon for the CP of TMV and an in-frame sequence encoding the 12-amino acid peptide, angiotensin-I-converting enzyme inhibitor (ACEI), led to the production of both free TMV CP and fused CP-ACEI at up to 100 µg/g fresh tissue (Hamamoto et al. 1993). A similar strategy was used to express a 15-amino acid malarial epitope fused to the C-terminus of TMV CP. Mutant virus was recovered at yields up to 1.2 mg/g fresh weight in systemically infected tissue (Turpen et al. 1995). In all cases above, the ratio of modified CP/unmodified CP was between 1/200-1/20.

As mentioned previously, peptide fusions to the C-terminus of TMV CP can exhibit severe inhibitory effects on particle assembly and intraplant movement (Takamatsu et al. 1990), and promoter duplication (gene insertion) vectors result in soluble foreign protein levels up to approximately 1% of total soluble protein (TSP). Likewise, C-terminal readthrough strategies result in reduced, non-stoichiometric yields of the CP-foreign peptide fusion product.

The search for a virus-based system for high level production of large foreign peptides or even whole, functional proteins, in plants, led to the design of a new expression vector incorporating a "free-or-fused CP" co-expression strategy. This system is the so-called OVERCOAT® protein technology.

OVERCOAT® protein presentation/release system

PVX, like TMV, is a rod-shaped virus, hence, no *a priori* constraint is imposed on the size of inserted genes due to RNA packaging. Despite the lack of an X-ray-derived structure for PVX, serological and other evidence indicated that the N-terminus of each PVX CP subunit was exposed on the outer surface of the virus particle. Wild-type PVX has approximately 1300 CP subunits per mature virion.

To avoid genetic instability via duplicated promoters, as with gene insertion strategies, and steric packaging constraints of CP-foreign peptide or protein fusions, a novel vector was engineered to provide spontaneous auto-cleavage of the fusion protein (i.e. between the newly inserted sequence and the N-terminus of PVX CP). To achieve this autocleavage, a short (16-amino acid) sequence from foot-and-mouth disease virus (FMDV), the 2A "protease", which normally disrupts peptide bond formation during FMDV RNA translation (Ryan et al. 1991) was fused to the N-terminus of the PVX CP gene. Theoretically, 100% co-translational cleavage should occur, resulting in the release of high and stoichiometric levels of the soluble foreign peptide or protein (1:1 with CP).

To study the characteristics of OVERCOAT® as a "protein release" system, a chimaeric open reading frame (ORF) encoding the 27 kDa *Aequorea victoria* jellyfish green fluorescent protein (GFP), the FMDV 16-amino acid 2A sequence, and the 25 kDa PVX coat protein was constructed in a full-length infectious PVX clone (Santa Cruz et al. 1996). The results showed that this construct allowed virus assembly, as well as local and systemic movement *in planta*. However, in infected plants, this modified PVX vector produced not only the predicted free GFP-2A and free PVX CP subunits, the latter to function in virus assembly and cell-to-cell movement, but also high levels (50%) of an approximately 54

kDa GFP-2A-CP fusion protein. Even more unexpected was the discovery that these 54 kDa "fusion" protein subunits co-assembled efficiently with free PVX CP into viable PVX particles. The progeny particles were more than twice as wide (29.7 nm) as wild-type PVX (12.6 nm) and attained high levels in plants (10-20% of total soluble protein) (Santa Cruz et al. 1996). Free PVX CP could also be supplied *in trans* from CP transgenic *Nicotiana benthamiana* plants, thereby complementing (rescuing) defective PVX 2A-CP fusion vectors.

The general utility of the OVERCOAT® approach described here, for assembly of foreign protein-CP fusion containing virions, required that proteins other than GFP could be fused to PVX CP and retain the ability to assemble into virions and move systemically. Fusions between PVX CP and neomycin phosphotransferase II (31 kDa), chloramphenicol acetyltransferase (25.6 kDa), a *ß*-galactosidase fragment (8.5 kDa) or a single-chain antibody (scFv; 27kDa) have all resulted in assembly and virus movement. The assembly of PVX.GFP-CP virions is not therefore a unique attribute of the GFP-CP "subunit". The PVX system can be used to express a wide range of unrelated peptides, polypeptides and proteins (so far, up to 33 kDa).

The fact that PVX CP can tolerate a high proportion of N-terminal fused foreign peptide without altering virus movement and accumulation makes OVERCOAT® an efficient epitope presentation system. Actually, the 2A peptide is extremely versatile and the proportion of CP subunits fused to the foreign protein or peptide can be modulated through minor modifications of the 2A "linker" sequence, to alter "cleavage" efficiency. This permits exploitation of different expression or purification strategies for either the CP-fused or free form of the foreign target protein, polypeptide or peptide. Numerous antigenic peptides expressed via OVERCOAT® have resulted in movement and high yields of virus. High level accumulation, lack of invertebrate vectors, no pollen or seed transmission, a wide host plant range, and possible ease of virus purification make PVX an ideal vector for this purpose.

A similar approach can be developed for other (rod-shaped) viruses. Zhang and French used BSMV to fuse *uid*A [encoding the 60 kDa *ß*-glucuronidase (GUS) from *E. coli*] or *gfp* sequences in-frame at the 5'-terminus of the CP gene, separated by the FMDV 2A auto-proteolytic peptide. GUS activity and green fluorescence were detected in infected barley protoplasts (30% and 10% of protoplasts, respectively, expressed the corresponding reporter gene) and approximately 50% of the GFP-CP fusion protein was cleaved (Zhang and French, 1997). These results are promising and may demonstrate that rod-shaped viruses in general have the ability to accommodate large CP fusions through their N-termini and an FMDV 2A linker. Further experiments on plants infected with BSMV CP-fusions will show if virus movement and yields are affected.

The use of OVERCOAT® technology has already provided new insights into the mechanism of PVX movement in plants, allowing subcellular localisation of the virus itself (Oparka et al. 1996). The OVERCOAT® technology has also been applied to another rod-shaped virus, TMV, which accumulates to higher levels than PVX in infected plants, and consequently should produce even higher yields of recombinant protein than PVX (Table 2).

Table 2 Prospects for plant virus OVERCOAT® protein technology

Vaccines	(dis)continuous or multiple B or T cell epitopes
Therapeutic proteins	cytokines, hormones, antibodies
Industrial enzymes	proteases, lipases
Phyto-bioremediation	environmental clean-up of heavy metals, pesticide residues, chlorinated hydrocarbons, waste streams
Biocatalysis	bioreactors, metabolic engineering
Added-value food crops	high lysine/methionine proteins
Crop protection	expression and screening of possible insecticidal, nematicidal, anti-viral proteins

Conclusions

It is clear that plant RNA viruses, in particular, present attractive, safe, efficient and high yielding alternatives with which to screen and develop valuable biomolecules in green plants. Viruses for which there is no seed, pollen or natural vector transmission by insects, fungi or nematodes, are clearly useful candidates from a biosafety and environmental containment perspective. Indeed over the last 3-4 years, large-scale (up to several hundred-acre) releases of genetically engineered TMV have been made into open field plots in Kentucky with no deleterious effects on adjacent commercial tobacco crops, or neighbouring weed species. In fact, the fibrous biomass and many hundreds of kilograms of recombinant virus particles are returned to the soil as compost following sap extraction at the commercial-scale facility. Extensive (USDA-monitored) trials, RT-PCR testing for TMV survival in the soil, and alert tobacco growers in adjacent farms all concur that the processes being used are biologically safe and successful.

Both isometric and helical plant virus particles, propagated in living plant tissues, have been used successfully as multivalent particulate carrier system. Peptides up to approximately 25 amino acids can be fused to 60 identical sites in the coat protein of cowpea mosaic virus, and peptides of 36 amino acids up to complete proteins of 31 kilodaltons have been attached to $\leq$ 1,500 sites (but more typically several hundred sites) on potato virus X or tobacco mosaic virus capsids. Most, if not all, these peptides and proteins continue to function specifically either as fluorophores, antigens or even single-chain antibodies, targeted to their specific epitope.

In Europe, it is probable that these recombinant RNA virus particles will be grown only under closely monitored containment conditions, despite the fact that they have no invertebrate vectors and soon revert to the parental virus. In contrast, in the USA, and elsewhere, large-scale commercial field releases are already a feature of local production agriculture, turning farms into “phactories” for the burgeoning biomedical, therapeutic and diagnostic industries. There are numerous ethical and biomedical safety advantages in developing plants and plant virus-based expression vector systems to produce new medicines, neutraceuticals etc. Not least is zero risk of contamination by mammalian pathogenic agents such as prions, HIV or hepatitis B, for example, from blood products. No plant virus causes any disease in mammals. They are a regular feature of much of our dietary intake. Moreover, as with other recombinant production systems, plants avoid the distasteful and high risk activity of extracting human biopharmaceuticals from cadavers or human placental tissues.

In short, the many advantageous features of horticultural glasshouse, or even open field crop plants as bioreactors using plant RNA viruses as transient expression vectors (particulate or non-particulate), the balance of biomedical safety, their ready availability for developing countries and their ease of application (in edible plant material) make it clear that this field of endeavour and application will grow.

The development of high-copy-number plant RNA virus vectors for foreign gene expression represents a dramatic breakthrough in plant biotechnology. Because of their versatility, high yield and ease of application, these tools may supplement or replace the conventional transgenic plant approach. It is difficult to predict whether a given virus will be useful as an autonomously replicating gene expression vector. However, from the growing information available, it seems that CPMV, PVX and TMV peptide epitope presentation vectors are the most efficient candidates for large-scale production of small peptides and, more significantly, that rod-shaped, single-stranded plus-sense RNA viruses, such as TMV and PVX, can be adapted to express larger peptides or much larger fully-functional proteins. The latter may be released or secreted as soluble moieties in or from the infected plant cell, or now may even remain attached to virus particles, creating a multivalent carrier, the so-called OVERCOAT® protein strategy.

Plant virus vectors may produce proteins with therapeutic or industrial applications that do not need to be purified from their host plants; for example in phyto-bioremediation, crop protection, metabolic pathway engineering, the nutritional improvement of food crop species, or the use of OVERCOAT® epitopes as edible vaccines (Chapman and Wilson, 1997). Plant virus-based vectors can also be used to study a wide range of basic plant functions including plant metabolism (Kumagai et al. 1993), gene *trans*-regulation (Sablowski et al. 1995) and defence mechanisms (Hammond-Kosack et al. 1995) as well as cell biology and intra-plant transport processes (Oparka et al. 1996; Boevink et al. 1996). In addition, chimaeric viruses have contributed to a better understanding of virus infection and the stability of viral genomes through *in vitro* and *in vivo* studies on plant viral protein functions.

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Validating the biosafety of genetically modified crops

Nickson, T.E., Fuchs, R.L., McKee, M.J.

Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198, USA

Abstract

It is apparent that the world's population will increase significantly in the near future resulting in greater demand for healthy, affordable food. Given that the area of available arable land will not expand significantly, new environmentally superior technologies that enable farmers to sustainably produce more food on the same amount of land must be developed. The development of genetically modified crops through biotechnology is one of several technologies now available to begin to address the world's demand for food grown in an environmentally better way. Genetically modified *Roundup Ready*® plants (soybean and canola), tolerant to *Roundup*® herbicide, have been available to farmers in North America for 3 years affording superior weed control with environmental benefits. Crops such as *Bollgard*® (*Ingard*®) cotton, *NewLeaf*® potato and *YieldGard*® corn (maize), protected from insect predation while posing negligible risks to beneficial insects, are now a commercial reality in many areas of the world. The benefits of insect protected plants to the environment are a significant reduction in the use of less selective pesticides. Prior to commercialization, these genetically modified crops were evaluated in detail to assure their biosafety. This presentation summarizes the Monsanto products currently on the market, their rapid adoption and high approval ratings in the market place, and an overview of the benefits that have been realized. In addition, a brief overview of trait and biosafety considerations for the future will be given.

The need for a new environmentally superior technology for food production

Some of the most important factors affecting the environment today are food production practices and trends in land utilization among the growing number of world inhabitants. Population projections, which vary widely, are almost irrelevant when viewed against the environmental risks resulting from the current trends in land use and some of the practices commonly used in food production. Some farms are being converted from agricultural use while in other regions of the world forests, deserts and wetlands are being converted into food production areas. Since only a small percentage (approximately 10%) of the world's land is suitable for producing food (almost all of the world's food is produced in an area about the size of North America) the balance between land utilization and food production has significant environmental consequences. The great challenge is to develop environmentally superior means to produce the food for this growing world population on the limited land resources available.

Economically developed areas of the world are seeing stable to declining population growth, have less prevalent poverty and food is relatively abundant. One trend in economically developed areas is to convert productive land from farm use into recreational or housing use. Since much of the better farm land is found in these industrialized countries, conversion of good farmland into other uses places additional burdens on the productive acreage remaining. For example, since less land is available to accommodate rotation into fallow, higher inputs of energy and chemicals are required to maintain productivity. These current trends in food production are not sustainable.

Land use and population trends are quite different in the developing areas of the world where poverty and starvation are common. Numerous reports clearly show that the population increase is greatest among the poorest people of the world. Because food production is tied to economic and social development, many developing countries are seeing forests, deserts and wetlands being converted into farming. These practices are not sustainable since they destroy valuable environmental resources which would take a long time to recover.

Several new strategies employing promising technologies are being developed to address the need for sustainable food production. A short list of these approaches includes improved plant breeding through genomics and marker-assisted breeding, development of biocontrol and new chemical agents with environmentally improved characteristics, enhanced farming techniques, as well as biotechnology. Advances in genomics (mapping genes and genetic combinations) is an exciting new area that will enhance the ability to develop new and more productive crops. Plant breeders are also using marker-

assisted breeding to facilitate the development of new varieties and reduce the time required to bring these varieties to market. Agrichemical discovery remains an area of important research. Environmentally superior crop protection agents are needed immediately to control pests and promote the growth of crops. In addition to chemical agents, biocontrol offers the opportunity for enhancing safe and efficient agricultural productivity. Important improvements in farming practices such as precision farming, conservation tillage and water management will enable farmers to be better stewards of their land. These technologies combined with advances in biotechnology like genetic engineering of plants are the foundation upon which the important improvements in food production are currently based.

Monsanto is committed to developing the techniques of genetic engineering of plants to develop products for the sustainable production of food in a manner consistent with conserving a healthy environment. New products must enable farmers to produce food in a cost effective, socially acceptable, and environmentally sound manner. In other words, to be sustainable, an agricultural product must meet the economic as well as the environmental needs of the increasing world population. The increasing concern and demand for sustainable agricultural products, particularly those derived through the techniques of modern biotechnology, has resulted in significantly more detailed assessments of the safety of new products. The following is a description of our products and the benefits they have yielded and some future needs in terms of the biosafety of these products.

Insect protection

Insecticides and associated management practices cost approximately $10 billion annually worldwide. Despite these inputs, estimates are that 20 to 30% of the total crop product is still lost due to insect pests. The microbe *Bacillus thuringiensis* (*Bt*) has long been know to produce insecticidally active proteins, and *Bt*-based products have been used safely in agriculture since 1961. They currently account for less than one percent of all pesticides used in the USA because they are expensive to produce and apply. Other drawbacks to include the need for repeated applications, inactivation by sunlight and being washed off by rain all of which have been overcome by putting these genes encoding these proteins directly in the host plant. Due to the importance of effective insect control and the availability of *Bt* proteins, insect protected genetically modified plants based on genes derived from the bacterium *Bt* were the first to be developed. Successful introduction into plants required modifying the gene to increase the level of expression of the insecticidal protein in the plant. Levels of the protein were raised as much as 100 fold compared to expression of the native bacterial gene in the crops, and were now sufficient to provide commercial protection against the targeted insect pests. Other key factors related to the biosafety of *Bt* proteins are their specificity and selectivity, due to specific receptor-mediated binding in the insect gut.

The effectiveness of this delivery system is reflected in the successful launches of insect protected potato, cotton and corn products. Monsanto's current portfolio of insect protected crops includes: *Bollgard*® Cotton, *NewLeaf*® Potatoes and *YieldGard*® Corn (Table 1).

Approximately 730 thousand hectares of genetically modified insect protected cotton varieties were grown in the USA in 1996, accounting for approximately 13% of the US cotton crop, with approximately 900 thousand hectares planted in 1997 and 1.2 million hectares projected in 1998. In addition, insect protected cotton varieties were planted in Australia and Mexico. These plants provide protection against the three major insect pests in cotton: tobacco budworm (*Heliothis virescens*), pink bollworm (*Pentinophora gossypiella*) and cotton bollworm (*Helicoverpa zea*). US cotton growers planting Bollgard significantly reduced their use of chemical insecticides. Increased effectiveness in control of these insect pests resulted in an average yield increase of 7% in cotton production while also resulting in increased numbers of beneficial insects due to increased specificity of the pesticidal protein. Despite heavy infestations of bollworm, one of the targeted insect pests, 60% of the growers needed no chemical insecticide applications to control these pests. Most growers who applied insecticides to the *Bollgard* cotton varieties used only one application compared to an average of four to six applications for the non-genetically modified cotton varieties. Chemical insecticide use was reduced by approximately 1,000,000 liters in 1996, due to the introduction of insect protected cotton varieties.

Table 1 Total area of commercialized Monsanto biotech crops (hectares in thousands)

Region **Product**	**Canada** **1996/97/98**	**Mexico** **1997/98**	**Argentina** **1997/98**	**Australia** **1997/98**	**USA** **1996/97/98**
Insect Protected					
Bollgard® Cotton		19/55			730/900/1,200+
Ingard® Cotton				60/83	
NewLeaf® Potato	0/2/?				4/10/16
YieldGard® Corn	0/10/120				300/1,000/4,050
Roundup Ready®					
Soybeans	0/1/40		0/1,400/4,000		400/3,600/10,100+
Cotton					0/320/1,000+
Canola	20/200/1,300				
Corn	0/0/1				0/0/320
YieldGard/					0/0/12
RoundupReady					
Corn					
Bollgard/Roundup					0/0/24
Ready Cotton					

Note: *Ingard*® is a product name for a Monsanto *Bt* cotton production Australia. 1998 values are projections with a total estimate of over 8M hectares worldwide.

Approximately 4,000 hectares of genetically modified *Bt* potato plants protected against Colorado potato beetle, the most damaging potato insect pest, were planted commercially in 1996 in the USA. Growers planting the genetically protected potato plants used approximately 35% less insecticide to control the Colorado potato beetle compared to growers planting non-genetically protected potato varieties. Planted acres more than doubled in 1997 (10,000 hectares) with another doubling in acres projected in 1998 (16,000 hectares).

Approximately 300,000 hectares of YieldGard corn plants protected against corn borer were planted commercially in 1997. This number increased significantly to over 4 million hectares in 1998. In addition to *YieldGard* corn, a number of additional insect protected, genetically modified corn products entered the market in 1997, grown on over 3 million hectares, and accounting for approximately 10% of the US corn crop. This area is projected to double in 1998 and over 20% of the total corn production will be genetically modified for insect protection.

Research at Monsanto continues to explore the possibility of expanding utility of *Bt* proteins in agriculture through insertion of genes from a variety of *Bacillus* species. With this diversity of *Bt* genes, many additional insect pests will be controlled and crops protected. Future research at Monsanto will continue to be based on the fact that plants expressing the *Bt* proteins have improved biosafety due to the selectively active protection against the targeted insect pests. This selectivity enables growers to maintain beneficial predatory insects in their fields that provide additional protection against insect pests and against some diseases that are transmitted by insects (e.g. viruses) in contrast to systems that use the broad spectrum insecticides. Within these improved agricultural practices, Monsanto's primary focus is to assure the biosafety and long-term durability and effectiveness of their products. Toward this end, numerous strategies have been developed and implemented to assure that these products maintain their effectiveness long term. Furthermore, these strategies are being reviewed and refined based on new information gained from our field experience.

In addition to the genes from *B. thuringiensis*, many other sources are being evaluated as potential insecticidal proteins at Monsanto. Leads include the use of cholesterol oxidase to control lepidopteran insect pests of cotton as well as cotton bollweevil (*Anthonomus grandis*), another major insect pest in cotton. These next generation genetically modified plant products have the potential to provide improved control against additional important insect pests with enhanced safety.

Roundup Ready® crops

Weeds are one of the major agricultural pests that can devastate a crop if not controlled or managed properly. Weeds compete with crops and reduce yield, decrease harvest efficiency, decrease seed quality and serve as a reservoir for crop pests. Prior to the introduction of herbicides about 50 years ago, agricultural practices were limited to labor intensive hand weeding, plowing which contributed to soil erosion, and rotation into alternative crops. Greater farming flexibility and productivity was realized with the development of chemical weed control. Today herbicides are used on almost 100% of the acreage of the major agronomic crops in industrialized countries.

Biotechnology provides an opportunity to make another improvement in weed control by modifying crops to tolerate specific herbicides. *Roundup Ready*® crops allow farmers to apply *Roundup*® herbicide, whose active ingredient is glyphosate, with its favorable environmental properties to control weeds while leaving the planted crop unaffected. The benefits to the farmer are numerous and included enhanced flexibility for weed control at a reduced cost. In addition, Roundup has favorable environment characteristics such as low toxicity, tight binding to soil and rapid soil degradation, all of which fit well in a sustainable program. Because the use of Roundup is amenable to conservation tillage practices, many farmers are using *Roundup Ready*® and conserving valuable topsoil.

Today Roundup Ready soybeans, canola (oilseed rape/colsa), cotton and corn have entered the marketplace in North America and Argentina for soybean (Table 1). Glyphosate tolerant soybeans were planted on over 1.5% of the US soybean acres in 1996 by over 10,000 growers. Because of exceptional weed control and crop safety, these soybean varieties yielded an average 5% more per hectare than soybean fields treated with conventional herbicide programs. Approximately 75% of the growers used only one herbicide application, providing a significant reduction in the amount and number of herbicides used compared to soybeans treated with conventional herbicide programs. In 1997, approximately 15% of the US soybean hectares were planted with glyphosate tolerant soybeans. Over 10 million hectares of glyphosate tolerant soybeans are projected for 1998, accounting for over 30% of the US soybean production. Approximately 1.4 million hectares of glyphosate tolerant soybeans were grown in Argentina in 1997, with significant increases projected in 1998. Farmers in Canada are also producing *Roundup Ready* soybeans.

Roundup Ready technology is rapidly being adopted in canola, cotton and corn production (Table 1). Use of Roundup® herbicide over the top of canola in Canada has increased from its first year of introduction in 1996 (approx. 20,000 hectares) to well over 1 million hectares in 1998. Canadian farmers have reported yield benefits and cost savings. Likewise with *Roundup Ready*® cotton, farmer satisfaction has been high (approx. 90%) and the acreage is growing rapidly. Limited acreage of *Roundup Ready* corn was planted in the USA and Canada in 1998, 320,000 and 1,000 hectares respectively.

Monsanto scientists are currently assessing additional crop production systems where the *Roundup Ready* technology will provide benefits to farmers and the environment. Each potential new use of glyphosate tolerance technology is thoroughly studied for its potential impact in agricultural systems and the environment. Our focus is to provide an efficacious, long-lasting, weed control program that will serve farmers and the environment through improved economics and biosafety.

Traits and biosafety considerations for the future

Numerous traits introduced into crops through biotechnology will be available from Monsanto in the near future in addition to those currently in the market. One example is viral-derived genes to provide effective control of viruses in potato. Resistance to the most economically important viral disease in potato, potato leafroll virus (PLRV), was achieved by introducing a gene involved in viral replication. Antifungal traits will also be important for improving the sustainability and biosafety of food production. Recent efforts in several labs have focused on isolating and expressing in plants more effective hydrolytic enzymes (e.g., chitinases and glucanases) that degrade fungal cell walls, thereby increasing their fungal resistance. It appears that there remains a need for more potent enzymes to further enhance anti-fungal activity. Expression of plant defensins or other proteins induced upon fungal infection are especially promising. These products have enormous potential to reduce the volume of insecticides (to control aphids that vector viruses) and fungicides applied to crops. Savings will also be realized in reduction of fossil fuels used to make frequent applications of these chemical pesticides.

The agronomic traits (herbicide, insect, virus, and fungal tolerance) can be considered the first wave of biotech products. Furthermore, commercial experience to date with herbicide, virus and insect protection has clearly demonstrated environmental benefits without increased biosafety concerns. All available information have shown that the modified plants are not altered in their weediness potential. Secondly, the introduced trait affords no significant competitive advantage in the environment or toxic effect when compared with practices currently used in agricultural systems.

The second wave of biotech products are becoming available including quality traits and utilizing plants to produce key materials on a large scale (biofactories). Many of the product concepts have been proven to some degree in products like the delayed ripening tomatoes, soybeans and oilseed rape with modified oil compositions, potatoes and tomatoes with improved processing characteristics. Others are still in the research phase like sugarbeet and sugarcane with increased sucrose content, and crops with modified protein and amino acid levels or expressing pharmaceutically important proteins. Each trait, modified plant and novel protein will have to be evaluated for its biosafety using a science-based risk assessment procedure. Monsanto has continued to develop and improve upon the methods originally formulated through scientific organizations like OECD. Information obtained using this model will be appropriate for submission under any global environmental regulatory requirements (e.g. 90/220). Our risk assessment model is designed as an iterative approach involving problem formulation, data collection and analysis, risk characterization, and risk management (Figure 1).

The first step in conducting a risk assessment is to formulate the problem using information available in the literature about the biology, weediness potential, use and agronomic characteristics of the plant that has been genetically modified. With over 25,000 field releases occurring to date, the experience and information gained from these releases as well as other data from the literature serve as the basis for determining the important measurements that must be taken and the accepted range of experimental results. During problem formulation, careful analysis is also given to the nature of the trait, its potential to confer a selective advantage and to produce harmful effects to nontarget species. The potential for enhanced toxicity of the modified plant must be assessed by understanding the acceptable levels of known toxicants (e.g. glucosinolates in oilseed rape). An appropriate strategy also takes into account secondary genetic effects such as gene instability and pleiotropic effects. For crops expressing agronomic traits, genetic instability would be clearly evident in a loss or sudden change in the plants phenotype. Also, all the gene products including the marker proteins used to produce the modified crop must be evaluated for their biosafety.

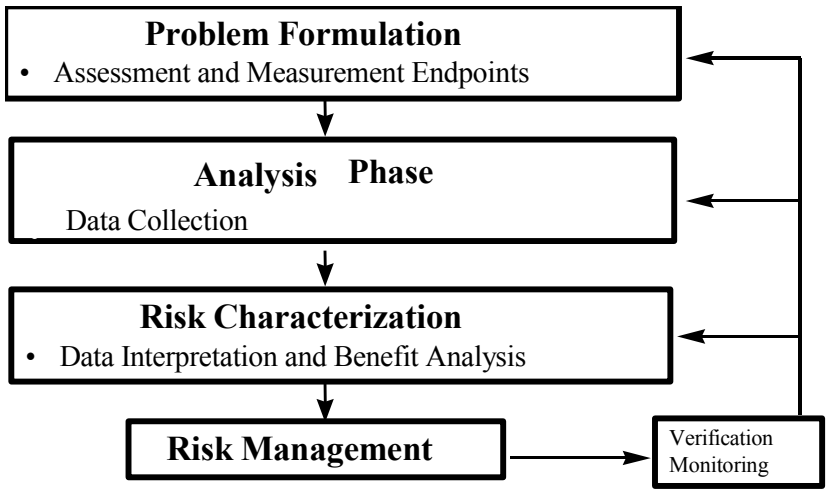

Figure 1 Environmental Risk Assessment Model

The second phase of the risk assessment, is the data collection and analysis phase where experimental protocols are developed and implemented to address the critical, "need-to-know" factors affecting risk (hazard x exposure). Based on the factors considered during the problem formulation phase and the experimental designs developed, endpoints are selected which will provide the necessary information to characterize risk to the environment. Experimental endpoints must be carefully and scientifically chosen to reduce uncertainty in the characterization of the risk. The analysis process should be repeated when new information and knowledge is gained from experimental results obtained during risk analysis.

In the third phase of the risk assessment process, all experimental data and observational information are integrated into a risk characterization. The data are reviewed to assess whether the modified plant has not been changed in any substantive way in terms of its impact on the environment allowing for the presence of the novel trait. We have termed this conclusion as Biological Equivalence which is similar to the concept of substantial equivalence developed for foods and feeds. Furthermore, the trait is assessed separately for its potential ecological and environmental impact. This is an important aspect of risk assessment as it accounts for the consequences of outcrossing of the novel trait. Lastly, the potential toxicity of the introduced protein(s) toward nontarget organisms (e.g. beneficials) is characterized. If the experimental data confirm that the plant is unchanged in its ecological and environmental properties (allowing for the presence of the novel trait which is assessed separately), it can be concluded that the modified plant is as safe as the traditional plant. Depending on the characterization of the risk from the scientific data, appropriate risk management actions are determined.

The philosophical basis for risk management at Monsanto is founded in product stewardship. Examples of risk management for our current products are resistance management programs, academic collaborations, customer satisfaction surveys and our quality control program. Risk management and other aspects of the risk assessment procedure are modified based on new information, and the process is repeated for as long as the product is commercially available.

Conclusions

Biotechnology is one of several important tools being developed to enhance the ability to produce food sustainably. Farmers and the environment in many parts of the world are currently realizing the benefits of using crops developed through biotechnology. Each Monsanto product has undergone a thorough science-based risk assessment to determine the biosafety of the plant, the novel trait and the introduced protein, and where appropriate risk management programs have been designed and implemented. All the information collected to date have demonstrated that the risks of the new crops are manageable and that they provide benefits compared to other agricultural systems in use. Furthermore, products that are developed under a philosophy of stewardship, where the safety and benefits of the product are weighed against all the risks present, will meet the requirements of the sustainability.

Pragmatic approach to farmer release of transgenic rice in Africa

Hull, R.

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

Summary

The world population is estimated to double in the next fifty years and much of this increase will be in developing countries. For food sustainability crop production in those countries would have to at least double in that time. One of the contributors to this increase will be genetic modification of local crops to reduce losses due to pests and diseases. However, it is likely that the public, rather than the private, sector will have to play the dominant role in the production and distribution of transgenic crops in poor developing countries, especially in Africa. This paper explores the linking public sector institutions in developed countries with those in developing countries to develop an uptake pathway for rice transgenically protected against rice yellow mottle virus right through to the resource-poor farmer in Africa.

Introduction

Most of the discussion on the uptake of transgenic crops is focussed on developed countries and transgenic lines produced by national and multinational companies. However, the major impact of the genetic manipulation technology on food security will be in developing countries where the crops are produced on a subsistence and/or local cash crop basis. The world population is expected to double by the year 2050 and most of this increase will take place in developing countries, especially Africa. From Table 1, which shows the population predictions in certain African countries (those relevant to this paper) over the next 50 years, it can be seen that populations in some countries will triple or even quadruple. Many of these developing countries, especially in Africa, have low per capita gross national product (Table 1) and much of their populations is classed as poor (<$1/day).

Table 1 Population predictions for selected African countries

Country	Population (x 1000)[a]	Predicted population increase[b]		World per capita GNP position[c]
	1998	2020	2050	1996
Southern Africa				
Madagascar	14,463	+1.80x	+3.34x	142
Malawi	9,840	+1.22x	+1.72x	151
Mozambique	18,641	+1.63x	+2.56x	157
East Africa				
Kenya	28,337	+1.20x	+1.55x	133
Tanzania	30,609	+1.53x	+2.50x	152
West Africa				
Burkino Faso	11,266	+1.71x	+3.10x	146
Côte d'Ivoire	15,446	+1.64x	+2.88x	107
Ghana	18,497	+1.43x	+1.86x	127
Mali	10,109	+1.95x	+4.00x	144
Niger	9,672	+1.86x	+3.50x	148
Nigeria	110,532	+1.66x	+3.05x	144

[a] Source: US Bureau of the Census; [b] Proportional increase (+) or decrease (-) over 1998 figure; [c] Source: World Bank. Out of 157 countries; position >103 with per capita GNP of <$750 and regarded as low income.

It is estimated that about one third of crop production is lost to diseases, pests and weeds, characters against which single transgenes can be and have been successfully deployed. Thus, genetic transformation could play a major role in narrowing the gap of food supplies. However, the current economic structures and farming systems are not conducive to the commercial introduction of transgenic crops. Most of the attempts to improve agriculture are through the efforts of public sector governmental and non-governmental organisations. These organisations do not have the capabilities or facilities for developing transgenic crops but, in many countries there is the infrastructure for introducing and transferring new crop varieties to the resource-poor farmer.

The Plant Science Programme (PSP) of the UK Department for International Development (DFID) is funding research on the use of transgenes to control rice yellow mottle sobemovirus (RYMV). The aim of this project is, not only to produce transgenic lines of rice in an advanced UK laboratory but also, to eventually transfer promising lines to resource-poor farmers in Africa. This paper reports on a study of the possible pathways to effect such a transfer which could be a model for the wider use of this approach to increasing crop yields.

Rice yellow mottle virus

RYMV was first described in Kenya (Bakker, 1970) and in West Africa in 1973. Since then it appears to be spreading rapidly in lowland rice in several parts of sub-Sahelian Africa (Figure 1) but has not been reported from outside that continent. The disease occurs as sporadic, and often severe outbreaks especially in irrigated rice, a production system which is on the increase.

Figure 1 Distribution of RYMV in Africa

RYMV causes yellow mottling of the leaves of infected plants and stunting of plants which form few tillers and no panicles. Infection of seedlings of many varieties leads to plant death and early infection can result in the total loss of a crop. The virus is transmitted by chrysomelid beetles and is easily mechanically transmitted which probably leads to spread during transplantation. However, there is lack of detailed understanding of the epidemiology of this virus.

There is some evidence for variation of RYMV over its distribution. Various serological differences have been reported between isolates from different countries (Mansour and Baillis, 1994; Konate et al. 1997).

The main means of virus control is by insecticide spraying against the beetle vector. The locally grown rice varieties in most parts of Africa are highly susceptible to the virus as are most other Indica varieties. There is some resistance or tolerance in Japonica varieties and resistance in *Oryza glaberrima*. However,

it has proved very difficult to introgress this resistance or tolerance into locally acceptable rice cultivars by conventional breeding techniques. There are numerous examples of the use of virus-derived transgenes giving protection against the donor virus in a wide range of crops. This approach depends upon detailed knowledge of the viral genome and an efficient transformation system for the crop.

RYMV belongs to the sobemovirus group having isometric particles of about 25 nm diameter containing a genome of a single (+)-strand RNA species. The genome has been sequenced (Ngon A Yassi et al. 1994) and its genome organisation determined (Figure 2). Biolistic transformation of rice is now relatively easy and is cultivar independent (Christou et al. 1991). This latter feature is important in that there is considerable conservatism in the acceptability of rice varieties in African and other developing countries and thus popular varieties can be transformed to protect against the virus.

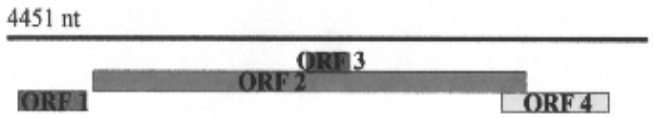

Figure 2 Genome organisation of RYMV. The top single line represents the single-strand RNA genome and the shorter single line the subgenomic RNA. The boxes indicate the four open reading frames (ORFs). ORF 1 is thought to encode the cell-to-cell movement protein, ORF 2 the polymerase and other proteins involved in viral replication and ORF 4 the viral coat protein; the function of ORF 3 is unknown. Based on Ngon A Yassi et al. (1994)

In the DFID PSP project, the ORF2 sequence of a Nigerian isolate of RYMV has been transformed into several West African rice varieties and at least two of these lines of the widely grown Bouaké-189 variety show resistance to the virus. As the RYMV transcript and gene product cannot be detected in these lines it is considered that the resistance is due to a gene suppression mechanism (Pinto et al. 1998).

The study

Uptake pathways for transgenic crops are well defined in many developed countries and, among other factors, commercial pressures are refining the procedures. The issues have been extensively discussed and, in an increasing number of these countries, the use of transgenic crops is becoming widely accepted. In contrast to this, in many developing countries, and especially in the very resource-poor countries, there has been little or no consideration of the use of transformation technology to help overcome current or looming food security problems. This is due to the lack of understanding by senior decision makers of the potential benefits of this technology and also the lack of trained scientists who could inform these decision makers and educate the farmers and public. There are, however, some developing countries which could effect transgenic releases with relatively little few further inputs and which could act as examples to show neighbouring countries the potential of this approach. Thus, in identifying target countries for the possible release of the RYMV-protected transgenic rice lines a pragmatic approach had to be taken to allow for various factors which could affect the smooth uptake of the product. In conducting this study three questions were addressed.

1. The virus. Is it present in the country and if so, what is its importance and economic impact? Is there any evidence for variation of the virus?
2. Biosafety. Does the country have a functional biosafety regulatory structure or, if not, are there moves towards establishing one? Are the local people capable of implementing it?
3. Uptake of new cultivars. What is the structure for uptake of local cultivars? How efficiently do new cultivars reach the small subsistence farmer?

For the purposes of this survey, Africa was divided into three regions, Southern Africa, East Africa and West Africa.

Southern Africa

Madagascar is the major rice producer in Southern Africa with 1.2 million ha under production, the only other significant producer being Mozambique. RYMV was first reported in Madagascar in 1989 (Reckhaus and Randrianangaly, 1990) and is found in most rice growing areas of the country. It is most prevalent in the important producing lowland areas of the north and north west (Reckhaus and andriamasintseheno, 1997) where up to 30% of the crop may be infected in the wet season and is increasing in the south. Outbreaks of RYMV in the north are causing farmers to move from the irrigated lowland areas to adjacent forested upland sites which they open up by slash and burn. RYMV has recently been recognised in Mozambique but its significance is not yet known.

There are no biosafety structures adopted yet by Madagascar but their close association with France and the recent recognition by the Ministries of Science, of Research Applied to Development and of the Environment that such guidelines might be needed are leading to moves to adopt such regulations. However, there is a lack of trained local people who could implement controlled field releases but the cooperative programme between FOFIFA (National Center for Applied Research for Rural Development) and IRRI might help to overcome this problem.

There is a working system on the uptake of new cultivars derived from the FOFIFA-IRRI project which would be suitable for acceptable transgenic lines. This system involves a three- to five-year multilocational testing procedure followed by a three- to five-year farmer evaluation leading to general release. Farmer evaluation is important as it leads to a demand-led uptake of useful new varieties.

East Africa

Tanzania is the major rice producer in East Africa accounting for 75% of the production in the area; much of the rest is produced in Uganda, Malawi and Kenya. Rice production in Tanzania is mainly in the lowland (wetland) ecosystem and there is intense rice growing on the islands of Zanzibar and Pemba. Rice production in Kenya is mainly around Lake Victoria but there are recent extensive irrigated rice schemes in Central and Coastal Provinces.

There are only scattered references to RYMV in Tanzania but recent surveys are revealing that the virus may be causing serious problems in some areas. Although RYMV was first described from Kenya it is currently not regarded as important. However, the very recent increase in irrigated rice schemes indicates that there is the potential for serious outbreaks. There are limited records of RYMV in Malawi and none in Uganda; the virus has also been reported from Rwanda.

Kenya adopted Biosafety Regulatory guidelines in 1995 but these have not yet been implemented due to indecision as to who has the final responsibility for granting permission for initial field releases. There are no biosafety guidelines in Tanzania although very recently there have been some moves towards considering them. Kenya has the infrastructure and personnel who could undertake initial field releases of transgenic crops and also has the seed distribution system for getting useful lines to the poor farmers. Tanzania, at present does not have an infrastructure suitable for either controlled field releases or for widespread seed distribution.

Thus, in East Africa Tanzania has the virus problem but not the immediate possibility for suitable pathways for the uptake of transgenic plants whereas Kenya which has the pathways does not have an immediate problem with the virus.

West Africa

West Africa is the most important rice growing region in Africa producing more than 60% of the total annual output. About 50% of the rice production in this region is in Nigeria with Côte d'Ivoire, Guinea, Mali and Sierra Leone being also important producers. There is also increasing production in Burkino Faso and Niger with new irrigation schemes. The West African Rice Development Association (WARDA), which is an autonomous intergovernmental association, has it headquarters and main research facilities in Côte d'Ivoire. Its mandate, which currently covers most rice producing countries in West Africa, is to strengthen the region's capability in rice production science; it is likely to soon expand its geographic focus to other rice producing countries in Africa.

RYMV has been reported from most countries in West Africa and probably occurs even in those countries for which there are no reports. It is of major importance in Burkino Faso, Côte d'Ivoire, Mali and Niger, especially in the new irrigation scheme areas. RYMV is currently less significant in Nigeria and Ghana. Overall WARDA estimates RYMV to be the second most important constraint on irrigated rice production in the region and the third most important for lowland rice.

Biosafety regulations were adopted by Nigeria in 1994 but, as with Kenya, have not yet been fully adopted as the National Biosafety Committee has not been convened. Côte d'Ivoire and Ghana are close to adopting biosafety regulations. None of the other countries in this region appear to be making moves to set up a regulatory structure for transgenic organisms. Nigeria has the infrastructure and personnel in both national and international institutes (International Institute for Tropical Agriculture) to conduct controlled field releases and WARDA in Côte d'Ivoire will soon be able to undertake such experiments. In both Nigeria and Côte d'Ivoire seed distribution to resource-poor farmers is reasonably well organised.

Conclusions

RYMV is of current importance, especially in Madagascar, Tanzania and some West African countries. As attempts to improve food production in these and other countries by upgrading agriculture, and especially in the adoption of irrigated rice schemes, it is likely that the virus will be of increasing significance. Thus, it is important that means of controlling the disease be found and adopted as soon as possible and the use of transgenic protection has great promise but raises various biosafety issues. These are primarily at the controlled release level as various local potential risks have to be addressed before farmer release can be considered.

Because of the economic and social structures in these countries it is unlikely that the large agrochemical and seed companies will be involved in supplying the resource-poor farmers with transgenic seed. Furthermore, these countries do not have the capabilities of producing transgenic crops themselves. The best route to these farmers would appear to be through the public sector and this study has explored possible routes linking an advanced laboratory in a developed country to the seed distribution systems in the developing countries. The most important features of this route are a realistic approach to risk assessment based on standards generally acceptable to other countries, regionalisation of biosafety regulations to enable smooth and legal transfer of promising lines between adjacent countries, the transformation of varieties acceptable to the consumers in the target countries and farmer evaluation of potentially useful lines which would result in demand-led uptake. This uptake system involved close and long-term collaboration between the advanced laboratory and institutions in the developing countries. It is only during field evaluation of promising lines that the durability of the resistance will become fully tested and any shortcomings in this would have to be overcome by the advanced laboratory. Thus, if strains of RYMV were found to overcome the transgenic protection new constructs based on these would be made and added to the transgenes in the rice lines.

Because of its central position in rice research in West Africa, WARDA is well placed to assist interested mandated countries in adopting biosafety regulatory structures. At an international meeting in Abidjan in 1996 it was suggested that WARDA coordinated biosafety issues in West and Central Africa (Mulongoy and Tacchini, 1996). The current suggestion is that after Côte d'Ivoire has adopted and implemented its national biosafety regulations leading to the first field releases there will be strong moves towards regionalisation of biosafety regulations so that transgenic crops effective in one country can be tested in other countries with the minimum of regulatory bureaucracy.

Acknowledgements

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Recent experience with commercialized genetically modified crops

Tabei, Y.

Innovative Technology Division, Agriculture, Forestry and Fisheries Research Council Secretariat
Ministry of Agriculture, Forestry and Fisheries, Japan

Introduction

Transformation technology has a high potential to develop novel varieties. Many companies and institutes in the world have already developed many genetically modified (GM) crops having herbicide resistant, insect resistant genes and other useful traits. Until 5 December 1997, the Committee of Food Sanitation in the Ministry of Health and Welfare (MHW) in Japan established that 20 GM crops were safe as food (Table 1). They had been tested in accordance with the "Guideline for the Safety Assessment of Food and Food Additives Produced by Recombinant DNA Techniques". Accordingly, these GM crops have passed all regulatory requirements and can be safely introduced into the Japanese market. In Japan, some public institutes and private companies are also investigating to develop and commercialize new varieties. I will describe novel GM crops developed by gene engineering and the procedure for risk assessment for environment, food and feed in Japan.

Table 1 Current status of commercialization of transgenic crop plants in Japan

Organism/Phenotype	Company	Purpose
1. Herbicide tolerant Soybean (40-3-2)	Monsanto	Food, Feed
2. Herbicide tolerant Canola (HCN92)	AgrEvo	Food, Feed
3. Herbicide tolerant Canola (PGS1)	Plant Genetic Systems	Food, Feed
4. Herbicide tolerant Canola (GT73)	Monsanto	Food, Feed
5. Lepidopteran resistant Corn (Event176)	Ciba-Geigy	Food, Feed
6. Lepidopteran resistant Corn (Bt11)	Northrup King	Food, Feed
7. Coleopteran resistant Potato (New Leaf Potato)	Monsanto	Food
8. Lepidopteran resistant Corn (Yield Gard Corn: MON810)	Monsanto	Food, Feed
9. Coleopteran resistant Potato (New Leaf Potato)	Monsanto	Food
10. Lepidopteran resistant Cotton (Ingard Cotton)	Monsanto	Food, Feed
11. Herbicide tolerant Corn (T14,T25)	AgrEvo	Food, Feed
12. Herbicide tolerant Hybrid Canola (PHY14,PHY35)	Plant Genetic Systems	Food, Feed
13. Herbicide tolerant Canola (PGS2)	Plant Genetic Systems	Food, Feed
14. Herbicide tolerant Hybrid Canola (PHY36)	Plant Genetic Systems	Food, Feed
15. Herbicide tolerant Canola (T45)	AgrEvo	Food, Feed
16. Herbicide tolerant Cotton (Roundup Ready Cotton)	Monsanto	Food
17. Herbicide tolerant Cotton (BXN Cotton)	Calgene	Food
18. Herbicide tolerant Canola (MS8RF3)	Plant Genetic Systems	Food, Feed
19. Herbicide tolerant Canola (HCN10)	AgrEvo	Food, Feed
20. Ripening delayed Tomato	Calgene	Food

Risk assessment procedure in Japan

Risk assessment with respect to the influence on the environment

Environmental risk is evaluated through four steps, involving a closed greenhouse, a semi-closed greenhouse, an isolated field and open field. Risk assessments at a closed greenhouse and semi-closed greenhouse are performed as an experimental phase and conducted by Science and Technology Agency (STA). Risk assessments at an isolated field and open fields conducted by Ministry of Agriculture, Forestry and Fisheries (MAFF) are carried out later on a commercial basis. If safety of GM crops to the environment were confirmed through these steps, the transgenic crops can be cultivated under completely the same conditions as ordinary crops.

Safety assessment of food

MHW is responsible for the risk assessment of GM food. The Committee of Food Sanitation under MHW established the essential guidelines. The Committee assesses the safety of GM food exclusively on the basis of the data provided by the applicants. Thus all data submitted to the Committee were compiled in the country of their origin. The Committee can request additional or complementary data if the first data submitted cannot prove the food safety.

Safety assessment of feed

The Livestock Industry Bureau in MAFF organized the Agricultural Material Council for the risk assessment of GM crops as feed. The Committee assesses feed safety according to the "Guideline for Safety Assessment of Feed Produced by the Recombinant DNA Techniques". Most of the items that are tested are basically similar to the procedures for food safety assessment.

According to the guideline for environment risk assessment, as in July 1998, the Minister of MAFF issued approval to 89 applications for field trials to be conducted at an isolated field, and to 34 applications including 51 transgenic lines for non-regulated cultivation. Eighteen transgenic crops out of 34 applications have already been confirmed safe for food and 22 out of 34 were confirmed safe as feed until January 1998.

Field release and commercialization of GM crops

Blue color carnation

Suntory Ltd. and Froligen Ltd. jointly worked on the development of blue color carnation and introduced Flavonoid 3',5' hydroxylase (F3'5H) and Dihydrofravonol-4-reductase (DFR) gene into carnation from October 1995. F3'5H added a new metabolic pathway for the production of dihydromysetin, and introduced DFR catalyzes from dihydromysetin to blue pigment, Delphinidin-3-glucoside. As a result of the integration of the new two genes, blue colored of carnation was obtained in July 1996. The field trial was carried out in an isolated field in 1996, and confirmed its environmental safety compared to non-transgenic carnation in 1997. All requirements for risk assessment have already been completed in GM carnation, and a new blue color carnation named "Moondust" has been sold since last November in Japan as first transgenic floricultural crop. Suntory Ltd. is developing darker color carnation, and it will be evaluated in an isolated field from October 1998 and will be marketed soon.

Virus resistant tomato, melon and rice

Several national institutes also developed useful transgenic crops. These include virus resistant tomato, melon and rice which were developed by introducing viral coat protein gene. For these transgenic crops their environmental biosafety was already confirmed, and they can be cultivated in ordinary fields now. These crops, especially transgenic tomatoes, are used as breeding material because genetic resources of CMV resistance were not found in tomato yet.

Insect resistant azuki bean

The larvae of most bruchid beetles feed and develop on a limited number of legume species. Some bruchids are obviously economically important because they develop on grain legumes and cause serious postharvest damage. Azuki bean (*Vigna angularis*) is an important grain legume in East Asia,

particularly in Japan. Its cultivation and production is severely hampered by the widely distributed azuki bean weevil (*Callosobruchus chinensis*) which inhabits most of Japan, except its northern part, Hokkaido. The larvae of this bruchid and the cowpea weevil attack seeds of cowpea, mungbean and sometimes soybean, in addition to azuki bean. These larvae can be raised experimentally on nonhost species such as pea and broad bean, but not on common bean. Secondary compounds present in the common bean seeds including saponins, heteropolysaccharides and lectin play a protective role against bruchid pests. These compounds as such are not individually responsible for bruchid resistance, because they do not inhibit larval development at the same concentrations as those found in common bean seeds. Ishimoto et al. (1988) purified and identified a proteinous α-amylase inhibitor (αAI) as a bruchid-resistance factor, and introduced the gene encoding αAI into azuki beans to enhance bruchid resistance (Ishimoto et al. 1996).

The risk of transgenic azuki bean plants was evaluated in a closed and semi-closed greenhouse from June 1996 to December 1997. Subsequently, the transgenic azuki beans were evaluated for their influence on the environment in an isolated field from May 1998 to December 1998.

Powdery mildew resistant strawberry

Strawberry cv. Toyonoka is a main variety in Japan, but it is susceptible to powdery mildew, *Spaerotheca humuli*. To develop novel powdery mildew resistant strawberry, a group of researchers at Nara prefecture tried to introduce chitinase gene isolated from rice into the strawberry plants (Asao et al. 1997). Leaf discs (4,000 explants) and petioles (4,128 explants) were co-cultivated with *Agrobacterium tumefaciens* carrying the modified pBI121 having a rice chitinase gene, and were screened for transformation by 50 mg/l kanamycin resistance. In total 544 explants developed kanamycin resistant calli and 123 calli out of 544 calli formed green adventitious shoots within 10 weeks after transfer to shoot induction medium. Introduced gene was confirmed by polymerase chain reaction. Powdery mildew resistance was examined by artificial inoculation of spores of *S. humuli* (10^5 spores/ml). Disease development on the surface of the leaves was measured and the percentage of colony area to total leaf area was calculated 30 days after inoculation. When the transformed strawberries were infected with *S. humuli*, disease development was substantially reduced, and the development of transformants to that of non-transformants was compared. The average colony area to total area of leaf was 22.0±2.5% (2.5-53.2%) in transgenic strawberry plant, although that of non-transgenic strawberry plants was 40.0±2.7% (15.5-64.7%).

Transgenic strawberry plants were cultivated in a closed greenhouse, and their influence on the environment was evaluated from November 1994 to April 1996. Moreover, they were also evaluated for their risk to the environment in a semi-closed greenhouse from November 1996 to April 1998. During cultivation in a closed and semi-closed greenhouse, a highest resistant strawberry line was also selected, and the resistant line will be evaluated for its risk to the environment in an isolated field from November 1998.

Herbicide resistant rice

Public institute of Iwate prefecture developed herbicide tolerant rice having the *bar* gene that gives herbicide (gluphosinate) tolerance. The herbicide tolerant traits in rice make it possible to lower the quantity of herbicide applied, and avoid the plowing of field thereby reducing the labor and cultivation expenses. The bar gene driven by ubiquitin promoter was introduced into protoplasts from rice variety "Kakehasi" by electroporation. A total of 11 herbicide tolerant rice plants was recovered and *bar* PCR and Southern-hybridization detected gene from all regenerated rice. During cultivation of the transgenic rice plants in a closed and semi-closed greenhouse, inheritance of introduced gene and stability of herbicide tolerance also was assessed with other risks.

Transgenic herbicide tolerant rice plants were cultivated in a closed and semi-closed greenhouse to evaluate influence on the environment from May 1996 to September 1997. The environmental risk assessment in an isolated field is carried out from May 1998 to September 1998.

Gray mold resistant cucumber

Rice chitinase gene was introduced into this cucumber. Some transgenic cucumber lines exhibited high resistance to Gray mold (Tabei et al. 1998), and a field trial of these cucumber lines in an isolated field was started in May 1998. Details of environmental risk assessment are described in the poster session.

Conclusion

GM crops commercialized in Japan were developed in foreign countries, and no GM crops developed in Japan were commercialized except blue color carnation because there are some consumer and/or environmentalist organizations which excessively object to utilize GMO and are agitating consumers not to use GMO. Most Japanese companies are cautiously developing and commercializing GMO. However, the Society for Techno-Innovation of Agriculture, Forestry and Fisheries (STAFF) carried out a questionnaire for better understanding of consumer's impression of GMO in November 1997. The questionnaire results informed that 60% of the respondents (657 persons) judged that GM crops will be useful for their better life, while 7% of the respondents think GMO will not be useful. Moreover, more than 60% of the respondents have no objections to buying GM crops cultivated by no or less agricultural chemicals than ordinary crops. Next, more than 50% of the respondents support the purchase of GMO if they represent the more nutritious food, more delicious and cheaper than ordinary crops. On the other hand, over 90% of the respondents wish to have detailed information related to food safety, and about 70% of them are interested in its influence on the environment.

This questionnaire reveals that many consumers would rather purchase GMO with desirable traits than rejecting GMO and/or processed food derived from GMO. These results seem to reflect typical impressions, anxiety and requests of consumers concerning the utilization of GM crops. Consequently, both the development of novel GMO that the consumers wish, and the transmission of accurate information seem to be essential for GMOs' acceptance by Japanese consumers.

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Field release and commercialization of transgenic organisms in China

Zhangliang, C.

National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871, China

In the past few decades, considerable research advances have been made in the field of genetic engineering of agriculturally important crops, animals, and microorganisms in China. The aims of genetic manipulation include introduction of resistance genes into transgenic plants against virus, fungi and bacterial pathogens, insects, and improved protein or fiber quality, and other characteristics such as modified microorganisms for nitrogen fixation with high efficiency, as well as transgenic fish, pig and sheep with a high growth rate. New varieties produced with this non-conventional technique are moving through the small field test toward large-scale releases and commercialization. China is one of the world's major agricultural countries with a huge population, and this technique has already been explored upon helping Chinese farmers for increasing crop production and decreasing yield losses caused by disease and insects.

Since 1988 scientists in China have obtained this technique and produced many transgenic plants for various purposes. Since the Administration Regulation on Genetic Engineering was taken into action in 1996, 86 applications either for field releases or commercialization of transgenic organisms have been reviewed and 70 of them have been approved. Among these 70 approved applications, 6 of them are for commercialization, and the rest of them are for field releases. The species of genetically engineered crops included rice, wheat, maize, cotton, soybean, potato, tomato, sweet pepper, pepper, tobacco, petunia, and poplar tree (Tables 1 and 2). Transgenic TMV and CMV resistance tobacco plants and transgenic cotton plants with modified *Bt* toxin gene have been tested in several locations in China with more than thousand hectares. The transgenic cotton with modified *Bt* toxin gene was developed by Chinese scientists. The transgenic cotton with *Bt* toxin gene developed by Monsanto company was also commercialized in China (Table 1). The genetically modified microorganisms for enhancing nitrogen fixation and transgenic fish for big size were developed (Tables 3 and 4). The genetically modified microorganisms for enhancing nitrogen fixation were released in field tests.

Table 1 Approved commercialization of transgenic plants in China (up to May 1998)

Plants	Traits	Institutions
Tomato	Antisense ACC	Central China Agri. Uni.
Tomato	CMV CP	Peking University
Sweet pepper	CMV CP	Peking University
Petunia	CHSA	Peking University
Cotton	*Bt* toxin	Biotech. Center, Agri. Acad. Sciences
Cotton	*Bt* toxin	Monsanto Company

Table 2 Approved field releases of transgenic plants in China (up to May 1998)

Rice	RDV S8	Peking University
Rice	Trypsin Inhibitor	Peking University
Rice	Anti-bacterial blight	China Rice Research Institute
Rice	*Bt* toxin	China Rice Research Institute
Rice	CPTI	Institute of Genetics, CAS
Cotton	CPTI	Institute of Genetics, CAS
Tobacco	TMV CP	Peking University
Tobacco	CMV CP	Peking University
Tobacco	CPTI	Institute of Genetics, CAS
Potato	PVY CP	Institute of Microbiology, CAS
Potato	10kDa Zein	Peking University
Potato	IPT	Peking University
Potato	Xa21	Biotech. Center, Agri. Acad. Sci.
Potato	PSTV Ribozyme	Institute of Microbiology, CAS
Wheat	HMW	Beijing Agri. Acad. Sci.
Wheat	PPT	Beijing Agri. Acad. Sci.
Maize	*Bt* toxin	Monsanto Company
Soybean	Barnase	Agri. Acad. Sci.
Poplar	*Bt* toxin	Institute of Microbiology, CAS
Papaya	PRSV CP	Tropical Agr. Inst.

Table 3 Approved field release of genetic modified microorganisms in China (up to May 1998)

Organisms	Traits	Institution
Nitrogen Fixation Bacterial NG 13	nifA/hup	Institute of Plant Physiology, CAS
Nitrogen Fixation Bacterial Ac 1541	ntrC/nifA	Institute of Plant Physiology, CAS

Table 4 Approved release of transgenic fish in China (up to May 1998)

Species	Traits	Institution
Carp	Growth hormone	Wuhan Hydrobiology Institute, Chinese Academy Sciences

Agricultural biotechnology in Mexico: Release, research and commercialization of GMOs

Alvarez-Morales, A.

CINVESTAV/IPN, U. Irapuato, Department of Plant Genetic Engineering,
Apdo. Post. 629 Irapuato, Gto., 36500 Mexico

Abstract

During the period 1988-1998, Mexico has approved 117 proposals for field trials that include 12 different transgenic crops. The most widely tested crops were maize, tomato, cotton, soybean and potato, the most commonly tested traits being insect resistance, delayed ripening, herbicide tolerance and virus resistance.

Research in Mexico is very limited and in fact there are only three places concerned with the development and testing of these materials: Cinvestav, a federally funded research institution, the International Center for Maize and Wheat Improvement (CIMMYT), and a large Mexican biotechnology company, Grupo Pulsar.

In terms of commercialization, only the FLAVR SAVR tomato of CALGENE and ZENECA have been totally deregulated and thus can be grown and sold in Mexico without any restriction. *Bt* potato and rape-seed modified in its oil content can be imported and consumed, as they have been approved by the Secretary of Health, but are not yet allowed to be freely grown in Mexico.

Field releases have not provided much data concerning the biosafety issues as they are mostly intended to assess agronomic performance, and therefore there is a serious gap of information regarding the possible effect of the release of transgenic maize in Mexico and its effect on the wild teocintle population and the land races. A compromise between continuing the field trials with this material and obtaining relevant data on biosafety issues has been proposed through tightly controlled experiments and monitoring.

Introduction

The first request to introduce genetically modified organisms (GMOs) into Mexico for a field trial came from a large American biotechnology company in 1988. Due to the lack at the time of formal guidelines to address these issues, the Secretary of Agriculture, through its General Office for Plant Health (Direccion General de Sanidad Vegetal, DGSV), set up an *ad hoc* committee to review the application, and provide technical advice as to the potential risks involved in the proposed release of this material.

The newly created committee rapidly evolved from a mixture of politicians, regulators and scientists which founded a forum for discussions over the new technologies, to a five-member technical advice panel formed by three researchers from government research institutions, one representative from the Seed Certification and Inspection Service, and a coordinator from the DGSV. Through the years, this committee has invited several experts to present their opinions on specific issues and, in some cases, have been invited to form part of this committee.

The actual committee, known as the National Committee for Agricultural Biosafety (CNBA) is formed by 16 members, and coordinated by a representative of the DGSV (Table 1). The committee continuously invites experts from different fields, participates in different events aimed to discuss the possible implications of the release of transgenic materials in Mexico, and participates in events where the concerns of the people and non-government organizations (NGOs) can be addressed.

The members of the CNBA do not constrain themselves to the discussion of the proposals presented to them. They constantly visit the trial sites and participate together with the large companies and research institutions in the search for results or projects leading to the acquisition of the data that sometimes is urgently needed to properly assess the risks involved with particular field releases.

Table 1 Members of the National Committee for Agricultural Biosafety (CNBA)

Institution Members	Type of Institution	Area of Expertise of Members

DGSV 4 members	Federal Government General Office for Plant Health	Coordinator of CNBA (1) Technical staff (3)
Secretary of Health 2 members	Federal Government	Regulatory affairs (2)
Cinvestav 2 members	Federal Government Biotechnology Research Institution	Biotechnology Biotechnology and Virus resistance
I NI FAP 2 members	Federal Government Agricultural Research and Extension	Biotechnology Biotechnology and Virus resistance
Col. Postgraduados 2 members	Federal Government Agricultural Research Institution	Bacterial Biotechnology Entomology
National University 2 members	Autonomous Research and Academic Institution	Plant Biotechnology Ecology
Agri. Univ. Chapingo 1 member	Autonomous Agricultural Research and Academic Institution	Agricultural Biotechnology
Private sector 2 members	Private Biotechnology Companies	Commercialization and Technical services (2)

Release of GMOs

As mentioned before, the first experimental release of a GMO took place in 1988, after it was approved by the *ad hoc* committee set up by the Secretary of Agriculture to review it. After this first field trial, which ended in June 1989, no requests were received for further testing until the end of 1991. During this interval, work began to formally establish this committee within the legal framework of the Secretary of Agriculture, to establish the role of its members, and to homogenize scientific and technical criteria with the corresponding instances in the USA and Canada, in preparation of the North American Free Trade Agreement (NAFTA), then a possibility, now a reality.

Field releases took place again at the beginning of 1992, and during that time, and up to 1995, a substantial amount of tests were conducted by CALGENE with tomato which had been modified to extend its shelf life. 1992 was also the year when Cinvestav, a national non-profit research institution, began conducting field trials testing potatoes with coat-protein mediated virus resistance.

The requests for field releases of transgenic plants increased over the years up to a total to this day of 117 approved proposals (Figure 1). However, this number does not reflect the actual number of field trials that had taken place, since an approved proposal may cover multiple-site testing, or a number of trials over a certain period of time.

Up to 1995, none of the requests for field trials had posed special problems to the CNBA, except perhaps for the deregulation of the FLAVR SAVR tomato of CALGENE, which was agreed after a one-year discussion. In 1994, however, and due to the importance of Mexico as a center of origin of maize, and to the historical importance of the crop itself in this country, it was clear that a bigger problem could arise when companies wanted to request permission to release transgenic maize in Mexico. This did not seem a long-term possibility since transgenic varieties of this crop were already extensively tested in the USA.

Therefore, in September 1995, a symposium was organized in Mexico by the National Institute for Research on Agriculture, Forestry and Livestock (INIFAP), the International Center for Maize and Wheat Improvement (CIMMYT**),** and the CNBA, to address the issue of the possible consequences of the release of transgenic maize in Mexico. The objectives of this meeting were:

1. to identify possible risks involved with the release of transgenic maize;
2. to find ways to reduce the risks involved without hampering research; and
3. to decide on the critical data required to properly assess the risks, and how to obtain such data.

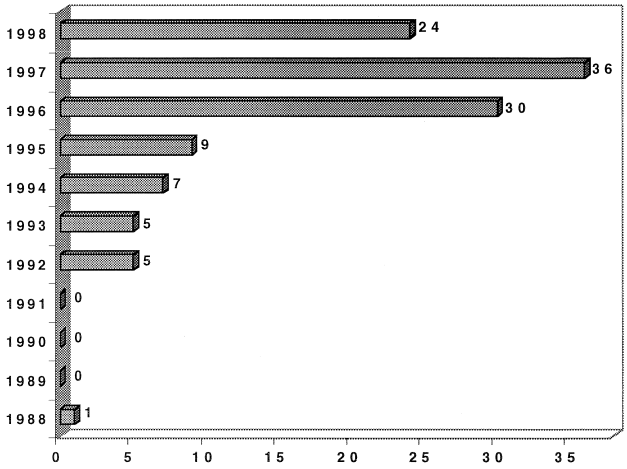

Figure 1 Approved requests per year (to May 1998)

Prior to this symposium, only five requests for field trials with transgenic maize had been reviewed by the CNBA: one from a company wishing to test transgenic maize in the state of Nayarit, which was rejected because in that state native teocintle can easily be found; one from Cinvestav involving 10 plants with genetic markers, and three from CIMMYT involving mainly green-house work with maize transformed with genetic markers. However, in January 1996 transgenic corn was deregulated in the USA, and during that same month the CNBA started to receive requests from companies wanting to carry out field trials with transgenic maize in different parts of Mexico. Out of the total of 117 field releases approved to May 1998, 90 (76.9%) have taken place in the period 1996-1998, and of these, 34 (37.7%) have involved maize, and the increasing trend is likely to continue (Figures 2 and 3).

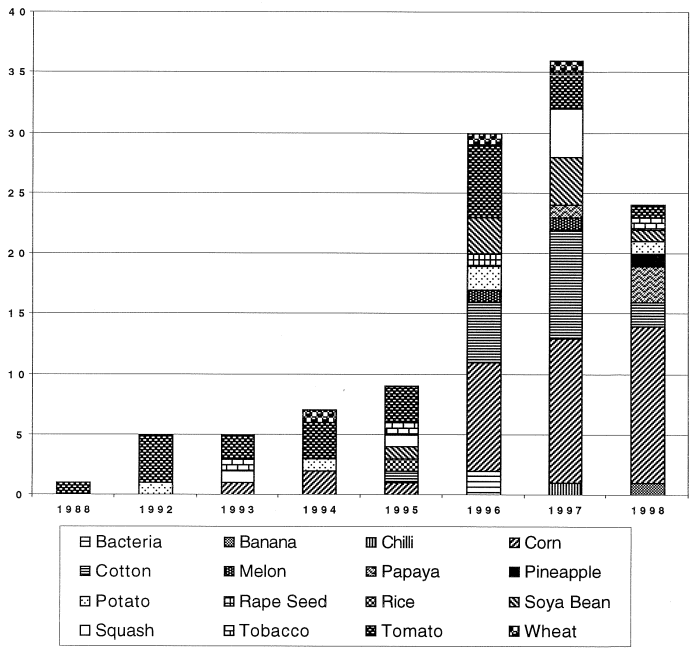

Figure 2 Crops tested by year

Insect-resistant cotton is another crop that has been extensively released, in terms of sites, number of times, and acreage. In this case, pre-commercial scale releases were approved, linked to a program covering the following points:

1. Training of the growers in terms of the strategy devised by the company to minimize the appearance of *Bt*-resistant insects.
2. Monitoring the insect population to look for the possible appearance of resistant individuals.
3. Training local entomologists to assist with the monitoring program.
4. Ensuring total control of seed stocks.

Besides corn, tomato and cotton, there are 12 other crops that have been tested, plus two limited field trials conducted with recombinant bacteria carrying *cry* genes from *Bacillus thuringiensis* (Figure 3).

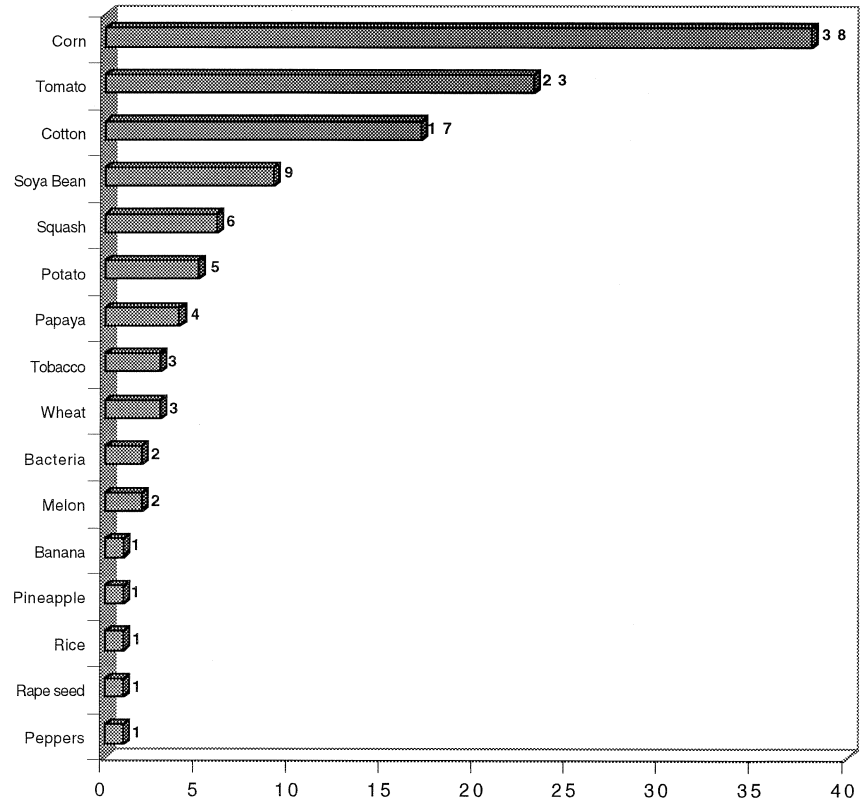

Figure 3 Number of approved requests by crop type

With respect to the traits that have been tested in Mexico, insect resistance mediated through the use of *cry* genes from *B. thuringiensis,* both in corn and cotton, has been tested extensively. This is followed by ripening, herbicide tolerance and virus resistance (Figure 4).

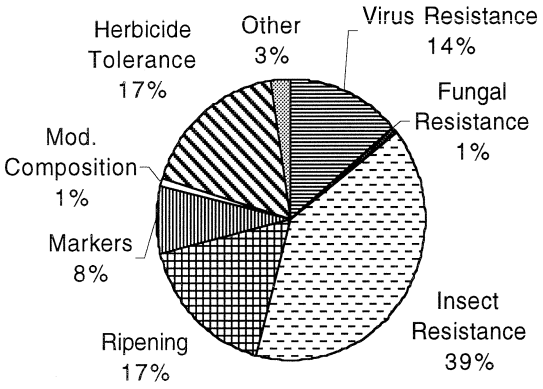

Figure 4 Phenotypic traits

Research with GMOs

In Mexico there are very few places where research with GMOs is taking place. The Center for Research and Advanced Studies at Irapuato (Cinvestav), is the only national institution that has produced new crops through genetic engineering. These are PVX and PVY resistant potatoes intended to be commercially released during 1999. Cinvestav is also conducting basic and applied research in the areas of plant physiology, disease resistance, modification of metabolism, plant pathogen interaction and plant genomics. This is the only national institution that has conducted field trials for research and commercial purposes.

CIMMYT has also conducted field trials with both maize and wheat, for research and to validate possible products aimed to eventually be released through their international programs.

The Mexican Consortium known as Grupo Pulsar has also been conducting field trials with GMOs in Mexico, with banana, pineapple, and papaya in the southeastern State of Chiapas, through CIICA, and maize through Asgrow Mexicana. However, most of the research and product development takes place in the USA.

In terms of biosafety-oriented research, there have been few initiatives, mainly directed towards investigating the possible effect of the release of transgenic maize on the teocintle populations. These initiatives have been, in some cases, promoted by members of the Biosafety Committee in conjunction with institutions such as CIMMYT, or INIFAP. However, in most cases the research proposed has been based on model systems, such as interactions between white and colored-seed maize, rather than transgenic maize and teocintle or the land races. This research, although useful, does not provide the means to determine what could be the possible effect of a transgene on the wild teocintle population or the land races.

Even though during the maize symposium of 1995 it was proposed to obtain more data before allowing large-scale releases of transgenic maize in Mexico, it was also predictable that many companies would try to test their material in this country. To this end, a map of Mexico was divided into high, medium, and low risk areas, depending on the abundance of wild relatives such as teocintle. These, and other considerations, have made it possible, at least for the majority of interested parties, to test their material in Mexico. Most of this material is maize that has been modified with *Bt* genes to confer resistance to the European corn borer, which is not a significant problem in Mexico, or herbicide-tolerant varieties.

Commercialization of GMOs

In November 1993 CALGENE requested from the Secretary of Agriculture the deregulation of the FLAVR SAVR tomato. This was granted in March 1995. During the same year, CALGENE also

received the approval from the Secretary of Health regarding the commercialization of the FLAVR SAVR tomato in Mexico. This product is intended for the fresh produce market. Deregulation of a tomato variety intended for the food processing industry, and containing the same genes as the FLAVR SAVR tomato, was granted to ZENECA in September 1996 by the Ministry of Agriculture and by the Ministry of Health.

To this date, these are the only transgenic crops that can be grown and sold in Mexico without any restrictions, although in fact these products are not intended for the Mexican market and cannot be easily obtained in Mexico, if at all.

However, the Secretary of Health granted permission to a company wanting to import, as a commodity, a variety of transgenic potato grown in the USA and containing *Bt* genes which confer resistance to the Colorado potato beetle. This potato is being used by the fast food market. A similar situation arose with transgenic herbicide-tolerant rape seed, which can be imported, processed, and the oil and by-products sold in Mexico. Nevertheless, none of these transgenic varieties can be used as seed or planted in Mexico as they do not have been deregulated by the Secretary of Agriculture.

In terms of new products being deregulated in the near future, the most likely candidate is PVX/PVY resistant potato from Cinvestav. There is a strong interest to deregulate *Bt* cotton and *Bt* corn, however, for these two crops there are wild relatives in Mexico and, some people doubt whether the strategies proposed by the companies to avoid, or at least delay, appearance of *Bt* resistant insects could work in Mexico.

Discussion

With respect to the topic that brought us together to this meeting, "The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms", and looking back to the 10-year experience in Mexico, unfortunately it is not much that can be said.

Companies wanting to test their material in Mexico have usually performed previous tests in the USA and/or Canada, and when they come to this country, their aim is usually to observe the agronomic behavior of the varieties that they have previously selected, through prior testing in their country of origin.

In terms of biosafety, field trials have been requested based heavily on the inferences that could be drawn from the biology, ecology, agricultural practices, previous practical knowledge of the particular crop species, and current knowledge about the molecular biology of the transgene and the manipulations involved. Very rarely there have been supportive data from particular experiments aimed to answer a specific question for a biosafety issue of a transgenic crop.

Furthermore, when companies present their report concerning a particular test, the information they provide has been directed to show that no escapes were detected, what was the treatment given to the site after the trial, what was the fate of the seed or vegetative material, etc. These issues are of course important and should be reported, however, no new data comes from these tests to support or confirm the initial data provided as evidence that the transgenic variety being tested is not more likely to cause harm than the original non-transgenic variety.

It can always be argued that limited field trials, and limited conditions as those sometimes imposed over field trials with certain transgenic varieties, can only yield limited data that can hardly be extrapolated to the actual production-field scale and conditions.

Even more problematic can be the fact that usually one would be looking for low probability events such as the transfer of genes from maize to teocintle, and therefore it may not be possible in a limited experiment to find that this transfer actually happens. However, if it does occur and the event is identified, one would need to study the long-term effect on the wild population. The time required to gather this data could be so long as to prove unpractical, if we were required to have it before making a decision on terms of the large-scale or commercial release of transgenic maize in Mexico.

Therefore, a different approach should be taken to obtain the data needed to make a rational decision as to the possible deregulation of transgenic maize in Mexico, and to avoid delaying the possible benefits

that this introduction may bring about. In my opinion there may be two possibilities to achieve these goals:

1. to begin conducting well-planned and controlled experiments using the actual material that is intended for commercial releases, and placing it in close proximity to the wild populations of teocintle or land races, and implementing short-, medium- and long-term monitoring of the site, and
2. to begin to establish a short-, medium-, and long-term monitoring program of key places and situations, to ensure that there is not a significant change in the population of either wild-relatives of maize or, as in the case of *Bt* corn, changes in the populations of target and non-target insect species, etc.

All this could be done through collaboration among industry, international agencies, local and federal government, and the local research institutions, universities, or agronomy schools.

What is important is that something should be done. And the first step has been taken, at least in Mexico, by a biotechnology company who has submitted a research proposal to the CNBA on the lines of the first possibility mentioned before. They propose conducting an experiment using herbicide tolerant maize, grown alongside a wild population of teocintle. They will be providing all the means to continuously monitor the site for the possibility of gene transfer to teocintle, and to analyse the face of the transgene and its effect on the population, should transfer occur. This proposal was recently approved.

To many colleagues this seems as a futile experiment because they say the outcome is very predictable: transfer will occur at a very low rate, if at all; the transgene should not spread or have any effect on the population because selection is not going to be present; eventually the gene will disappear from the population or could be maintained without any significant effect.

What the lay people and those concerned with the conservation and health of our ecology want, is not for us to stop and throw away this technology, but in the particular case of maize in Mexico, many people do not want to rely on the predictions of the scientists or regulators, they want something done because they have learnt to be skeptical, for whatever reason. Not doing anything could only lead to suspicion and mistrust. Implementing a monitoring system could only benefit everyone, whether by confirming that the predictions were right, or by allowing us to find a possible source of a problem and correcting it on time and in a responsible way.

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Conditions for commercialisation of GMOs in Central and Eastern Europe

Twardowski, T.

Institute of Bioorganic Chemistry, Polish Academy of Sciences, 61-704 Poznan, Poland

Summary

The knowledge of the state of art and the status of the biotechnology in Central and Eastern Europe (CEE) countries is very limited for several reasons. As the most important we have to take into account the following: the modern biotechnology has started in this region in the 1990's, at the same time as the political and economical transition. Tremendous changes have occurred in this decade. The legislation (for example, intellectual property rights) needs to be changed, or a brand new (like in the case of biosafety) has to be introduced.

The development of modern biotech industry based on genetic engineering technology is still in its infancy. The future depends on the legislation formulation harmonised with the European system. There are several common parameters characterising the situation in CEE; the most significant are: the economy under transition and the past legal and political systems. We can recognise the similarities between three CEE countries: Hungary, Poland, and the Czech Republic, and common elements in the characteristics of others. The report discusses the present state, trends and perspectives of biotechnology in CEE based on the legal aspects.

Introduction

For the development of biotechnology and conversion of molecular biology and genetics into an industry there are several factors of key importance. The most important are: human resources, legislation, intellectual property rights, and public perception.

In the case of Central and Eastern European (CEE) countries the available data are very limited and based (mostly) on personal contacts. We have to remember that after 1990 we observed conversion to free market economy. Eight years after "the revolution" we cannot compare the economy of CEE to well established free market systems, e.g. Germany.

Overview of biotechnology in CEE

Modern biotechnology including genetic engineering started in CEE in the late 1980s. Of course, biotechnology-related issues had been discussed long before, however, only among scientists. In the late 1970s and throughout the 1980s, the view on biotechnology was dominated by the American biotechnology. After 1990, applications of several CEE countries for the membership of the EU determined the priorities of the national politics. That also means acceptance of the European legislation, e.g. Directives of EC (90/219 and 90/220) related to biotechnology. In the 1990s, we have seen international conventions significantly influencing national legislation, e.g. Convention on Biological Diversity, membership of OECD, the Budapest Treaty and protection of intellectual property rights. This influence was at different levels of significance.

In the case of CEE the commercialisation of the modern biotechnology, particularly genetic engineering is very limited. We can differentiate three groups:

- Hungary, the Czech Republic, and Poland,
- other post-COMECON states,
- former Soviet Union's states (Baltic's republics should be recognised as a separate group).
- At the moment, in August 1998, we do not have novel food on the market.

However, there are several significant economical successes in introducing more "classical" biotech to the industry. For example, most technologies of *in vitro* fertilisation (animals) and multiplication of ornamental plants (micropropagation) are done according to the very modern technology (particularly in Hungary and Poland).

The first releases of genetically modified plants (GMP) to the environment were carried out with the permission and under strict supervision of experts in 1996 (in Hungary and Bulgaria); in 1998, it is

estimated that there are about 100 experiments with genetically modified organisms (GMO) in the environment (most of them in Bulgaria, Hungary, the Czech Republic, Poland, and Russia).

NGOs are in the process of formation. There are well established and strong consumer organisations. The new Eastern Greens have support from EU organisations. However, they are not significant and do not have much influence on the governments' decisions. They are mainly interested in the environmental issues and animal rights.

Very important but difficult to evaluate is the effect of the presence of giant companies on the market (Monsanto, AgrEvo, Pioneer etc.)

Conditions for commercialisation of biotechnology in CEE countries

Human resources

In the case of human resources we observe high similarity in all regions:

- high level of basic science,
- lack of transmission of scientific achievements into the industry,
- underinvestment of scientists and government officers,
- inefficient state administration,
- lack of a continuous capacity building process.

Legislation and intellectual property rights

In the table (in an "abstract" form) the following data concerning CEE countries are presented:

- Membership of the International Organisations,
- UNEP - CBD = ratification of United Nations - Environment Programme Convention on Biodiversity (Rio de Janeiro, 1992);
- OECD - Organisation of Economical Co-operation and Development;
- European Union association;
- National legislation related to the biotechnology;
- Establishment of the national "gene law";
- Establishment of the national Biotechnology Committee;
- System which regulates the release of GMO into the environment;
- GMO released into the environment.

Country parameter	Bg	Cz	H	Pl	Ro	Ru	Sl	Sv
1. UNEP - CBD	d	d	d	d	d	d	d	d
2. OECD		d	d	d				
3. EU		d	d	d				d
4. Gene Law	l		l			d		
5. Biotechnology Committee	d	d	d	d	d	d	d	d
6. System:								
-registration	l	l	d	d		d		l
- guidelines	l	l	l	l		l		l
- permits	l	l	d	d		d		l
7. GMO:								
- microorganisms						d		
- plants	d	d	d	l		d		
- animals				l		d		

d – done; l - done with limitations or preparation in advanced progress; former Soviet Republics follow more or less the legal status of Russia.

Public perception

The society is oriented towards the Western Hemisphere. Modern technology, e.g. genetic engineering, makes a significant effect on the public opinion and reflects the public perception of the future prospects and the conversion of attitudes. The public in general takes biotechnology to be one of the key technologies for the future; however, with a big respect concerning biosafety and legislation. Terms like "Frankenstein food" are well known, and many people are afraid of transgenic food.

Investment

The private entrepreneurship is encouraged but various factors influence the development of the commercial biotechnology. These are:

- entrepreneurial attitude,
- technical feasibility and infrastructure,
- availability of capital,
- public acceptance,
- legislation and regulations,
- governmental initiatives in biotechnology.

It is important to recognise that venture capital is available to a very limited extent (in Hungary, the Czech Republic, and Poland). Initial public offering (IPO) does not exist in CEE countries. There are several specific aspects, for example,

- In the Czech Republic: the stepwise privatisation of agricultural area brings about new legislative problems. Almost 50% of previously state (govermental) agricultural ownership is in the hands of private individuals or companies. This fact brings about the necessity to specify their rights concerning formerly constructed genotypes and production of the new ones.
- In Hungary: due to the privatisation of large pharmaceutical companies and the stabilisation of the Hungarian industrial sector some biotechnology programmes were initiated in industrial biotechnology. Priorities are given to agrobiotechnology, pharmacy, and environmental biotechnology programmes including biomonitoring of fresh water.
- The future prospects of biotechnology in Poland are connected with agrobiotechnology, health industry, and services. The first field experiments with 3 transgenic plants, potato, corn and beet, were performed in Poland in 1997; in 1998, about 20 are in progress. All the field experiments are performed with the permission of the Ministry of Agriculture and under strict supervision of experts. However, commercialisation of these achievements is a question mark.
- In Romania research and industrial applications of biotechnology are developing especially in the fields of pharmaceutics, plant protection, agriculture, food industry and textile and other industrial sectors which utilize biotechnological methods or/and products.
- Biotechnology in Slovenia is mostly developing in medicine, pharmacy, medical diagnostics, agriculture, veterinary and food industries.

Integrating commentary

The scientific society have recognised three key components of the "value added chain" represented by modern biotechnology: science + technology + public perception. However, in the light of common interpretation we can expect that the future of biotechnology will depend on moral dogmas much more than on science and technology.

The value-added chain in biological developments can be long and/or intricate. Each step along this chain can lead to intellectual property rights rewards, easily derivable from existing legislation and related to a given form of protection, such as patents, designs, trademarks, plant variety protection, copyright, or database protection. These rewards are strong incentives which justify, and to some extent cover, the investments required.

International cooperation is of special importance. For example, the United Nations Environment Programme (UNEP) plays a key role in the sustainable development of the environment, particularly in the situation of the dynamic development of modern technologies of economic significance, such as

biotechnology. In compliance with Agenda 21 of the UN and following the Biodiversity Convention, Poland as a Member State has decided to strengthen its cooperation on biosafety.

Economic and socio-cultural importance of "bio" information is strictly related to public perception, policy, and mass media. Sustainable development of all these three modules is considered today to be a prerequisite for sustainable social and economic development, biotechnology included.

Trajectories for the future

The future prospects of biotechnology in CEE are connected with agrobiotechnology, health industry and services. These major directions of development are directly related with the sustainable development of biotechnology as covered by the "Convention on Biological Diversity". Non-governmental organisations emphasized socioeconomic constraints in biotechnology development. In this context the legal regulations: biological safety of human being and environment protection as well as the protection of intellectual property rights, are of basic significance. Since the last changes in Eastern European countries from centralised to market-oriented economy, a large number of possibilities for doing highly profitable business in this region have arisen. Agricultural research and plant biotechnology improvement in this regard are, and will continue to be, a prerequisite for increasing agricultural productivity and the economy as a whole in CEE. However, a number of biotechnology applications are now strongly dependent from: 1) How many of them are new and original - generally they are patented and are owned primarily by private sector corporations in industrial countries; 2) How quickly the institutional, political, infrastructural and financial constraints will be oriented and adjusted to the western standards from CEE countries; 3) The possibilities for the effective seed production and sales; 4) Novel food market for producers and consumers.

In the CEE region the biotech companies are highly active. The big firms recognise at first the new market of about 70 millions people (without former Soviet Union) and the new connections with a possible gate to the European Union.

Conclusions

Biotechnology has been identified as one of the key technologies for the decades to come. In the opinion of EU and OECD experts biotechnology presents an enormous potential for further growth, competitiveness and employment. The European Commission as well as other international organisations, particularly OECD and UNEP, provide impetus for the international implementation of the integrated European policy framework. However, the safeguarding of human, animal and plant health, and protection of the environment is a duty of national governments. National legislation plays a key role in this process.

Nowadays, all industries are trying to provide customers with products whose properties meet all the expectations of the market. Safe technologies using biological methods are being introduced. The quality of the end product is becoming top priority for a majority of manufacturers. They are now commonly using organisms and enzymatic preparations obtained with the use of genetic engineering technologies (GMOs). Modern biotechnology and, in particular, genetic engineering play a very important role in the industrial development today. On the other hand, we observe that societies are very reluctant to approve transgenic products, especially food.

In my opinion, today, we should stress the positive and fruitful aspects of modern biotechnology to accelerate its further progress, particularly in the so-called "transition" states. We have to take into account the transformation to the market economy, the government programme of privatisation and the long-distance goal of joining the United Europe. Legal aspects are particularly important for the cooperation and integration with the European Community. In the last years we observed significant modification of the CEE law towards the West European standards and norms.

In any case, the following issues have to be dealt with:

- Harmonisation of national legislation with EU directives;
- Priority of international regulations over national ones,
- Obligatory licensing for any activities involving GMO,
- Free access to information,
- Public safety being top priority.

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Commercialization of a genetically modified symbiotic nitrogen-fixer, *Sinorhizobium meliloti*

McClung, G.

Office of Pollution Prevention and Toxics, US Environmental Protection Agency, Washington, DC

Abstract

In 1997, the US Environmental Protection Agency (USEPA) approved the limited commercialization of a genetically modified microorganism under Section 5 of the Toxic Substances Control Act (TSCA). The microorganism, RMBPC-2, is a strain of *Sinorhizobium meliloti* for use as an alfalfa (*Medicago sativa*) inoculant. The strain was modified by the insertion of a gene cassette containing the *nifA* gene, *dct* genes, and the *omega* (Ω) fragment containing the *aadA* gene encoding resistance to the antibiotics streptomycin and spectinomycin.

The inoculant strain had been tested in small-scale field trials for four years prior to the limited commercialization approval. RMBPC-2 was shown to improve alfalfa yields in some soils where RMBPC-2 exhibited efficient nodulation of the host plant, particularly in those soils low in organic matter and low in numbers of indigenous rhizobia. In other soils in which there was a high degree of competition from indigenous rhizobia, RMBPC-2 failed to efficiently nodulate the host plants. Therefore, no effects on yields were observed with RMBPC-2 applied as the inoculant. The most important biosafety issues that required thorough examination and consideration throughout the years of field testing and through the commercialization request decision process included the nitrogen-fixing potential of RMBPC-2, the competitiveness of RMBPC-2 for nodulation, the effects on non-target legumes, and the potential for transfer of antibiotic resistance markers in the environment[1].

Introduction

In the USA, certain genetically modified microorganisms used for various purposes are subject to review by EPA under Section 5 of the Toxic Substances Control Act (TSCA). TSCA Section 5 regulates "new" microorganisms that are manufactured or imported for commercial purposes. New microorganisms are defined as those that are intergeneric, meaning that the introduced DNA is from source organisms of different taxonomic genera. During the review period, EPA conducts a risk assessment using the paradigm, Risk = Hazard X Exposure.

Characterization of RMBPC-2

The parental strain, PC, was a naturally competitive strain isolated from an alfalfa root nodule in Wisconsin. The genetic modification consists of a 10.9 kb cassette: *dctABD/*Ω*/nifD:nifH* leader::*nifA*/T_1 T_2, inserted by homologous recombination into the pRmSU47b megaplasmid of strain PC (USEPA, 1994a). The *dctA* gene, which is regulated by the products of the *dctB* and *dctD* genes, encodes a permease that allows for transport of C4-dicarboxylic (succinate, malate, fumarate) acids across the bacterial membrane. The *dct* genes were obtained from *Rhizobium leguminosarum*. The omega fragment (Ω) was a gene cassette containing the *aadA* gene derived from the R100 plasmid of *Shigella flexneri* (Prentki and Kirsch, 1984). The *aadA* gene product is the enzyme aminoglycoside adenyl transferase which encodes resistance to the antibiotics streptomycin and spectinomycin. The *S. meliloti nifA* gene was fused to the *Bradyrhizobium japonicum nifD* promoter to enhance expression of the *nifA* gene product. An untranslated leader RNA from *S. meliloti nifH* used to join the two also enhances the expression of the *nifA* gene. The gene product of the transcriptional activator gene, *nifA*, controls the suite of *nif* genes involved in the synthesis of the nitrogenase enzyme complex which fixes atmospheric nitrogen in rhizobia. T_1 T_2 are terminator sequences from the *Escherichia coli rrnB* gene. This gene cassette was inserted into a presumably symbiotically silent location, the *ino* locus, which controls the

[1] All data and related EPA assessments and supporting documents, including the final risk assessment, are available for review through the TSCA (OPPTS) Public Docket located at 401 M St., S.W., Washington, DC 20460, Room NEB607, telephone 202-260-7099.

pathway for utilization of the sugar alcohol, *myo*-inositol, as a carbon source under free-living conditions (USEPA, 1994a).

Background of field testing

Beginning in 1988, Biotechnica International submitted several voluntary Premanufacture Notice[2] applications to the USEPA to field test a number of strains of genetically modified *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*). In the early recombinant strains, only the *omega* fragment and the *nifA* gene fusion were introduced into one of three parental strains, PC, RCR2011, or UC445. Initially, most of the field testing consisted of assessing the ability to monitor the introduced strains with the antibiotic resistance marker. Later tests were conducted to investigate whether addition of the *nifA* or *dctABD* genes enhanced nitrogen fixation. Strain RMBPC-2 was developed later than the initial strains, and it was not field tested until 1992. All field testing conducted on the various strains since the spring of 1992 has been conducted by Research Seeds, a company that purchased the recombinant strains from Biotechnica. Table 1 presents brief descriptions of all the *S. meliloti* strains tested. Various of these recombinant strains were tested in a number of field tests conducted over seven growing seasons as shown in Table 2.

Data collected from the small-scale field tests through 1995 included assessment of alfalfa yield, which is an indirect measure of the nitrogen fixation ability of the rhizobia, and nodule occupancy, an indication of the competitiveness of the recombinant strains for nodulation. Testing done on the earlier strains in 1989-90 also included monitoring for vertical and horizontal movement in the soil profile, and aerial dispersion at the time of inoculation. No monitoring for dissemination was conducted with RMBPC-2.

Table 1 Parental and recombinant *S. meliloti* strains tested in small-scale field trials

Strain	Parental Strain	Genes/Modification	Integration Site
PC	-	-	-
RMB7201	PC	Ω	*ino*
RMB7203	PC	*nifA* / Ω	*ino*
RMB7240	PC	*nifA* / Kn^r	P3
RMBPC-2	PC	*dctABD/ nifA* / Ω	*ino*
RCR2011	-	-	-
RMB7101	RCR2011	Ω	*ino*
RMB7103	RCR2011	*nifA*/Ω	*ino*
RMB7135	RCR2011	*nifA*/Kn^r	P3
RMB138Ω710A	RCR2011	*dctABD*/Ω	P3
RMB139Ω710B	RCR2011	2 (*dctA*/Ω)	P3
UC445	-	-	-
RMB7401	UC445	nifA/Ω	*ino*

[2] These reviews, including the commercialization request, were "Premanufacture Notices" reviewed under the 1986 Policy Statement. The current regulations for genetically modified microorganisms are: Microbial Products of Biotechnology; Final Regulation under the Toxic Substances Control Act; Final Rule, 40 CFR Parts 700, 720, 721, 723, and 725, April 11, 1997, which are electronically available at http://www.epa.gov/opptintr/biotech/.

Table 2 Field test sites and *S. meliloti* strains tested

1989-1990 (2-years)	PCRCR2011	UC445		
Sun Prairie, WI	RMB7201	RMB7101	RMB7401	RMB7103
1990 (1-year)	RCR2011			
Sun Prairie, WI	RMB7103			
Rio, WI	RMB7135			
1992 (3 - 4 years)	PC	RCR2011		
Hancock, WI	RMB7201	RMB7103		
Arlington, WI	RMB7203	RMB138Ω710A		
Lancaster, WI	RMB7240	RMB139Ω710B		
Marshfield, WI	RMBPC-2			
1993 (2 - 3 years)	PC			
Hancock, WI	RMB7201			
West Salem, WI	RMB7203			
Becker, MN	RMBPC-2			
Grand Rapids, MN				
1994-97 Test Marketing	RMBPC-2			
North Dakota				
South Dakota				
California				
Nebraska				

Summary of yield results with RMBPC-2

Since alfalfa is a perennial crop, it is inoculated only once at the time of planting, and then grown in stands lasting for four to five years. Although typical management practices are to harvest several times annually for three or more years beginning the year following seeding, yield measurements were also taken the first year in the small-scale field tests. Table 3 (adapted from Scupham et al. 1996) presents alfalfa yield data from the field trial of longest duration (the Strain Comparison Tests initiated in 1992 at several sites in Wisconsin) since longer-term studies are the more representative of typical agricultural growing practices. Although data were collected for four growing seasons at the Hancock site, trials were terminated after three years at the other sites, so only 1992-1994 data are summarized in Table 3. RMBPC-2 resulted in a statistically significant yield increase of 7.4% compared to the PC parent strain at this site. The Hancock site is characterized by sandy soils low in organic matter and low in numbers of indigenous rhizobia. Due to the lack of competition, RMBPC-2 exhibited good nodulation of the plants which was measured by determining percentage of nodules occupied by the intended inoculant strains. Nodule occupancy values of 82-97% by RMBPC-2 were observed over the three-year period at this site.

Table 3 Average annual yield of alfalfa (Mg dry forage/ha) inoculated with parental and recombinant strains in the Strain Comparison Trials over three years (1992-1994) at the Hancock, Lancaster, and Marshfield, Wisconsin sites

Strain	Hancock	Lancaster	Marshfield	All Sites
PC	8.75 de	9.44 b	6.25 abc	8.14 c
RMB7201	8.97 cd	9.70 ab	6.41 a	8.36 ab
RMB7203	8.84 cd	9.53 ab	6.36 a	8.24 bc
RMB7240	9.18 bc	9.68 ab	6.31 ab	8.39 ab
RMBPC-2	9.40 b	9.73 a	6.22 abc	8.45 a
Control	8.81 d	9.46 ab	6.23 abc	8.16 c

Data followed by the same letter within a column are not significantly different at the 10% level of confidence as determined by Tukey's least significance test.

RMBPC-2 also resulted in a statistically increased alfalfa yield of 3.1% compared to the parent strain PC when averaged over all three years at the Lancaster site (Table 3). Nodule occupancy by RMBPC-2 was also quite high at this site, with values ranging between 73-90% during the 3-year period.

There were no statistical differences in alfalfa yields among the rhizobial inoculants in the Marshfield soil which is characterized by high organic matter and large indigenous rhizobial populations (Table 3). The nodule occupancy measurements at this site showed that RMBPC-2 was not competitive with the indigenous rhizobia, with nodule occupancy values of only 12-59% over the three years. Therefore, the yields reported at the Marshfield site are not reflective of the nitrogen-fixing ability of RMBPC-2 since the indigenous rhizobial strains predominated in the nodules. Alfalfa yield for RMBPC-2, when summed over all sites for all years (Table 3), was significantly greater than the yield obtained with the parental strain (3.8%) and with the uninoculated control (3.6%).

Yield data from many other field tests demonstrated similar results (USEPA, 1994b; USEPA, 1997a). In the absence of high populations of indigenous rhizobia, RMBPC-2 was efficient in nodulation, and increased yields were often observed. RMBPC-2, while competitive in some soils, could not out-compete well-established indigenous populations in others.

Biosafety issues

In early stages of review, a number of potential concerns with recombinant rhizobia were identified:

- effects on the nitrogen cycle through increased nitrogen fixation
- detrimental effects on alfalfa yield
- competitive displacement of indigenous rhizobia
- effects on nontarget legumes
- transfer of antibiotic resistance genes to pathogenic microorganisms.

These concerns were addressed directly through field testing or through analysis of existing literature.

Effects on the nitrogen cycle

In the early stages of evaluating the potential effects of introduced genetically modified rhizobia, it was postulated that the use of enhanced nitrogen-fixing strains of rhizobia may lead to over-enrichment of soil nitrogen, which may then lead to development of other problems such as increased nitrate pollution of groundwater, establishment of new weeds, and increased flux of nitrous oxides in the atmosphere (Tiedje et al. 1989). Throughout four years of small-scale field testing of RMBPC-2, and several additional years of testing with related rhizobial strains containing the same introduced genes, it was demonstrated that the genetic modifications in RMBPC-2 resulted in nitrogen-fixing ability that was not dramatically different from that which might be obtained with naturally-occurring rhizobia (USEPA, 1994b; USEPA, 1997a; Peterson and Russelle, 1991). Thus, relative to existing rhizobial inoculants, no overproduction of nitrogen was expected from the commercialization of RMBPC-2.

Effects on alfalfa yield

Data obtained from small-scale field testing did not reveal detrimental effects on alfalfa yields during a number of experiments at different sites, and with various alfalfa cultivars (USEPA, 1994b; USEPA, 1997a). As discussed previously, alfalfa yields with RMBPC-2 as the inoculant were sometimes increased, by as much as 13%. Often yields using RMBPC-2 were not different from other strains or treatments. In these cases, however, there was usually poor nodulation by RMBPC-2, and yield differences were not observed because indigenous rhizobia predominated in the nodules (USEPA, 1994b; USEPA, 1997a).

Competitive displacement of indigenous rhizobia

Competitive displacement of indigenous organisms has long been a potential concern with the environmental release of any organism. Increased competitiveness in conjunction with ineffective

nitrogen fixation could be ecologically and economically harmful. Throughout several years of small-scale field testing, RMBPC-2 was not shown to be excessively competitive with indigenous rhizobia (USEPA, 1994d; USEPA, 1997b). In soils where there were small populations of existing rhizobia, nodulation by RMBPC-2 was good (e.g., the Hancock site with nodule occupancy values of 82-97% over three years). In soils with high indigenous rhizobial numbers, RMBPC-2 was not very competitive (e.g., the Marshfield site with nodule occupancy values of only 12-59% over three years). Therefore, the concern over potential competitive displacement of indigenous rhizobia by RMBPC-2 was alleviated through field testing.

Effects on non-target plants

In addition to alfalfa, *Sinorhizobium meliloti* nodulates sweet clover (*Melilotus*), fenugreek (*Trigonella*), and mesquite (*Prosopis glandulosa*). The concerns about commercialization of RMBPC-2 with respect to non-target legumes were alteration of host range, increased weedness of known non-targets, and decreased growth of some of the known non-targets that are intentionally grown as crops or are of economic or ecological importance (USEPA, 1994b). No direct data on the effects of RMBPC-2 on non-target growth were obtained during the small-scale field testing. However, after a thorough examination of the genetic modifications, there was no reason to suspect any alteration of host range, since there were no modifications of the *nod* (nodulation) or *hsn* (host specificity nodulation) genes involved in nodulation which is a highly specific process controlled by products of both the rhizobia and the legume host (Havelka et al. 1982; Kondorosi et al. 1984; Horvath et al. 1986). Potential effects on fenugreek and mesquite were mitigated by a lack of geographic proximity to alfalfa-growing regions, since fenugreek is grown only in small amounts in Florida and California, and mesquite is confined to arid regions in the south-western parts of the USA.

The non-target sweet clover required additional consideration since it may be grown intentionally as a crop or it may occur as a weed in close proximity to alfalfa-growing regions. As a crop, sweet clover is inoculated with high numbers of specific strains of *S. meliloti* known to be both efficient in nodulation and effective in nitrogen fixation in sweet clover. It is unlikely that small numbers of RMBPC-2 that may move off-site from an alfalfa field would out-compete the high numbers of rhizobia intentionally applied as a sweet clover inoculant. The potential for RMBPC-2 to cause a weedness problem with sweet clover growing in the wild also was deemed unlikely. The well-known phenomenon of rhizobia-host specificity suggests that rhizobial strains demonstrated to be efficient in nodulation and effective in nitrogen fixation in alfalfa are probably not so in another legume host (Brockwell and Hely, 1961; Brockwell and Hely, 1966). Even if RMBPC-2 could nodulate and fix nitrogen in sweet clover, the nitrogen-fixation ability of RMBPC-2 as assessed through four years of field testing did not appear to be dramatically different from that which could be obtained with other effective naturally-occurring *S. meliloti* inoculants (USEPA, 1994b). Therefore, there was little concern for alteration of sweet clover growth with RMBPC-2 relative to changes that could occur with other *S. meliloti* alfalfa inoculants. In addition, concern over increased weedness of sweet clover is mitigated by the typical alfalfa management practice of low mowing height which restricts the re-growth of sweet clover (USEPA, 1994b).

Transfer of antibiotic resistance markers to pathogenic microorganisms

The genes introduced into RMBPC-2 were inserted into pRmSU47b, one of the two megaplasmids in *S. meliloti*. Extensive analysis of the literature (USEPA, 1994a) indicated that gene transfer of this megaplasmid was highly unlikely due to both size (1600 kb) and stability. The most likely mechanism of transfer would be through conjugation, however, the megaplasmids of *S. meliloti* are not self-transmissible at detectable levels (Maoui et al. 1985; Pretorius-Guth et al. 1990). Although the potential for transfer in the presence of a helper plasmid to strains of the same species is theoretically possible, and has been demonstrated under ideal laboratory conditions (Maoui et al. 1985), the megaplasmid would probably not be maintained in the recipient (USEPA, 1994a).

Neither streptomycin nor spectinomycin is of critical importance in clinical usage in the USA, although the former does have some agricultural uses and also may be used in combination with three or four other drugs for treating tuberculosis (USEPA, 1994c). Streptomycin is sometimes used in livestock for brucellosis, although this disease is well-controlled through vaccination in the USA. In addition, some poultry respiratory diseases have been treated with streptomycin, however, other drugs are more

commonly used (USEPA, 1994c). Streptomycin, in several forms, is registered for use as a pesticidal agent in control of *Erwinia, Pseudomonas, Xanthomonas, Agrobacterium tumefaciens* and a few other plant pathogens for crops such as apples, pears, tobacco, ornamentals, and some vegetable crops. Spectinomycin has very limited use in the USA which is predominately veterinary (USEPA, 1994c). The combination of low exposure and the low likelihood of gene transfer to those pathogens which are treated with either streptomycin or spectinomycin mitigated the concern for antibiotic resistance development in pathogens from use of RMBPC-2 (USEPA, 1994c).

Conclusions

The limited commercialization of a genetically modified strain of *Sinorhizobium meliloti,* RMBPC-2, as an alfalfa inoculant was approved by the USEPA in 1997, allowing production of a specified amount over the course of the first three years. EPA retains the option to reevaluate any potential effects of RMBPC-2 in several years if necessary. Much data have been produced over the course of many years of small-scale field testing of RMBPC-2 and related recombinant rhizobial strains. The biosafety issues deemed of most importance for evaluating the release of RMBPC-2 at the commercial scale were (1) its nitrogen-fixing ability, (2) its competitiveness for nodulation, (3) its effects on non-target plants, and (4) the potential for transfer of its antibiotic resistance genes. Field testing results demonstrated that its nitrogen fixing ability was not substantially different from that of effective naturally-occurring strains. In some soils where good nodulation was achieved, RMBPC-2 produced substantial increases in alfalfa yields. In other soils in which there was a high degree of competition from indigenous rhizobia, RMBPC-2 did not compete well for nodulation which alleviated the concern for competitive displacement of native rhizobia. In these situations, no yield effects were observed due to the lack of presence of RMBPC-2 in the nodules. Since field testing data could not alone resolve all the biosafety issues identified, decisions were made using thorough examinations of the literature and of the genetic construction of the microorganisms as well. The widely known concept of rhizobia-host specificity lessened concern for alteration of host range. Considerations of exposure, along with analysis of the genetic construct, limited concerns for effects on non-target plants. Lastly, a thorough examination of the potential for gene transfer of the streptomycin and spectinomycin antibiotic resistance marker gene to bacterial pathogens for which these antibiotics are used as treatment concluded that this scenario was highly unlikely. Therefore, EPA concluded that there are no significant risks associated with the use of RMBPC-2 as an alfalfa inoculant (USEPA, 1997c).

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Commercialisation of transgenic linseed: International regulations and economic considerations

McHughen, A.

Crop Development Centre, University of Saskatchewan, Saskatoon, Canada

Abstract

International trade in genetically modified crops is being hampered by different jurisdictions imposing onerous and often scientifically questionable regulations. Using linseed flax as an example, this paper examines some of the issues and arguments raised in the restriction of commodity GMO access to international markets, and what might be done to facilitate the orderly introduction of these commodities while respecting sovereign rights to regulate imported products.

Introduction

Plant breeders over the course of history have used their ingenuity to create new and improved crop cultivars through "conventional" or "traditional" methods. All of these methods were at one time novel, unconventional, non-traditional and, for the most part, untested in terms of their environmental, human and animal health risks. The methods and the products of these breeding efforts were, and are, accepted by scientists as being "low risk" and by the public as being "safe". Note the distinction between the relative "low risk" and the absolute "safe".

Until recently, a new cultivar for a field crop was bred, evaluated by the breeder primarily for agronomic parameters, and released for commercial production with international distribution. Although regulations governing new cultivar registration vary across different jurisdictions, the international commodity market was not too interested in those technicalities; the market was more concerned with the quality and the price of the product. Seed growers and farmers would buy seed of the new cultivar, grow a crop and sell the resulting seed into the commodity market, blended with seed of the same species grown from many different cultivars on the market. This bulk commodity seed would be shipped to customers locally and around the world. The variety names of the cultivars, or the breeding methods used to develop the cultivars present in the shipment, were largely irrelevant. If the quality was good and the price was fair (especially the latter), it was assumed that the product was "safe".

Today we live in a New World. People in general are more concerned with their environment and health. They are demanding (and willing to pay for), and getting (and certainly paying for), more regulatory control over products that might pose increased risk to health or environment. This is not limited to products of biotechnology, but across the board of consumer products; I believe it is good that people are taking greater interest in such important scientific issues as health, nutrition and ecology.

Using the example of two recent Canadian linseed cultivars, let us explore some of the regulatory processes and financial costs involved in their commercial development.

Canada regulations

The two exemplary cultivars are CDC Normandy and CDC Triffid. CDC Normandy was evaluated and supported for variety registration in 1995 by the "expert committee" system, a national group consisting of public and private breeders, pathologists, grain chemists and agronomists (Rowland and McHughen, 1995). CDC Normandy is the highest yielding, earliest maturing linseed cultivar in Canada. It is not transgenic, so it is allowed freely into the EU and around the world. The file on CDC Normandy, as required to allow the new variety to be grown commercially and marketed worldwide contains about 30 pages of documentation.

CDC Triffid, on the other hand, is a transgenic line carrying the ability to grow on soil contaminated with sulfonylurea herbicide residue (McHughen and Holm, 1994; McHughen et al. 1997). It was evaluated and supported for variety registration by the expert committee in February, 1994, one year earlier than CDC Normandy. However, in addition to variety registration, CDC Triffid needed environmental clearance and animal feed clearance before it could be commercialised in Canada. It took an additional 27 months to complete these requirements; it was May, 1996, before CDC Triffid was cleared for

commercial production in Canada (McHughen et al. 1996, 1997; see also internet documents relating to CDC Triffid). In addition to the same documents as required by CDC Normandy, the registration of CDC Triffid in Canada required a substantial data package, largely for environmental clearance in Canada.

What did the two cultivars cost to develop? I work in a public plant breeding institution, the Crop Development Centre (CDC). My colleagues have developed many registered cultivars of a range of species over the years. It is difficult to calculate the development cost of a new plant variety, anywhere from about $200,000 to $2,000,000. A typical conventional breeding programme in the CDC results in the release of one new cultivar each year. It takes about 10 years to develop, and each programme costs the taxpayer about $US200,000 a year, so the admittedly simplistic calculation suggests about $200,000 to breed and put a new cultivar, such as CDC Normandy, on the international market.

In contrast, cultivation of CDC Triffid in Canada is limited and segregated because it, being transgenic, requires additional regulatory approvals. As well, although linseed is not grown commercially as a food product, we sought food use clearance from Health Canada because some people do eat the seeds. It is not typical for new linseed varieties to undergo scrutiny for human consumption, so it became the first linseed variety (conventional or GMO) specifically cleared as human food in Canada. The cost for regulatory compliance in Canada alone was over $1,000,000.

International regulations

The major international customers for Canadian linseed are the USA, Europe and Japan. In order to comply with US regulations to market CDC Triffid there, we consulted with FDA for food and feed use of this transgenic linseed (FDA, 1998), and also with USDA for permission to market the seed in the USA. These are now complete and CDC Triffid is clear for US marketing. The paperwork required for the US clearances was appropriate and reasonable to address legitimate questions and concerns of potentially hazardous risk. The EU and Japan applications are currently pending. As a result, no seed of CDC Triffid is being shipped overseas. CDC Normandy is, however, being shipped worldwide.

As scientists, it is our responsibility to ensure regulatory agencies employ scientifically sound principles in the analyses of consumer products. These principles include both strategic and tactical approaches to regulation. Many current regulations of products of biotechnology are tactically based; that is, they target the individual product attributes without considering the strategic relevance of the tactics. This is analogous to industrial regulators approving a new screen door based on its construction quality, mesh size, compositional materials, etc., without considering it was intended to be fitted to a submarine. Scientific credibility with the public requires strategic as well as tactical evaluation, or else the public will come back and ask, “Why didn’t you tell us the ship would sink?”

When we move from screen doors to crops, strategic evaluation must include, at least initially, all crop technologies. This does not mean that we should start scrutinising ordinary plant varieties with the same vigour as for transgenics, but rather that we should determine the degree of tactical scrutiny based on a strategic assessment of increased risk over what is currently considered “safe”. This will mean some products of traditional breeding will receive greater scrutiny, while some transgenics will require reduced scrutiny. CDC Triffid is transgenic. It is essentially the older cultivar Norlin with a known piece of DNA inserted into its genome. CDC Normandy is a somaclonal variant. I have no idea what happened to make CDC Normandy mature earlier or yield higher. Conceivably it might have new or enhanced undesirable traits, but the assumption is that it is “safe”. From a personal and scientific perspective, I know CDC Triffid is a lower risk to health and safety than CDC Normandy, and that both cultivars are “low risk”; I have no concern over my family eating either one, yet the differing regulatory requirements, supposedly science-based, are dramatic (Table 1).

It seems the current regulatory scrutiny targets only some products, in what appears to be an arbitrary manner, as the triggers for and extent of scrutiny can differ markedly in different jurisdictions. Surely a potential hazard to the health of citizens of one country would be a similar hazard to citizens of a neighbouring country. Table 2 presents a series of hypothetical linseed cultivars, broken somewhat subjectively into risk groups, from lowest to highest. In this list, only the highest risk group (which includes examples of both transgenic and “conventional” breeding) should require regulatory scrutiny beyond that required for ordinary new cultivars. However, current regulations in most countries target low-risk products of biotechnology, and exempt higher risk products of “conventional” breeding

technology. International trade in GMOs will remain unstable until these discrepancies in regulation and their justifications are addressed.

Table 1 Regulatory requirements and current approvals for national and international marketing of two recent linseed flax cultivars from the Crop Development Centre, University of Saskatchewan

	Canada	USA	EU	Cost ($US)
CDC Normandy Somaclonal variant	Variety Registration	None	None	$250,000
CDC Triffid transgenic	Variety Registration Environmental clearance Feed clearance Food clearance *	FDA (Food and Feed) USDA (importation)	Pending	> $1,500,000 and climbing

* optional and voluntary

Table 2 Hypothetical linseed cultivars developed using different breeding methods, in approximate ascending order of hazard to health and/or environment. In each case, the starting genotype is a commercial cultivar (rDNA = recombinant technology, "genetically engineered")

Group 1: least hazard
rDNA linseed with silent or inactive linseed DNA from the same plant.
rDNA linseed with an additional active linseed gene from the same cultivar (e.g. stacking).
rDNA linseed with a gene from a different linseed cultivar (e.g. L6 for rust resistance).
Cross-pollination with another linseed variety. The new cultivar may have an improved agronomic trait (e.g. rust resistance), but contain many genes and gene combinations never before present in the same genome.
Group 2: modest increase in risk
Somaclonal variation from the known cultivar, regenerated *in vitro*, a plant with higher seed yield than the parent.
rDNA linseed with inactive DNA from another species.
Spontaneous mutation a linseed plant found growing in a farmer's wheat field after spraying with herbicide used to kill volunteer linseed.
Induced mutation: with ionizing radiation or chemical such as EMS. Select a progeny line with, e.g. a novel oil profile.
rDNA linseed with a gene homologous to L6 but from a different species.
Group 3: requires appropriate additional scrutiny
Embryo rescue: using substantial human and chemical intervention, pollinate between distant relatives, rescue the hybrid embryo and develop into a new cultivar.
rDNA linseed with active DNA for a novel trait from another species (e.g. *Bt*).
rDNA linseed with active gene for a known toxin or allergen.

For products of related industries, there is a relatively high degree of international harmonisation. Pesticide residues are rarely a concern in commodity grain shipments, for instance.

International commerce occurs, apparently in spite of the fact that differing jurisdictions have legitimately differing ideas of regulatory oversight and degree of comfort with potentially hazardous commodities. Farmers around the world use chemicals. Residues from these chemicals inevitably end up in the commodity. As each country has its own set of tolerances for residue of a particular chemical, how can a load of grain, complying with local residue tolerances, comply with those of all of the potential destinations? Obviously, there is a place for international regulatory oversight to determine, if not absolute harmony, then reasonable concordance. This has developed for the more mature farm chemical industry, which has allowed reasonable flow of grain internationally, even with differing tolerances for various pesticides.

This maturation is still occurring for the agbiotech industry. Currently, each jurisdiction has its own regulations (or not) and all biotech products are scrutinised individually or tactically. This inefficiency is costly and can potentially lead to mischief.

For example, all Canadian canola is currently kept out of the EU market because not all GMO cultivars grown in Canada have EU approval. Only after all GMO cultivars are approved in the EU can the market be re-opened. Now, if I develop a GMO linseed in Canada, have it approved in the EU, I would be able to market my new cultivar and (hopefully) make a business. If I make too much business, a competitor could register in Canada a new GMO linseed, but not bother to pursue approval in the EU. All of a sudden, the EU shuts the door to Canadian linseed because not all registered cultivars in Canada are approved in the EU.

Eventually our able representatives will negotiate a harmonisation of regulations to allow the international flow of products, including products of biotechnology. Simon Barber from OECD is speaking to this issue later in this Symposium.

Until we achieve harmonisation, we deal with products tactically, individually, which introduces unnecessary delays, costs and denies not only the breeders, but also the farmers and consumers the benefits of the products.

Economic considerations

Cost of development of transgenic versus cost of conventional cultivar.

Needless to say, the costs associated with regulatory compliance for commercialising CDC Triffid are enormous compared to those for CDC Normandy. Why? What is the scientific justification?

The costs of additional regulatory burden placed on the targeted products of biotechnology are disproportionate to their degree of increased risk, compared with products of conventional breeding. The seed business is not a particularly high margin/high profit enterprise. This is why seed companies are being bought by chemical companies, not vice-versa. One of the arguments against biotechnology is that "Only the big multinationals will benefit from it…" This has become a self-fulfilling prophecy because the opponents, those largely responsible for the establishment of unnecessary regulations, effectively eliminated the public and small private enterprises from playing the game. The public and small private plant breeding and seed companies do not have the financial resources to cover these additional regulatory costs.

And who pays these additional costs? Directly or indirectly, the consumer always pays. Political realities, not always scientific principles, will ensure that for many years to come, transgenic cultivars will cost substantially more than "traditional" cultivars. This will not only eliminate public and small private companies from using biotechnology to breed new cultivars, it will drive the technology shift from transgenic cultivars of gross commodities to transgenic plants that produce higher-value specialty products, such as pharmaceuticals or industrial chemicals, thus avoiding the additional regulatory burdens imposed on the bulk commodities.

So, what can we do to promote appropriate regulation of new crop cultivars? First and foremost, we need to insist that scientific principles prevail in regulations claimed to be "science-based", not just on the tactical, but on the strategic. We also have to re-establish science as a source of objective information. There is no point having a science-based system if people do not believe the science. Finally, we need to have international harmonisation of regulations.

Scientific credibility

On both sides of the public debate, we hear "Let the people (i.e. consumers) decide" - a noble enough position, and one with which I personally agree, on the condition that it is an informed choice. While this might be implied or self-evident, it is not. What would happen if an uninformed public were asked in a binding referendum to reduce personal taxes by 20%? It is irresponsible for a society to pose such questions without first providing sufficient information - from protagonists as well as antagonists - so people can feel confident they are making an informed determination.

Currently in Europe, the general public does not have full information to make an "informed" decision. Where does one obtain objective information on a scientific subject?

In most of the world, the most credible source of scientific information is from scientists, especially academic or public sector scientists. In Europe, scientists, rightly or wrongly, do not enjoy the same level of credibility as elsewhere.

So, where do European people get the information to formulate an "informed" choice?

The void is often filled by sincere people with little or no scientific training, or insincere people claiming to want to help provide objective information, but actually driven by a hidden agenda.

Often, a person wanting scientific information, but distrustful of scientists, will head to the Internet, where there is a plethora of sites expounding either the virtues - or evils, of biotechnology. Few are scientifically impartial. One website I came across gave me great hope, the opening screen stating "This website was created to provide a scientifically based and impartial information on genetically engineered food …" until I scrolled down, as the sentence finished with "…in order to counterbalance the highly biassed (sic), distorted and incomplete information provided by the proponents for biotechnology." (Suurküla, 1998). No doubt the author sincerely believes he is impartial and objective himself.

Are scientists pandering to public paranoia by agreeing to participate in pseudo-scientific methodology? This is a main route by which credibility is lost and the public becomes suspicious of scientists and the scientific method. One example concerns the environmental assessments. Most jurisdictions have a regulatory process to scientifically determine the likelihood of escape of a particular transgene into the environment. Usually they require the proponent to provide direct data on the incidence of outcrossing in the species, on the frequency of local populations of wild relatives, on the degree of outcrossing between these related species, on the possibility of the cultivar having become more "weedy" due to the presence of the transgene through increased dormancy, aggressiveness, flowering characteristics, etc. These data are invariably required to be submitted in a scientifically valid format. The scientist proponents can conduct the experiments and collect the data, honestly say they have answered the questions fully and truthfully and usually conclude that the risk of transgene escape is low. The public is told of the stringency and scientific validity of the data and that the risk of escape is low. The public interprets this as an absolute, not as a relative, and so the conclusion may therefore be acceptable.

Credibility is lost when someone realises that nowhere in this process is any question concerning the chief means of genetic escape - seed spillage by the farmer, which happens on every cultivated field. The scientists and bureaucrats involved in the process are then discredited, the public feels deceived and more suspicious.

In another example, scientific credibility is claimed by opponents to technology to discredit "scientists". From another website comes this release, dated March, 1998: "Genetically engineered products that have proven to be damaging to health, even fatal, include: …a strain of soybeans was genetically engineered with a Brazil nut protein. Only recently was it discovered that the soybean caused a marked reaction to many people allergic to Brazil nuts." (Natural law party, 1998). The addition of a gene for an allergenic protein is, of course, a legitimate concern, but the implication that it had already caused damage to consumers, and that it was opponents to biotechnology that pointed it out, is not only incorrect, it is a gross manipulation of public perception. The concern over transgenic legumes carrying the brazil nut storage protein as potentially allergenic has been in the scientific literature for over ten years (McHughen, 1988).

It is obvious to me that the public can acquire credible scientific information only from credible scientists and credible science journalists.

Conclusions

The first transgenic crops are on the international market and more are coming. The temporal, emotional and financial costs of regulatory compliance is complicated and has certainly hampered the process, but it is moving forward; international harmonisation and rationalisation is occurring, albeit more slowly than many of us would like, while too quickly for others. The data collected from the thousands of stringent environmental releases of the past ten years provides assurance that, while individual products may require management or abandonment, the technology itself is not inherently hazardous. However,

the additional and often unnecessary costs associated with regulatory compliance ensure only the biggest and richest companies can commercialise their products. This deprives consumers and smaller organisations from the benefits of this technology, as the larger players abandon commodity crops and focus on less regulated products.

In order to establish and maintain public trust in science and scientists, it is important that the process used is scientifically sound. While we scientists have little expertise or influence over other factors - political, legal, ethical, etc., currently engaged in the debate over transgenic crops, we are responsible for scientific integrity. While sometimes reality requires us to comply with regulatory requirements, we must be careful not to jeopardise our integrity for political expediency by appearing to support scientifically unsound processes.

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Global commercial experience with transgenic cotton

Deaton, W.R.

Monsanto Company, 700 Chesterfield Parkway North, St. Louis, MO 63198, USA

Abstract

Over ten years after Monsanto made a commitment to develop transgenic cotton products, the *Bollgard* and *Roundup Ready* genes were brought to the market. The *Bollgard* gene was based on the coding sequence for the insecticidal protein from *Bacillus thuringiensis*. *Roundup Ready* gene gave the cotton plant the ability to tolerate over-the-top treatments with Roundup herbicide. *Bollgard* was first commercialized in 1996 in the USA, Australia and Mexico. It has since been commercialized in China, Argentina and South Africa. By the year 2001, it is anticipated that *Bollgard* will be commercially available in five more countries. *Roundup Ready* was first commercialized in 1997 in the USA and is expected to expand into seven more countries by 2001. In growers' hands, both provide a broad range of valuable benefits. As a consequence, farmers have accepted these products at unprecedented levels. Monsanto's experience with transgenic cotton has not been without its difficulties. When issues are raised in an open dialogue and regulations are based on science, progress can be made. Monsanto is committed to bring these and other valuable new transgenic products to cotton producers and those that use the products of their labors.

Business approach to transgenic cotton business

Monsanto began investing in plant biotechnology in the late 1970s. By the mid-1980s, Monsanto made a commitment to develop transgenic products for cotton. Cotton was obvious choice. It is grown on more than 30M hectares worldwide and is plagued by insects and weeds. On a per hectare basis, no crop is treated with more pesticides (insecticides, herbicides and fungicides) than cotton. Using results from the years of biotech research that preceded its commitment to cotton, Monsanto focused on two areas of interest: insect resistance and Roundup tolerance.

Cotton products

The key insect pests in cotton are members of the lepidopteran order. These include *Helicoverpa zea* (bollworm), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm) and *Helicoverpa armigra* (American bollworm). These insects are responsible for about 50-60% of the insecticide costs and treatments that are applied to cotton worldwide.

To address these pests with a transgenic product, Monsanto utilized a gene from *Bacillus thuringiensis* (*Bt*). The gene codes for a highly-active, lepidopteran-specific insecticidal protein. For the last 30-40 years, topically-applied insecticides based on *Bt* have been commercially available. The *Bt* proteins have been tested thoroughly and are known to be safe to humans, animals and non-lepidopteran insects. The proteins react with receptors specific to lepidopteran stomach and cause death as a consequence. Once developed and introduced into the cotton plant, Monsanto branded their *Bt* gene as *Bollgard*. With the *Bollgard* gene, the cotton produces its own insecticide.

Field testing of the *Bollgard* gene began in 1990. Since those initial efforts, the *Bollgard* gene has been evaluated in over 1,000 field tests in at least 12 countries. Based on these trials and commercial experience, *Bollgard* has demonstrated several significant benefits. These include:

1. Reduces or eliminates the need to treat with insecticides for these pests.
2. Controls insects that are resistant to current insecticides.
3. Allows beneficial insects to survive.
4. Increases potential for improved yields.
5. Reduces environmental impact of cotton production.

Uncontrolled, weeds are one of the largest threats to cotton production. Their importance forces growers to control weeds with either a broad array of herbicides or backbreaking hand labor.

Roundup is the most widely used herbicide in the world. It provides broad spectrum control with minimal environmental impact. However, without man's intervention, *Roundup* controls crop plants as well as it controls weeds. Before biotechnology, growers were forced to either treat *Roundup* when the crop was not present in the field or use application equipment that would keep sprays off the plants so it could be used once the crop was up.

With the tools of biotechnology, Monsanto identified a gene that gave crop plants the ability to tolerate *Roundup*. The gene codes for an alternate version of the enzyme targeted by glyphosate, the active ingredient in *Roundup*. The transgenic enzyme keeps on working in the presence of glyphosate, creating an alternate pathway to replace the deactivated native enzyme. This product is called the *Roundup Ready* gene.

The *Roundup Ready* gene was first tested in 1990. It has now been evaluated in over 500 field tests in more than eight countries. These trials along with grower experience have shown that *Roundup Ready* and *Roundup* yields the following benefits:

1. Increases weed control options.
2. Eliminates carryover issues.
3. Increases broad spectrum weed control.
4. Fits all cotton management systems.
5. Offers superior safety and environmental characteristics.

Commercial experiences

After years of product development research and safety testing, Monsanto obtained approval to commercialize *Bollgard* in 1996 in the USA, Australia and Mexico. Additional approvals to commercialize were given in China, Argentina and South Africa in 1998. By any measure, *Bollgard* was the most significant new product launch in the agricultural sector in the USA. Acreage and value created for growers far exceeded any product that had preceded it. Satisfaction and intent to repurchase was extremely high for a new product.

Similar results were observed with *Roundup Ready*. This product was first commercialized in 1997 in the USA. Additional countries are expected to approve *Roundup Ready* in 1999. Monsanto also began to offer cotton seed with both *Bollgard* and *Roundup Ready* genes in 1997. This combined product is also seeing great success by growers.

Prospects for the future

Additional regulatory approvals for *Bollgard* and *Roundup Ready* are anticipated in India, European Union, Brazil, Turkey and several other countries. Research efforts continue to identify and develop new transgenic traits for cotton. Monsanto is continuing to look for valuable agronomic traits like disease resistance and expanded insect resistance. However, there is also considerable effort aimed at improving cotton fiber to increase its value to processors and consumers of cotton goods.

Issues

The commercialization of transgenic cotton traits has not been without learning experiences. Monsanto needs to recoup its investment in biotechnology and invest in future products. As a consequence, it has been necessary to implement unique value capture mechanisms for these traits. These systems have generally been accepted in the marketplace, but not without some growing pains. Also, growers have accepted this technology, sometimes at a pace that exceeds their ability to understand how to manage it for the maximum benefit. Patience by growers and strong support from academics and extension have helped make the adjustment to these new production tools.

Perhaps the most important issues identified in the commercialization of transgenic cotton are in the public acceptance and regulatory arenas. It is obvious that the system works best when there is an open, transparent dialogue on the issues. Regulatory processes need to be science-based. It is important that they be free of political bias or abuse. When decisions are focused on providing the maximum benefits with reasonable risks, farmers can make the decision to use these products that work for them and bring competitive advantage to their country.

Summary

Bollgard and *Roundup Ready* were developed with great effort and investment by Monsanto Company. Both offer cotton growers excellent value by increasing and improving their crop management options. Commercial experience with these products has often led the way for other transgenic products, giving the market a glimpse of what biotechnology can and will do for agricultural production. There have been growing pains in commercializing these products, but commitment by industry, academics, regulators and growers has shown just what can be created when science works to the benefit of everyone.

Experience in the development of low-protein Japonica-rice

Maruta, Y.[1)], Ueki, J.[1)], Saito, H.[2)], Ohyama, A.[1)], Nitta, N.[1)]

[1)] Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., 700 Higashibara, Toyoda Iwata, Shizuoka 438-0802, Japan;
[2)] Applied Plant Research Laboratory, Japan Tobacco Inc., 1900 Idei Oyama, Tochigi 323-0808, Japan

Summary

To produce a rice variety suitable for both sake brewing and eating, we introduced a new antisense construct for glutelin, a major storage protein in endosperm, into rice by Agrobacterium-mediated transformation. Two transformed lines carrying the antisense glutelin gene in a homozygous state were selected. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the glutelin content of the transformed lines was lower than that of wild-type plants. The transgenic rice plants were evaluated for environmental safety according to guidelines set by the government of Japan. As greenhouse assessment and isolated field assessment of the transgenic lines and assessment of wild-type rice gave substantially equivalent results in all evaluation items, field trials have started this year.

Introduction

Highly polished rice grains are essential for the production of Japanese rice wine "sake". The extent of polish sometimes reaches 65% in superior classes of sake. The reason for the polishing is to decrease the protein content in the rice grains. Excess protein often gives sake a rough taste, deepens the color, and accelerates deterioration of quality after production (Yoshizawa and Kishi, 1985).

About 80% of rice storage protein is glutelin, which is localized in Protein Body II (Tanaka et al. 1980). During brewing, Protein Body II is digested more readily than Protein Body I, which contains prolamin, another type of storage protein (Kisaki et al. 1990). There is a negative correlation between the suitability of rice grains for sake brewing and the amount of total protein, and between the suitability for brewing and the amount of Protein Body II (Wakai, 1993).

Steamed rice is an essential food for the Japanese people. The taste of steamed rice is related to protein content (Ishima et al. 1974): low-protein rice tastes better.

To develop a Japonica-rice variety better suited to both brewing and eating, we have tried to suppress the accumulation of glutelin in rice grains. In a previous attempt (Ajisaka et al. 1994), an antisense construct with the glutelin promoter and cDNA was introduced into a Japanese variety by the electroporation method. However, the selected line did not show very much reduction in glutelin.

In 1994, we generated a new construct and introduced it into a Japonica-rice variety, Tsukinohikari, by Agrobacterium-mediated transformation. After three phases of safety assessment in greenhouses and in an isolated paddy field, the two transgenic rice lines are now growing in a normal paddy field this year. In this paper, we report the production process for the transgenic rice and results from each phase of safety assessment.

Gene construction and transformation of rice plants

Our preliminary study with *in vitro* translation implied that antisense RNA transcribed from 8 tandem repeats of the 5'-end fragment of the glutelin gene can inhibit the translation of the sense RNA more efficiently than a full-length, single-unit antisense RNA. Another study using stable transformants revealed that the reduction of glutelin can be enhanced by the first intron of the castor bean catalase gene (Ohta et al. 1990).

Rice glutelins are coded for by a small multi-gene family consisting of 2 subfamilies, A and B (Takaiwa et al. 1991). A full-length glutelin cDNA (1617 bp) belonging to subfamily A was isolated from a cDNA library constructed from immature endosperm of a Japonica-rice variety (*Oryza sativa* cv. Sasanishiki). A DNA fragment (312 by or 289 bp) at the 5' end of the full-length cDNA was cut out by restriction enzymes and assembled to make 8 fragments repeated in tandem. The glutelin promoter region (829 bp) was amplified by PCR with a pair of primers whose design was based on a subfamily A sequence (Takaiwa et al. 1987). The antisense construct was assembled from the glutelin promoter region, the first

intron of the castor bean catalase gene (Ohta et al. 1990), the antisense orientation of the tandem repeat, and the NOS terminator, in that order. The assembled gene was inserted into the T-DNA region of intermediate vector pSB24 (Komari et al. 1996). A hybrid vector was constructed by homologous recombination with acceptor vector pSB4 (Komari et al. 1996), which contained a hygromycin resistance (HPT) gene, and subjected to Agrobacterium-mediated transformation (Hiei et al. 1994) of calli derived from Japonica-rice (cv. Tsukinohikari). After transformation, the calli that proliferated on medium containing hygromycin were selected, and plants were regenerated as described by Hiei et al. (1994).

Selection process

S_o generation: The antisense gene and the HPT gene were located on distinct TDNAs in the hybrid vector; each gene can be integrated into the rice genome independently. All of the 106 regenerants showing hygromycin resistance were PCR-analyzed to detect the antisense gene. The 101 PCR-positive regenerants were further analyzed by Southern hybridization with the 5'-end of the full-size glutelin cDNA as a probe for estimation of the copy number. We selected 29 regenerants possessing fewer than five copies each.

Glutelin in the S_1 grains of the 14 fertile transformants was measured. Grains were ground to powder, and 50 mg of the powder was suspended in 1.5 ml of buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 4 M urea, and 5% 2-mercaptoethanol. Total protein, extracted by shaking overnight, was subjected to SDS-PAGE. Proteins were made visible by staining with Coomassie brilliant blue R-250. The density of the glutelin bands was measured by using a GS-670 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA). Out of 14 regenerants, H39 and H75 showed remarkably small amounts of glutelin, and their progeny were raised.

S_1 generation: Sixty descendants of H39 and 20 of H75 were analyzed by PCR to reveal the segregation of the antisense gene. The results implied that three copies in H39 segregated as two Mendelian factors and that four copies in H75 segregated as a single factor. H39-2, -56, and -59 were selected because of the existence of the antisense gene and the absence of the HPT gene. H39-59 was normal in growth and fertility and was selected for further study, but H39-2 and H39-56 showed low fertility. No H75 progeny showed segregation between the antisense and HPT genes, indicating that both genes were closely linked in the H75 genome. H75 progeny were further analyzed because of their remarkable decrease in glutelin content.

S_2 generation: Twenty plants were raised from H39-59. Progeny analysis by PCR and Southern hybridization showed that H39-59 was homozygous for the antisense gene and that H39-59 had lost one copy of the antisense gene as well as the HPT gene. Progeny from H75-3, -17, and -18 were also raised. Progeny analysis confirmed that these plants were homozygous for four copies of the antisense gene and for one copy of the HPT gene.

S_3 generation: Fifteen lines were raised from 15 S2 plants of H39-59 (H39-59-1 to H39-59-15). Three S3 bulks from H75-3, -17, and -18 were also raised.

Glutelin mRNA content in the immature grains of the transformants

S_1 progeny of H75 were grown, and panicles were harvested seven days after flowering. Total RNA was extracted and analyzed by Northern hybridization. Plants possessing the antisense gene expressed less mRNA. Northern analysis of 15 S_3 lines of H39-59 and 3 S_3 bulks of H75 grown in the isolated paddy field showed again an extreme decrease in glutelin mRNA.

Glutelin content in the grains of the transformants

In a typical SDS-PAGE of rice protein, several bands are strongly stained by Coomassie brilliant blue R-250: a glutelin precursor at 57 kDa, acidic subunits of glutelin at 37-39 kDa, globulin at 26 kDa, basic subunits of glutelin at 22-23 kDa, and prolamin at 1013 kDa. It appears that acidic and basic subunits of glutelin are composed of at least 3 bands.

The SDS-PAGE protein profiles of transformant seeds were different from those of wild-type seeds. A few bands in each acidic and basic subunit were thinner, but the lowest band in each subunit was the same or slightly thicker. Prolamin bands were also thicker.

The density of glutelin bands of H39 and H75 descendants was lower than that of the wild-type. S4 seeds, harvested from H39-59-6 and H75-3 in the isolated field, showed glutelin band density of 61% and 35%, respectively, of that of the wild-type.

Proteins from SS brown rice of H39-59 lines grown in the isolated field last year were fractionated according to Iida et al. (1993). Kjeldahl analysis revealed that the total nitrogen of the glutelin fraction of H39-59 lines was lower than that of the wild-type, although the total nitrogen in the whole brown rice grains of H39-59 lines was almost the same as that of the wild-type.

These results imply that glutelin synthesis and accumulation were clearly inhibited by the antisense gene and that prolamin possibly increased, resulting in little change overall in total protein content in the transformants. Iida et al. (1997) reported that a mutant line lacking the 3 minor bands of the glutelin acidic sub-units showed a 13% decrease in glutelin content but that total protein content did not change. It is possible that inhibition of synthesis of a protein results in a compensatory increase in other proteins.

Vector sequence integrated with T-DNA

Recently, some papers have reported that not only TDNA but also vector sequences are occasionally transferred into plants (Kononov et al. 1997). In such cases, transfer starting at the right border did not end at the left border but continued along the vector sequence downstream of the left border.

We investigated H39-59 (S_3) for the vector sequence by Southern hybridization and PCR. The plant was found to possess about 10 kb of the vector sequence downstream of the left border. The vector sequence accompanied one of the two copies of the antisense gene. Transcription of the genes in the sequence, which were controlled by bacterial-type promoters, was unlikely. Northern analysis with 4 probes, which covered the sequence, indicated no transcription of these genes at the ripening stage. H75 was concluded not to possess the vector sequence.

Safety assessment under greenhouse conditions

H75 S_1 progeny were examined by PCR for presence of the antisense gene, and positive plants were grown in a contained greenhouse for the first stage of safety evaluation experiments (Stage I). S_2 plants from H39-59 were raised and grown in a semi-contained greenhouse for the second stage (Stage II) and compared with wild-type plants for the following characteristics:

- Growth characteristics of transformed lines
  Culm length, panicle length and panicle number were measured. There were two differences: the average panicle length of H75 was shorter than that of wild-type rice in Stage I, and the average culm length of H39-59 was longer in Stage II. These variations are likely to be due to somaclonal variation.
- Reproductive traits of transformed lines
  S_1 progeny of H75 and wild-type rice were monitored in Stage I for anther size, pollen fertility, pollen dispersal, and frequency of outcrossing. Pollen fertility was judged by staining with iodine potassium iodidesolution. Pollen dispersal was measured at the time of pollen dehiscence as the number of pollen grains trapped on a glass slide at a given distance. Pollen dispersal was stimulated by an electric fan. For the assessment of the frequency of outcrossing, recipient plants were completely emasculated by hot water treatment, and the upper part of the glume was cut. Pollen from either transformants or wild-type plants was forced to disperse by an electric fan. After the forced pollination, the percentage of seed set was calculated. No differences were observed in any of these traits.

Production of new toxic substances

Leaves were homogenized in a mortar and pestle and steam-distilled. After addition of excess NaCl to the distillate, volatile components were extracted with ether. The ether was removed and the extract was dissolved in ethyl-acetate. The ethyl-acetate fraction was subjected to gas chromatography. The chromatogram did not show any peaks specific to S_1 progeny of H75 in Stage I.

For analysis of compounds that may be secreted from roots, two months old plants were transferred to a hydroponic system. After 30 days' culture, the medium was collected, adjusted to pH 3.0, and

fractionated with ether. After removal of the ether, the extract was dissolved in methanol and used for HPLC analysis. There was no appreciable difference in HPLC patterns between S_1 progeny of H75 and wild-type rice in Stage I.

For analysis of compounds that may be synthesized in leaves, leaves were homogenized in a mortar and pestle, mixed with 50 mM phosphate buffer (pH 7.0), and gently shaken. After removal of the debris by filtration and centrifugation, compounds larger than 10 kDa in the extract were removed by ultra-filtration. Small compounds of less than 10 kDa were analyzed by HPLC. The chromatogram did not show any peaks specific to S2 progeny of H39 in Stage II.

Soil on which either transformants or wild-type plants were grown for the entire growth phase was used to grow normal rice plants. In addition, leaves and culms from either transgenic or wild-type plants were mixed with normal soil, and seedlings of rice were transplanted onto it. In each experiment, there were no differences in culm length, panicle length, number of panicles, or dry weight of plants.

Ability to become weeds

Cold-tolerance of seedlings, germination ability, shattering habit, spikelet number per panicle, and fertility were compared in Stage II. There were no differences between transformants and wild-type plants in any of these traits.

Effects on microflora

Soil on which transgenic plants were grown was collected and mixed with water. The solution was applied to culture media for fungal or bacterial growth analysis. After five days' incubation, the colonies were counted. There were no significant differences between the numbers of colonies formed on these media and those formed on media prepared with soil from normal rice plants. This implies that no appreciable effects on soil microflora were exerted by growing the transgenic plants.

Safety assessment in an isolated field

After approval from the Ministry of Agriculture, Forestry and Fisheries (MAFF), S3 lines of H39-59-1 to -15 and S3 bulks of H75-3, -17, and -18 were transplanted to an isolated field in our laboratory in the summer of 1997 (Stage III).

Growth characteristics of transformed lines

The following traits were monitored: heading date, culm length, panicle length, panicle number, and fertility at reproductive growth stage. Heading dates were between two days earlier and four days later than in the wild-type. Lines H39-59-3, -9, and -11 appeared to be segregated in heading date and were excluded as candidates for the normal paddy field trial. Monitoring at the reproductive growth stage revealed that culm length of H39-59-15 and panicle length of H39-59-10 and H75-17 were significantly different from those of Tsukinohikari. These three lines were also excluded as candidates. The posture of H39-59-6 at ripening was most similar to that of the wild-type, and that of H39.59-1 was next, and these lines were selected. H75-3 was also selected because of slightly lower glutelin content than other H75 bulks.

Outcrossing frequency

Four transgenic (S_4 of H39-59) or wild-type plants were placed in the center of a plot. Male-sterile Tsukinohikari plants (12 clumps) were arranged in a cross. The fertility of the male-sterile Tsukinohikari plants in the H39-59 plot was 2.1%, and that in the wild-type plot was 1.7%; there was no difference in outcrossing frequency between them.

Ability to become weeds

Overwintering of rice stubble in an isolated paddy field, germination ability, shattering habit, and fertility were assessed. There were no differences between transgenic and wild-type plants in any of these traits.

Influence on the environment

Paddy soil in the isolated field was collected at 0, 4, 8, and 12 weeks after transplanting. Bacterial and fungal densities were estimated as described above. Insects were collected from transgenic and Tsukinohikari plots at heading and ripening stage. Weeds were also collected at heading stage. No differences were observed in any of these traits.

Field trial

After approval from MAFF in the spring of 1998, S_4 seedlings of the transformants were transplanted in normal paddy fields at three locations: the National Agriculture Research Center, Ibaraki; Mie Prefectural Agricultural Research Center, Mie; and our laboratory in Shizuoka. The paddy field at our laboratory is 300 m^2. The lines being grown there are H39-59-6, H39-59-1, H75-3 bulk, wild-type Tsukinohikari, and the control, Nipponbare. At the other locations, H39-59-6 and Tsukinohikari are being grown, in a field of about 30 m^2. In these locations, preliminary performance tests are under way; evaluated traits are heading and maturation dates, culm length, panicle length, panicle number, yield and others.

Conclusions

Results from these three phases of safety assessment indicate that this transgenic material is substantially equivalent to an ordinary rice variety developed through conventional breeding in all traits measured except the targeted trait, which is the reduction of glutelin in the grains. For other practically important traits such as productivity, we will analyze this year's products in field trials. Grain proteins will be further analyzed. The amount of glutelin will also be measured in grains with differing degrees of polish for the examination of suitability for sake brewing.

We will proceed with safety evaluation for food products according to the guidelines set by the Ministry of Health and Welfare in Japan.

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Cultivation of herbicide resistant crops: Weed management and environmental aspects

Hurle, K., Petersen, J.

Hohenheim University, Institute of Phytomedicine, Weed Science Department, 70593 Stuttgart, Germany

Abstract

Herbicide resistant crops will make weed control simpler and provide additional options which fit well in new concepts of weed control. The possible transfer of resistance genes to weedy relatives, the increased probability of selecting herbicide resistant weeds and the problem of volunteers need to be considered in the management of weeds, especially if such crops gain large acreages and only few compounds are used/available. For these reasons and for reasons of environmental contamination with herbicides it is important that the farmer does not depend on a one-herbicide-only control strategy.

Introduction

Today, the use of herbicides is by far the most widely used method of weed control in developed countries and has, to a large extent, replaced other control methods. But also in less developed countries, chemical weed control is gaining more and more importance. The reasons are: with herbicides, large acreages can be handled in a minimum of time at reasonable costs. Further, there are solutions available for most of the weed problems in all major crops, and, very important, in most cases herbicides work reliably, so the farmer can depend on it.

The herbicides presently used for weed control in crops are selective herbicides, i.e. they kill weeds and are safe to the crop. The selectivity of such herbicides often is limited to one crop or group of crop species and is based on natural tolerance of the crop(s) to the specific herbicide. However, this tolerance is not necessarily a common characteristic throughout the crop species, but can differ between cultivars, and thus limit the use of the compound. In general the efficacy of selective herbicides is not broad spectrum, i.e. depending on the compound certain weed species are not controlled. With the new technology of herbicide resistant crops (HRCs), a herbicide can be used in crops which have no natural tolerance but have been made resistant to the herbicide. This approach is mainly used for non-selective compounds in order to have a broader spectrum of weed control. The main difference between the conventional herbicide concept and the HRC-technology is that with the conventional concept an adequate crop safety for the herbicide has to be optimized in the screening process, while with the HRC-technology a target crop is tailored to the herbicide and usually achieved by means of genetic engineering. With this approach herbicide producers and seed companies are trying to expand their respective market share.

At present the non-selective herbicides glyphosate and glufosinate are the dominating compounds in this respect. For glyphosate, resistant varieties of soybeans, cotton and canola, and for glufosinate, resistant varieties of maize and canola are already available. Further resistant varieties are being developed as for oilseed rape, sugar beet and rice. It is quite clear that there is an enormous potential for this technology, and the rapid increase in acreage of herbicide resistant crops is evidence enough for the acceptance of this technology by the farmers. However, there is still an ongoing debate on the pros and cons of this new tool in weed control.

In the following the consequences of HRCs for weed control and the environment will be discussed.

Weed control in HRCs: What is the difference to conventional chemical control?

In general it is expected that with HRCs weed control will be simpler and more advantageous than with the conventional chemical control. The main reasons for that are:

- Weed species which either cannot be controlled effectively with the conventional system or only with additional efforts, e.g. herbicide mixtures, may no longer be considered as problem weeds. This includes also parasitic and crop-related weeds.
- With conventional weed control a proper timing of the herbicide application is essential, otherwise control efficacy is reduced and/or the crop is damaged. With HRCs the herbicide can be used at any

growth stage which provides more flexibility in timing. This can be crucial if, e.g., unfavourable weather conditions delay the herbicide application.

- Weeds which have become resistant to conventional herbicides can be controlled with the corresponding HRC-herbicide. HRCs thus provide an additional tool in the management of herbicide resistant weeds.
- Although conventional herbicides are considered as selective, their selectivity usually is not complete, i.e. the crop may suffer somewhat from the herbicide which can result in lower yields. With HRCs there is a potential for further improved selectivity and ultimately higher yields.
- With conventional weed control pre-emergence herbicides still have to be used in some crops as post-emergence compounds are not available, and therefore economic thresholds cannot be applied. With HRCs the herbicide is used post-emergence and the control decision can be based on economic thresholds.
- It is expected that the HRC-technology will contribute to further develop reduced tillage and no-till systems, since weed control and mulch management will be easier and more effective (Ammon et al. 1995). This could substantially help to reduce soil erosion which is one of the biggest problems in world agriculture.
- An aspect one has not paid enough attention to, partially because it is difficult to practise in conventional weed control, is the concept of period thresholds. With period thresholds, weeds are controlled at the time when they interfere with the crop and cause damage (critical period). At present the timing of the herbicide application is determined mainly by the growth stage of the crop and weeds, irrespective of the critical period. Due to a more flexible timing, HRCs will facilitate control according to period thresholds. Period thresholds have already been developed for some major crops as maize (Koch and Kemmer, 1980; Zimdahl, 1988), sugar beets (Scott and Mosey, 1972; Dawson, 1986), rice, soybeans and vegetables (Zimdahl, 1988).
- The new future-oriented development of precision weed control which takes into account the spatial variability of weeds via patch treatment or sensor-driven systems could promote HRCs, and *vice versa* (Hurle and Kunisch, 1997; Hurle and Walter, 1998).

In summary: In comparison to conventional chemical weed control the HRC-technology offers some additional benefits and fits quite well in new concepts and developments in weed management. Provided this technology is cost-effective, it could replace conventional systems to a great extent.

What are the risks?

Outcrossing of the herbicide resistance gene(s): A problem for the environment or the farmer?

Lateral gene transfer, i.e. hybridization via pollen between related plant species or between different varieties of the same species, is a common natural process. In plant breeding this can be a problem which breeders take into account in their breeding programme. It has been demonstrated that in absence of a specific herbicide selection pressure, plants with a herbicide resistance gene showed no greater fitness than plants without this gene (Crawley et al. 1993; Kareiva et al. 1996; Thill, 1996). Such plants are not weedier and more invasive than susceptible biotypes, and are no super weeds. Nevertheless it is important to know which species can hybridize with a herbicide resistant crop, as the outcrossing of herbicide resistance is a new way in creating herbicide resistant weeds.

A lot of research has been done during the last few years in order to find wild plant species which are able to hybridize with transgenic crops under field conditions. The Table presents some examples for relevant crop-weed combinations which produce viable and fertile F1-generations. However, the probability of the occurrence of F1-generations is very low. In addition, the survival rate of the hybrids is reduced and only a small percentage is able to reproduce. This applies also to hybrids of crops and their non-weedy wild relatives which *per se* are not of an agricultural importance (for an overview see Keeler et al. 1996). Although until now not all hybridization partners are investigated, the chance for crop-weed hybrids to appear as herbicide resistant weeds seems to be rather small. If crop-weed hybrids do appear it will reduce the advantages of HRCs, and we end up in a situation comparable to the conventional chemical weed control, where close relatives of the crop usually cannot be controlled by selective herbicides either. So far we have no experience of how much of an agronomic problem outcrossing of

herbicide resistance genes to wild relatives might be, and to what extent it will interfere with the HRC-concept.

Table Possible partners for hybridization between crops and weedy relatives

Crop	Weedy relatives	Reference
Oat (*Avena sativa* L.)	Wild oat (*Avena fatua* L.)	Dyer et al. 1993*
Oilseed rape (*Brassica napus* L.)	Turnip (*Brassica rapa* L.)	Downey, 1992
	Chinese mustard (*Brassica juncea* (L.) Czern.)	Downey, 1992
	Hoary mustard (*Hirschfeldia incana* (L.) Lagreze-Fossat)	Darmency et al. 1995
	Wild radish (*Raphanus raphanistrum* L.)	Darmency et al. 1995
	Wild mustard (*Sinapis arvensis* L.)	Leckie et al. 1993
	Dog mustard (*Erucastrum gallicum* O.E. Sch.)	Chèvre et al. 1997
Rice (*Oryza sativa* L.)	Red rice (*Oryza rutipogon* Griff.)	Dyer et al. 1993*
Sorghum (*Sorghum bicolor* (L.) Moench)	Johnsongrass (*Sorghum halepense* (L.) Pers.)	Dyer et al. 1993*
Sugar beets (*Beta vulgaris* ssp. *rapacea* (L.) Döll)	Wild beet (*Beta vulgaris* ssp. *maritima* (L.) Arcang.)	Boudry et al. 1993
Sunflower (*Helianthus annuus* L.)	Bolander's sunflower (*Helianthus bolanderi* A. Gray)	Heiser, 1976
	Prairie sunflower (*Helianthus petiolaris* Nutt.)	Heiser, 1976
Wheat (*Triticum aestivum* L.)	Wild wheat (*Aegilops cylindrica* L.)	Mallroy-Smith, 1996

*Review

In this context it is worth mentioning that for genes controlling the natural herbicide tolerance there are no reported cases of outcrossing in related weed species (Dyer et al. 1993). The reason probably is that the natural herbicide tolerance depends on more than one gene or is cytoplasmatically controlled, and thus a gene flow via pollen is improbable and not possible, respectively.

In summary: There is a limited chance for outcrossing of herbicide resistance genes to wild relatives. For species which have no relevance as weeds this is neither an ecological nor an agronomical problem. Wild relatives which became resistant and are occurring in crops cannot be controlled anymore with the corresponding herbicide. This will limit the advantages of the HRC-technology but does not pose a problem the farmer would not be able to handle.

Herbicide resistant weeds: An increasing problem with herbicide resistant crops?

There are three ways for herbicide resistant weeds to develop: a) selection of resistant biotypes, b) outcrossing of resistance genes, and c) survival of seeds or other propagules of resistant crops in the soil; b) and c) being specific to HRCs.

The number of weeds possessing herbicide resistance is increasing. More than 150 species are reported as resistant throughout the world (Rubin, 1996). The occurrence of herbicide resistance in weed populations is generally associated with a high selection pressure imposed by high frequency of use, high dosages, long-time use and long-lasting soil activity of the compound. Out of these factors, the repetitive use is the main cause for the selection of insensitive, i.e. resistant biotypes. In principle the selection pressure in HRCs is not bigger than with conventional chemical weed control. However, it will be increased if in a crop more than one treatment is needed to obtain sufficient control, and/or several HRCs resistant to the same herbicide are cultivated on a farm. Furthermore, the frequent use of a herbicide usually leads to a shift in the weed flora towards such species harder to control. In order to get these plants controlled, farmers often increase the herbicide dose, and thereby also increase the selection pressure. However, not all herbicides have the same potential for selecting resistant weed biotypes, and it is not possible to predict reliably how long a compound can be used in a field until resistant biotypes will occur (Subramanian et al. 1996; Heap, 1997).

Although the possibility of outcrossing of the resistance gene(s) to wild relatives is generally considered to be low (see above), it contributes to the occurrence of resistant species.

Crops can infest subsequently planted crops if their propagules get into the soil and develop into plants. Such volunteer plants are considered weeds. Some are posing real problems as, e.g. maize in soybeans, oilseed rape in sugar beets, and small grains in oilseed rape. A more serious volunteer problem could result from the transfer of the resistance trait to the same crop species especially if the recipient variety is resistant to another herbicide (multiple intraspecific resistance). This could happen with neighbouring fields of easily hybridizing crop species. Then the volunteers would be resistant to two herbicides. If herbicide resistant volunteers develop into a weed problem they need special consideration for their control, which in turn will increase the costs. To avoid these problems farmers have to make sure not to use different herbicide resistant crops with resistance to the same herbicide in crop rotations.

Finally, there are many possibilities for an unintentional distribution of seeds of HRCs, e.g. by agricultural machinery to uninfested fields, where these volunteers could create a problem if another HRC resistant to the same herbicide is cultivated. Oilseed rape could serve as a good example for unintentional distribution, since under practical conditions it is not very likely for the farmer to spend enough time for cleaning the combine from the small seeds of this crop.

It seems that in this context volunteers will be the main agronomic problem, followed by the "normal" selection of herbicide resistant weeds, and the emergence of herbicide resistant wild relatives by hybridization.

In summary: It is very likely that with HRCs herbicide resistant weeds, including resistant volunteer crops, will be an increasing problem. To avoid this problem the farmer must be aware of the possible risks, and take them into account in his weed management strategies. The main strategic element must be an appropriate crop and herbicide rotation.

Increased use of HRCs: A herbicide problem for the environment?

Due to the very high registration standards for pesticides, only environmentally benign compounds get on the market, and the specific herbicides for HRCs are no exception. However, there is a correlation between the intensity a compound is used and the probability of the compound to become conspicuous in the environment, e.g. in ground, surface and rain water, and in the atmosphere. In the context with HRCs this could be the case if for a single herbicide several major resistant crops are available, and farmers plant the crops and use the herbicide on large scale, because the system is cost-effective and provides good weed control. Atrazine is an example for it. But it could apply also to environmentally safer compounds if they were used year after year on large scale, and therefore finally do no longer meet the safety standards set by the society (Hurle, 1996). From this point of view it is highly desirable not to depend too much on a one-herbicide-only strategy.

In summary: With HRCs there is a risk for environmental problems if the system depends on a single/few herbicide(s) and such crops are cultivated on large areas.

Conclusions

Until now there is no long-term experience with HRCs. While the advantages for weed control are quite obvious, the risks in connection with hybridization, selection of herbicide resistant weed species and volunteers, and environmental contamination are clear in theory but need to be confirmed under practical conditions in order to obtain a real estimate of the risk imposed by HRCs to cropping systems and the environment. It seems quite clear that the risks increase with the extent to which a HRC is cultivated and the corresponding herbicide is used. Besides careful consideration of the possible risks involved with the introduction of HRCs during registration, it is recommended to follow HRC-systems in a post-registration monitoring programme in order to take action if necessary. This new technology requires more strategic planning of the management of weeds and of cropping systems in general. Especially for developing countries with less experience in chemical weed control this could be a crucial point.

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Bringing monitoring concepts into practice – case study: Post commercial release monitoring of glufosinate tolerant oilseed rape

MacDonald, R.L., Deschamps, R.J.A.

AgrEvo, 295 Henderson Dr., Regina, Saskatchewan, S4N 6C2, Canada

Abstract

By early 1995, AgrEvo had received all approvals required in Canada for the cultivation of glufosinate tolerant oilseed rape and consumption of derived products. AgrEvo conducted the first commercial release of a recombinant oilseed rape with the cultivation of 8,000 hectares under an "Identity Preserved" program in Canada. The fate of the oilseed rape following its commercial release was monitored indirectly through the interaction of AgrEvo staff, government agrologists, and other stakeholders within the farming community. In addition AgrEvo voluntarily conducted a systematic survey in 1996 and 1997 to assess the occurrence and fate of glufosinate tolerant oilseed rape volunteers and weedy relatives in production fields. The survey was conducted at 10 sites and included, at each site, the field where glufosinate tolerant *Brassica napus* cv. "Innovator" oilseed rape was grown in 1995, adjacent fields, non-cropped areas in the vicinity of Innovator fields, and transportation routes leading away from the survey site. A smaller number of sites where non-transgenic oilseed rape was grown were included in the survey for comparison purposes. Volunteer oilseed rape plants were effectively controlled by the weed control program employed by the producer regardless of whether they were transgenic or not. No weedy relatives possessing the glufosinate tolerance trait were found. This result indicates that within the surveyed area outcrossing did not occur, and certainly did not create any new weed control challenges. Very few oilseed rape volunteers were found growing in non-cropped areas such as fencelines or ditches along the margins of fields where oilseed rape was grown in 1995. Those found growing in non-cropped areas in the vicinity of the 1995 Innovator fields were shown to be glufosinate tolerant. Oilseed rape plants were found growing in ditches along roads leading away from survey sites, however, none were glufosinate tolerant. Based on the results of the survey and a total lack of any identified adverse effects following the commercial introduction, no evidence was found to suggest that glufosinate tolerant oilseed rape behaves any differently in managed and unmanaged environments than does standard, non-transgenic oilseed rape. Furthermore, no adverse effects or weed control problems with volunteer populations were identified by growers who had cultivated glufosinate tolerant oilseed rape. Proper management practices are the key to controlling or containing volunteer oilseed rape (transgenic or otherwise) and its weedy relatives. In light of the collected evidence further direct survey monitoring of subsequent glufosinate tolerant oil seed rape lines should not be necessary.

Introduction

Environmental risk assessments of transgenic crops are considered on a case by case basis. It is recognised that post commercialisation monitoring can form an integral part of ensuring the safe introduction of modified crops into the environment. Following an approval for the release of a transgenic crop, the scope and scale of any post commercialisation monitoring must also be considered on a case by case basis. Monitoring of transgenic plants begins early in the research phase and continues throughout the developmental phase utilising procedures to check and guarantee the high quality of transgenic oilseed rape seeds during the development of new cultivars. These processes include:

1. Specific PCR primers are used at each stage of plant breeding to assure the purity and identity of the seedlots.
2. Routine seed quality analyses are conducted at each stage of production both in-house and by independent third parties as part of the variety recommendation process to ensure quality standards are met or exceeded.
3. Seed certification (i.e. inspection of production fields, seed purity and germination) is carried out by independent third parties.
4. Monitoring of transgenic plant field trials in compliance with confined release field trial guidelines and seed trade association guidelines.

The sales and marketing department provides an important contribution to the monitoring of introduced transgenic crops. AgrEvo Canada Inc. maintains a sales infrastructure that conducts over 7,000 on site farm visits on an annual basis. In addition, approximately 5,000 farmers across western Canada participate in company-sponsored field days and training seminars. Approximately 40,000 farmers are contacted on an annual basis to discuss their farming practices and satisfaction with the products that they are using for crop production. All farmers have access to a toll free number which receives greater than 8,000 calls regarding crop production practices. Despite this high level of communication with customers, there have been no incidents or complaints documented regarding problems with the control of glufosinate tolerant oilseed rape in subsequent years. Farmers who feel reluctant to contact the company directly have access to provincial agrologists, who serve extension roles throughout the prairie. Agriculture Canada also conducts surveys of weed populations across the prairies to monitor the status of individual species in the prairies. This system allows for a transparency of any emerging weed control issues. Furthermore, it is also in the best interest of the farmer and the crop production industry to manage herbicide resistance issues as carefully as possible to ensure the long-term viability of its products.

There are many components to a program which monitors the development and release of a transgenic crop. The final tier to a monitoring strategy utilizes a systematic direct survey. Monitoring the release of low hazard GMOs with a history of cultivation and no adverse effects risks to the environment would not require a direct survey monitoring study. Objectives could often be met by the monitoring that is conducted routinely during the development and release as described above. The company is required under the terms of the release to immediately notify the regulatory agency of any unexpected or adverse effects as a consequence of the cultivation of the modified crop. A direct survey should only be required if it is necessary to confirm any significant assumptions that are made in the risk assessment, it continues to be necessary to confirm the validity of the risk assessment procedure or where significant risks have been defined and it is necessary to control the release.

Where it is deemed that monitoring is necessary, any monitoring plan needs first to be practicable and based on sound scientific principles with clear objectives tied to the risks identified during the risk assessment procedure. It must be further emphasised that the concept of monitoring should not be limited to direct survey and sampling techniques.

Strategy for monitoring: Release of glufosinate tolerant oilseed rape

In 1995, glufosinate tolerant oilseed rape (*Brassica napus* cv. “Innovator”) developed by AgrEvo was grown on an estimated 8,000 ha under an “Identity Preserved” program in the province of Saskatchewan. By 1996, the acreage of Innovator oilseed rape increased to approximately 150,000 hectares.

In the development of glufosinate tolerant oilseed rape cultivars, the potential for the crop to become a future weed problem has been expressed as a concern. Experiments conducted in the field by AgrEvo designed to investigate the potential for glufosinate tolerant oilseed rape to become a weed have shown that the genetically modified oilseed rape is no more invasive or competitive than standard oilseed rape. Therefore the transgenic crop is no more likely to become a problem weed than are standard oilseed rape varieties (MacDonald and Manning, 1996). Agriculture and Agri-Food Canada (AAFC), in assessing the environmental impact of glufosinate tolerant oilseed rape, have concluded that the herbicide tolerance trait has not bestowed any adaptive advantage on glufosinate tolerant oilseed rape in the absence of the herbicide as a selection pressure.

The potential for the glufosinate tolerance trait to outcross into wild species related to *Brassica napus* creating a new herbicide tolerant weed has also been expressed as a concern. Experiments conducted by AgrEvo and others have shown that *Brassica napus* will indeed outcross with related species (MacDonald and Manning, 1996). However, the frequency of outcrossing is extremely low and the viability or fertility of hybrids is very poor. In addition, the very low numbers of fertile hybrids can be controlled chemically by many other active ingredients or mechanically by cultivation. Given the above, AAFC has concluded that while gene flow from glufosinate tolerant oilseed rape to relatives is possible, it would not result in increased weediness or invasiveness of these relatives. Recent work by Snow et al. (1998) has also confirmed that the PAT protein in the absence of the selection pressure of the herbicide confers no fitness advantage to either the crop or to related species that it has been introgressed.

Furthermore, "Herbicide resistant plants are the GMOs that have been most widely released in all of the examined countries. No direct risk of harm to the environment has been identified for these plants in the examined countries". (A Comparative Analysis of Releases of Genetically Modified Organisms in Different EU Member States; Nöh, 1995).

Despite the overwhelming body of evidence indicating the safety of glufosinate tolerant rapeseed, it was decided to provide an additional confirmation of the risk assessment procedure by collecting direct survey monitoring data under a multi-site production scale scenario. Beginning in 1996, AgrEvo voluntarily undertook a survey of 1995 glufosinate tolerant oilseed rape production fields, adjacent non-agricultural areas, and transportation routes to document the occurrence and fate of *Brassica napus* volunteers and related species such as *Sinapis arvensis* and *Brassica rapa*.

The objectives of the voluntary survey conducted by AgrEvo were to assess the occurrence and fate of glufosinate tolerant *Brassica napus* volunteers, and to assess the occurrence and fate of weed species related to *Brassica napus*. Of primary interest were weedy relatives that may have received the glufosinate tolerance trait through outcrossing.

Methods

The project was separated into 3 specific modules focusing on:

1. cultivated areas where Innovator oilseed rape was grown in 1995 and adjacent fields,
2. non-agricultural areas in the vicinity such as shelterbelts, fence lines, sloughs, or abandoned farmyards,
3. storage areas and transportation routes.

The first module was conducted at specified intervals over the growing season (detailed below). Modules 2 and 3 were conducted later in the growing season when seed bearing plants could be identified. The rationale behind delaying the assessments for Modules 2 and 3 was that from an ecological point of view, the only individuals of concern were those that successfully reproduced resulting in the potential for re-establishment of the population in the next season.

Module 1: Cultivated areas

The approach chosen to assess the occurrence and fate of glufosinate tolerant *Brassica napus* oilseed rape and related weedy species was to systematically scout and sample 1995 Innovator fields and adjacent fields throughout the 1996 and 1997 growing season. The fields were planted to wheat, barley, flax or oats as is common for canola rotations, or were fallow. Ten (10) sites were selected where glufosinate tolerant oilseed rape (Innovator) was grown in 1995 (Table 1 and Figure 1). To provide a comparison, an additional five (5) sites where non-transgenic oilseed rape was produced in 1995 were surveyed in a similar manner.

Table 1 Occurrence of *Brassica napus* (BRANA), *Brassica rapa* (BRARA), *Sinapis arvensis* (SINAR), and *Thlaspi arvense* (THLAR) over the 1996 and 1997 growing season in ten fields where glufosinate resistant oilseed rape variety "Innovator" and non-transgenic cultivars were grown in 1995.

Transgenic (ten) Innovator sites									
Species	**Number of plants / m²**				**Species**	**Number of Plants / m²**			
BRANA	Survey #1[a]	Survey #2	Survey #3	Survey #4	BRARA	Survey #1	Survey #2	Survey #3	Survey #4
1996	208	18	1	0	1996	0	0	0	0
1997	11	13	2	1	1997	0	0	0	0
SINAR	Survey #1[a]	Survey #2	Survey #3	Survey #4	THLAR	Survey #1	Survey #2	Survey #3	Survey #4
1996	0	1	0	0	1996	3	1	1	0
1997	0	2	1	1	1997	11	19	1	2

Non-transgenic sites

Species	Number of Plants / m²				Species	Number of Plants / m²			
BRANA	Survey #1[a]	Survey #2	Survey #3	Survey #4	BRARA	Survey #1	Survey #2	Survey #3	Survey #4
1996	22	21	1	0	1996	0	0	0	0
1997	0	35	6	0	1997	0	0	0	0
SINAR	Survey #1[a]	Survey #2	Survey #3	Survey #4	THLAR	Survey #1	Survey #2	Survey #3	Survey #4
1996	0	4	0	0	1996	1	21	2	0
1997	2	1	1	0	1997	2	2	1	0

[a]Timing of surveys was as follows: #1 - prior to spring cultivation for seedbed preparation (early May); #2 - following emergence of crop but prior to post-emergent herbicide application (early June); #3 - following post-emergent herbicide application (July); #4 - following combining (October).

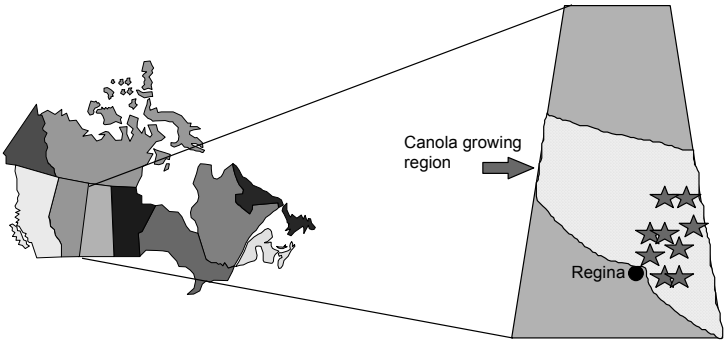

Figure 1 Map showing oilseed rape growing region of Saskatchewan and study area. Stars represent approximate locations of sites. Only nine stars?

The specific survey methods were similar to those used to obtain the annual weed surveys published in the province of Saskatchewan (Douglas and Thomas, 1986) with a few modifications indicated below. Briefly, the surveyor walked a predefined pattern (an inverted "V") in the field and took weed counts from 10 quadrats spaced 20 paces apart.

Assessments were taken at the following times: 1) early spring prior to cultivation and seeding (early May), 2) following crop emergence but prior to application of any herbicide (early June), 3) following herbicide application (July), 4) after harvest just prior to freeze-up (mid-October).

To determine whether a volunteer oilseed rape plant or weedy relative had the glufosinate tolerance trait, every second quadrat was marked with flags and treated with glufosinate ammonium herbicide. The field was re-visited 48 hours to one week later and survivors were documented.

Module 2: Non-agricultural areas in the vicinity of 1995 Innovator fields

Late in the growing season when *Brassica* and related species are in the reproductive stage, the non-crop areas were surveyed for their presence. The survey area was limited to 20 m from the margin of the field as it is unlikely for seed to be spread a greater distance by wind or harvest operations. If any were discovered within the sample area, green tissue from the plant(s) was sampled and tested for the presence of the PAT enzyme by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit.

Module 3: Storage areas and transportation route

Each transportation route was surveyed over a distance of 10 km measured from each location. A segment of 100 m out of each kilometre was walked and individual plants of *Brassica* or related weed species found along the roadside or adjacent ditch were counted. Green tissue samples were collected and assayed for the presence of the PAT enzyme by ELISA.

Results and discussion

Module 1: Cultivated areas

The 1996 and 1997 survey of the actual field where Innovator oilseed rape was grown in 1995 (Table 2) revealed similar results to that found in surveys of standard oilseed rape fields (Table 3). The following trends were common to both situations:

Table 2 Occurrence of *Brassica napus* (BRANA), *Brassica rapa* (BRARA), *Sinapis arvensis* (SINAR), and *Thlaspi arvense* (THLAR) over the 1996 and 1997 growing season in fields adjacent to 1995 Innovator fields. Values represent total individuals counted in 10 quadrats. If a particular area was not present in a site, "n.a." appears in the column. Values in parentheses represent the number possessing the PAT enzyme as determined by immunoassay.

	BRANA				BRARA				SINAR				THLAR			
Site	#1(T)[a]	#2	#3	#4	#1(T)	#2	#3	#4	#1(T)	#2	#3	#4	#1(T)	#2	#3	#4
Innovator sites mean plants																
1996	1(0)	0	0	0	0	0	0	0	0	6	0	0	3(0)	16	2	0
1997	5	7	1	0	n.a.	n.a	n.a.	n.a.	1	1	1	1	11	15	2	0
Non-transgenic sites mean plants																
1996	0	0	0	0	0	0	0	0	4	9	0	0	9	4	4	0
1997	0	0	1	1	n.a.	n.a.	n.a.	n.a.	3	2	0	0	2	1	1	0

[a]Timing of surveys was as follows: #1 - prior to spring cultivation for seedbed preparation (early May); #2 - following emergence of crop but prior to post-emergent herbicide application (early June); #3 - following post-emergent herbicide application (July); #4 - following combining (October).

Table 3 Occurrence of oilseed rape volunteers in non-crop areas adjacent to or in the vicinity of fields where oilseed rape (Innovator or non-transgenic) was grown in 1995. Values reported represent total individuals counted in 10 quadrats. If a particular area was not present in a site, "n.a." appears in the column. Values in parentheses represent the number possessing the PAT enzyme as determined by immunoassay.

Non-crop area	Fence lines		Ditches		Sloughs		Grassy areas	
	1996	1997	1996	1997	1996	1997	1996	1997
Innovator mean	0	1 (1)	1 (1)	3 (2)	0	0	n.a.	0
Non-transgenic mean	0	0	0	0	n.a.	0	n.a.	0

(1) There was a relatively high density of volunteer oilseed rape plants present early in the spring prior to any field operations in 1996 (mean 208 per m^2), where the population was greatly reduced in 1997 (mean 11 per m^2).

(2) The post-emergent weed control program employed by the producer was effective in controlling oilseed rape volunteers present in the 1996 and 1997 crop with mean populations of one and 1.7 plants per m^2, respectively.

(3) No oilseed rape volunteers germinated late in the season following removal of the 1996 crops and only one plant was detected in 1997.

(4) Among weedy relatives, *Sinapis arvensis* and *Thlaspi arvense* were most common and were effectively controlled by the post-emergent weed control program employed by the producer. Neither are sexually compatible with canola.

(5) There was no difference between the 1995 Innovator sites and the five non-transgenic sites used for comparison.

The glufosinate ammonium herbicide treatment of every second quadrat from the 1996 survey confirmed that oilseed rape volunteers counted in fields where Innovator was grown in 1995 were indeed glufosinate tolerant and that weedy relatives counted in these same fields were not glufosinate tolerant (data not shown).

Two conclusions can be made based on these two years of survey results. In cropland situations, glufosinate tolerant oilseed rape cv. "Innovator" appears to be no more persistent than standard oilseed rape varieties. This is in agreement with the small plot field studies conducted by AgrEvo (MacDonald and Manning, 1996). Of note is the fact that the herbicide program employed was entirely under the control of the producer at each site. No advice or special recommendations were given. This demonstrates that volunteer herbicide resistant oilseed rape can be controlled using cultural and chemical practices that producers currently employ.

Secondly, within the surveyed area, the transfer of the glufosinate tolerance trait to weedy relatives of oilseed rape through outcrossing does not appear to be a factor. While crosses between *Brassica napus* and *Sinapis arvensis* are possible (MacDonald and Manning, 1996) the potential of the cross occurring in the field is extremely low. The results obtained from this survey to date support these findings.

Module 2: Non-agricultural areas in the vicinity of 1995 Innovator fields

Unlike cultivated areas, adjacent non-agricultural areas are often unmanaged and therefore represent an opportunity for Innovator escapes or weedy relatives possessing the glufosinate tolerance trait through outcrossing, if present, to establish and perpetuate the population. Oilseed rape seed can be disseminated to these areas by a variety of mechanisms: oilseed rape windrows can actually be blown across or off of a field in a strong wind; or during harvest, the small fraction of seed that is not collected in the grain tank but is blown out the back of the combine with the oilseed rape straw and chaff.

Oilseed rape volunteers growing in non-crop areas in the vicinity of fields where Innovator was grown in 1995 occurred infrequently in 1996 and 1997 (Table 4). Those found, however, were shown to be glufosinate tolerant. In all cases, these plants were growing at the margin of the field alongside a road or trail that formed one boundary. Additional monitoring done in 1997 assessed the ability of these plants to perpetuate the population and the results demonstrated limited colonisation ability. Minimal destructive sampling was done in 1996 to identify the glufosinate resistance trait. This made it possible to return to these sites in 1997 to re-assess the population.

Table 4 Survey of roadside ditches leading away from oilseed rape production fields (10 transgenic sites and 5 non-transgenic) and/or storage sites. A single 100 m segment of ditch was surveyed per kilometer for 10 kilometers. Values reported represent total individuals counted. Values in parentheses represent the number possessing the PAT enzyme as determined by immunoassay.

Site	Total BRANA counted		Total other counted	
	1996	1997	1996	1997
Innovator mean	9.1 (0)*	0.9(0)	0	0
Non-transgenic mean	9.4	0	0	0

BRANA = *Brassica napus*; Other = *Brassica rapa*, *Sinapis arvensis*, *Thlaspi arvense*

Module 3: Storage areas and transportation routes

Additional areas where volunteer glufosinate tolerant oilseed rape or related weed species may establish include areas around storage bins and along transportation routes. Dispersal of Innovator escapes or weedy relatives possessing the glufosinate tolerance trait through outcrossing along transportation routes represents a mechanism for widespread introduction of a potential weed problem.

The production of Innovator oilseed rape under the "Identity Preserved" program in 1995 presented a unique opportunity to investigate this mechanism of dispersal since storage facilities were known and the entire oilseed rape production in the proposed survey area was transported by truck from the on-farm

storage facilities to a single crushing plant. Therefore, major transportation routes radiating from 1995 Innovator fields and/or in-field storage facilities could be surveyed for the presence of volunteer oilseed rape plants.

Oilseed rape volunteers were found growing along roadsides leading away from oilseed rape production fields. In no instance were any shown to be glufosinate tolerant. Considering that the survey area was in prime oilseed rape production territory, the fact that although several oilseed rape plants were found growing along roadsides yet none were glufosinate tolerant is not surprising. Since Innovator represented a small proportion of total acres seeded to oilseed rape in 1995 and 1996. Tonnes of oilseed rape seed from standard non-transgenic varieties are also trucked along these roads.

Although oilseed rape can and does grow in ditches, the probability of any plants producing seed to perpetuate the population is likely small. This survey was initially planned for late in the growing season so that any plants found would be well into the reproductive stage. This proved not to be the case. That is, nearly all of the plants sampled were still at the vegetative stage. Most ditches along rural roads in Saskatchewan are mowed periodically over the course of the summer. Plants that are not yet flowering by September or October are not likely to produce viable seed before the first killing frost and would not therefore perpetuate the population.

Conclusions

Based on the results of this two-year survey, no evidence was found to suggest that glufosinate tolerant oilseed rape behaves any differently in managed and unmanaged environments than does standard, non-transgenic oilseed rape. These results are in agreement with the findings of Crawley et al. (1993) who examined the fitness of transgenic oilseed rape in small plots which simulated managed and unmanaged environments. Furthermore our findings for western Canada are in concert with the study of Crawley and Brown (1995) which examined the distribution of feral canola populations along motorways in Great Britain, the authors concluded that seed spilled from trucks in transit from the fields to the storage/crushing facilities could germinate and produce plants in the next season. However, the population was not persistent without the influx of new seed. This confirms the validity of the risk assessment procedure and further demonstrates that a recombinant crop can be responsibly introduced to the environment in the absence of potentially restrictive “Statutory” monitoring requirements.

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New technologies in plant breeding

Grant, I.

Pioneer Hi-Bred International, 31840 Aussonne, France

Good morning. It is a pleasure to participate in the 5th International Symposium on The Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms.

It is also a pleasure to speak to you today about some of the new approaches to plant product development and the implications of these new approaches to product developers (plant breeders).

My presentation today will cover the following areas:

1. First I will provide you with a brief introduction on this subject.
2. Then I will describe some of the new technologies currently being used by product developers.
3. This will be followed by a description of the process of integrating new, novel traits into commercially useful germplasm.
4. I will then discuss some of the issues that impact the extent to which new technologies are utilized in the product development process.
5. I will finish with a few concluding remarks.

Clearly we have observed significant gains in the productivity of agricultural production systems over the past three or four decades. These gains in productivity can be attributed to a number of factors, including genetic improvements, improvements in crop management systems, training and education of agricultural producers and so on. Our agricultural production system feeds twice as many people now as in 1955, on the same amount of crop land. This is testimony to the significant gains in productivity that have been realized.

In order to continue to feed a growing global population on this fixed land base, we will need to continue to realize these productivity gains. We will continue to realize improvements in genetic gain for yield and other agronomic characteristics, but these gains will not likely be sufficient to meet the global food supply demand. Meeting the global demand for food will require the deployment of new genetic technologies. Technologies that improve the yield of plants, beyond that which can be achieved through conventional plant breeding; technologies that reduce crop production losses; and technologies that increase the value of the grain produced.

The goals of any seed product development/plant breeding program are typically very similar. If one surveyed a group of 100 plant breeders, who bred a wide variety of crop species, and asked them what the goals of their plant breeding programs are, one would likely receive the following responses: The first and most important goal, typically, is to “design” plants that produce more grain or forage per unit area. More grain or forage per unit area equates, directly, to more value being created for the producer of any particular crop. The second goal would be to design plants that resist attack by pests, such as insects, diseases and weeds. Crop species, typically, are susceptible to a broad range of pests. Genetic resistance to these pests is sometimes available in the base germplasm of a given crop species. However, there are many examples of pests that attack crop species, where there is no known resistance in the base germplasm (e.g.: European corn borer in maize; sclerotinia in sunflower; broadleaf weed susceptibility in canola). Today, new technologies are being employed to address susceptibility to pests, where there is no known resistance, or “natural” resistance is insufficient to adequately control the pest in question. In the previously mentioned examples, *Bt* technology for ECB; oxalate oxidase technology for sclerotinia resistance and glyphosate and glufosinate resistance for weed control, reducing crop losses through improved pest resistance and/or providing pest control options at a lower cost, both add value to the producer.

The third major objective of any plant breeding program would be to improve the value of the grain or forage a plant produces. Typically this involves modifying the components of grain, such as oil, protein and starch, such that they have higher feeding/nutritive value, or high utility in industrial processes. Both quantitative and qualitative improvements in the components of grain are significant. Similarly

improvements in the feeding value of forage are also important. Today a number of key modifications of the components of grain are being addressed through the use of new technologies.

E.g.: - amino acid modification of soybean protein
- modification of the structure of starch in the maize kernel
- modification in the FA composition of canola oil.

Ultimately, these three goals are centered on creating more value for the farmer, and for agricultural food production systems in general.

The above equation describes the total value created, expressed on a net income per unit area basis. Total value is a function of:

- (yield) x (the value of the grain) - (cost to produce the grain)
- value can be increased by:
--> increasing harvestable yield
--> improving the value of the grain/forage
--> reducing crop losses and input costs.

At Pioneer Hi-Bred International, the company I work for, everything we do in our research programs is driven by this value equation. Ultimately the purpose of any plant breeding research program should be to create additional value in food production systems.

The other main consideration is creating a safe and reliable supply of food. We have discussed how improving yield, reducing crop losses, reducing input costs and increasing end use value of grain and forage all contribute to creating a reliable supply of food. In addition there are genetic approaches to improving the safety of the food and feed supply. For example, engineering maize so that it is resistant to infestation from mycotoxin producing molds that infect the grain, or engineering canola so that anti-nutritional glucosinolates are eliminated from meal. There are also genetic approaches that support the use of more environmentally friendly crop inputs. For example, engineering crop species such that they are resistant to more benign herbicides.

A number of technologies have increased our ability to develop and deliver the high performing products we have on the market today. For the purposes of today, I will focus mainly on biotechnologies.

The first generation of products of biotechnology is made possible through a variety of technologies in the labs and the field. Largely, these technologies made the products we have today possible, for example, maize with the *Bt* trait was made possible via gene transfer technology. Typically single gene transfers are involved in first generation products and, typically, these genes are from other, non-plant sources. The integration of genes is typically “non-specific”, in that current gene transfer technologies are not able to specifically insert the gene into a known place in the plant genome. Finally, expression of genes can be directed into specific parts of the plant via tissue-specific promoters.

Products from this first generation technology mostly provide protection from pests or add another management options for farmers. For example,

European corn borer resistance:
ECB is estimated to cause $800 million in damage annually. Farmers experienced how this technology could protect and increase yields under ECB pressure largely for the first time last year. Some Pioneer customers saw up to a 12 bu./acre advantage, or more than 10% advantage.

Herbicide resistance:

- Herbicide resistance provides additional weed management options, but in most cases, does not contribute to increased productivity.
- Liberty Link - glufosinate. Provides alternative weed control for atrazine-sensitive watersheds.
- *Roundup Ready* - glyphosate. Reduces usage of residual herbicides and helps decrease crop input costs.

Technologies of the second generation give plant product developers sharper tools to develop better products. For example:

Genomics Research: Technologies associated with genomics research will allow plant breeders to mine our corn and soybean germplasm base, build an already strong knowledge of corn performance, and ultimately give farmers better products. Work in genomics will allow the identification and isolation of genes from plant genomes. These genes can then be used for gene transfer work.

Gene Transfer Technology: Will be focused on plant genes for the most part and site specific insertion into genome will be routine.

Gene Regulation: Genes will be regulated in specific tissues routinely and will be chemically induced.

Molecular Markers: Molecular markers will aid in the speed of gene introgression/trait integration and will speed up the plant breeding process, generally.

These are some of the complex, multiple-gene traits that newer technologies will help us develop into a total package of traits for the best product performance:

Yield: An understanding of the genetic basis for yield and heterosis will significantly benefit our ability to improve the productivity of grain production on plants.

Agronomic Traits: Standability, drought resistance, drydown and ear retention in maize are all examples of complex agronomic traits that can greatly influence the profitability of farmers.

Disease Resistance: Control of diseases such as gray leaf spot, anthracnose and ear mold is becoming more important as conservation tillage increases.

Quality Traits: These traits provide energy, amino acids enhancement and waste processing capabilities that add value to farmers, livestock producers, and end users.

Once technologies have been utilized to develop a specific trait that is stably and fully expressed, and is predictably heritable, the trait must then be integrated into elite germplasm. This can be accomplished via direct transformation of elite inbred lines - provided the transformation technology is sufficiently genotype independent, or via backcrossing - successive crosses to insert gene into a new line while recovering the genotype of the recurrent parent. In addition, traits can be "stacked", or combined together, either by co-transformation or by crossing two parent lines (each containing one gene/trait) together to form a hybrid possessing both traits.

Backcrossing: Backcrossing involves making an initial cross between a line carrying the gene of interest (the donor parent) and an elite commercial line (the recurrent parent). Subsequently a series of crosses are made with the recurrent parent with the objective of recovering the recurrent parent (or elite genotype) that carries and expresses the gene of interest (from the donor parent).

Advantages	Disadvantages
- predictable	- takes time (three generations/year)
- easy	- labour intensive
- conventional	- capacity

The other alternative is via direct transformation of elite lines.

Advantages	Disadvantages
- speed	- genotype dependency
- can be low cost	- can be costly
- large capacity	- new GMO created - regulatory

A typical scheme for the development of a commercial transgenic trait would have the following steps:

A. Transformation of gene constructs
B. Selection of transformed callus
C. Grow T0 plants
D. Verify gene is integrated into genomic DNA
E. Determine if and at what level the gene is expressing in appropriate tissues
F. Determine if there is a visible kernel phenotype associated with event
G. Begin backcrossing event into heterotic elite hybrid
H. Determine if event is transmitted to the next generation in a Mendelian fashion
I. Measure dosage effect
J. Identify any event x genotype interaction
K. Monitor expression level through successive generations
L. Backcross event into additional commercial lines
M. Determine the extent of GxE of event
N. Identify the impact of the event on yield and agronomic and grain traits
O. Narrow down to the single best event
P. Code lines containing trait
R. Begin backcrossing trait into all relevant parents
S. Make R2 hybrids
T. Test R2 hybrids, advance best hybrids and identify best code
U. Retest trait in R4C single cross hybrids
V. Commercialize hybrids containing trait.

There are several issues that could impact whether a product developer chose to utilize new technologies in a plant breeding program:

1. Access to technology:	- availability of gene or technology - acquisition, licensing in, in house development
2. Freedom to operation:	- product clearance process - contractual clearance - legal (patent) clearance
3. Cost:	- up-front investment: acquisition - license – royalty - in-house development cost - trait integration cost
4. Development timelines:	- time to market – cost - timelines vs conventional approaches
5. Intellectual property:	- patent position – competition - internal position - ability to protect investment
6. Stacking:	- ability to stack genes together - reduces development complexity - reduces number of products in line up - reduces inventory complexity / exposure.

Regulatory issues

Breeders are typically presented with many (>100) transgenic events to evaluate for each trait. Those must be reduced to one or two events for commercialization based on stability and agronomic performance in a variety of elite (commercial) genetic backgrounds. Events for evaluation are often presented to the breeder in non-elite (non-commercial) genetic backgrounds and must undergo repeated

cycles of breeding to introgress them into elite germplasm. Plants are carefully scrutinized at each stage to select those most resembling the desired commercial phenotype. Commercial products must have competitive performance across a wide variety of germplasms and environments. Breeders evaluate new products in hundreds of different locations before they reach the market. Any lines that show aberrant phenotypes or are not stable (i.e., not fixed) in their genetics are discarded since they cannot be commercialized (e.g., herbicide resistance genes which if not functioning effectively or poorly fixed, will show up as dead plants in the farmer's field).

Breeders conduct extensive field evaluations of transgenic crops to insure that they have the predicted phenotype and performance of their non-modified counterparts. Lack of any significant variation from the expected phenotype provides the starting point for a more in depth analysis of properties that are important in confirming the equivalence of the modified and non-modified crops. In addition, studies focus on the food and feed safety aspects of the newly expressed gene products and any environmental impact on non-target species or change in biological properties (e.g., invasiveness) that could adversely impact agriculture or the environment. At the end of the day, it is a balanced judgement based on all the data that provides assurance of the safety of new crop varieties and the basis for product advancement and regulatory approvals.

Regulatory requirements have evolved over the past few years as more products have been submitted to regulatory agencies for review. There is general consensus on the categories of data which should be generated. However, it is a significant challenge for developers to generate the necessary data and obtain essential approvals while maintaining an aggressive timeline for product development necessary to meet market expectations. Moreover, differences in regulatory processes and the time taken to review and approve products make it very difficult to ensure the coordination of all the approvals necessary to ensure free movement of foods and feeds derived from genetically modified crops. Other factors add to the challenge. Some of these could be simplified by reaching a consensus on issues such as the appropriate test materials to be used or focusing regulatory oversight on products most likely to pose food/feed safety or environmental risks. We have evolved a regulatory system that is difficult to reconcile with product development and commercialization. We must be cautious that we do not create additional complexity by inconsistent implementation.

Development time for new genetically modified plants (GMPs) is a critical factor in ensuring products remain competitive. Conventional crop improvement results in continuous increase in performance (e.g., yield). Products subjected to prolonged development and/or protracted regulatory reviews may be less competitive relative to conventionally derived counterparts when they finally reach the market. While individual traits may still add value, the value of the genetic package as a whole is diminished, especially if equivalent technology is already available in the market.

A critical challenge is determining when to start developing data for regulatory approval. Developers need to generate many events per transgene (e.g., >100). Breeders must make selections based on field data, collected over several seasons, to reduce those to one or two commercial events in competitive germplasm. Decisions as to when to start collecting regulatory data dramatically impact resource needs. Starting too early means generating and reviewing data on many events, most of which will never be commercialized. Starting later means fewer events must be analyzed and reviewed, but may delay regulatory approvals leading to reduced competitiveness in the market.

Regulatory compliance is complicated further when different countries take different approaches to the oversight of GMOs. Examples include:

- a lack of consensus on the appropriate test material with which to conduct food safety (equivalence) assessments,
- different regulatory scope with regard to the regulation of gene "stacks" derived by crossing two approved GMPs, using traditional plant breeding.

With respect to the most appropriate material with which to conduct food safety assessments, we would propose that the test material should be representative of near commercial hybrids or varieties rather than the earliest progenitor event(s), (inbreds) that gave rise to commercial products. The reasons for this recommendation are as follows:

- fewer events to analyze and review

- adequate seed supply available
- isogenic lines available from null segregants (hybrids)
- genetically closest to commercial material
- selected for genetic stability through multiple generations
- equivalence to progenitor material can be confirmed through molecular analysis.

Genetic modification is being increasingly used to introduce new traits into crop plants. It is necessary to combine traits in new varieties to deliver products with combination of traits demanded by customers. Gene stacking (or pyramiding) is also an important strategy to manage insect resistance to plant pesticides like *Bt*. Stacking can be achieved by co-transformation with several genes. A simpler approach is to cross two or more genetically modified lines to derive progeny with the desired combination (i.e., a gene stack). Many combinations (e.g., one crop with four traits could give 12 possible combinations) are possible. Same stacks will be formed naturally in the field as a result of open pollination, unless open pollinated crops are grown in isolation. Most combinations should pose no new safety issues. There is potential for gene interaction that might result in adverse reactions depending on the nature of the modification in the stack.

The primary responsibility for addressing the potential for interaction of traits lies with the developer. Assessments should focus on potential risks of interactions that may lead to unintended and adverse interactions - if any. A decision tree approach should be adopted:

- Two unrelated traits (e.g., herbicide resistance and insect resistance) - no new safety issues
- Two related traits (e.g., two pest resistances) - multiple pest resistance may raise issues
- Two related traits with same metabolic pathway (e.g., two oil modifications) - requires confirmation of no food safety issues arising from interaction.
- If regulatory oversight is deemed necessary, then oversight should focus on potential interactions that pose risk, not all stacks.
- Should not be necessary to re-review all the data that was developed for the parents, only such data as is necessary to bridge to the stack (e.g., showing unchanged gene expression relative to parental lines).

Until recently, the focus of genetic modifications in crop plants has been modification through the introduction of genes from outside the species - inter species transfers. These genes sometimes come from unfamiliar sources that may not be part of the normal human or animal diet - this has raised concerns over issues such as novel toxins and new allergens. The emerging science of genomics is aimed at understanding how organisms function at the molecular level. Companies like Pioneer are already accumulating libraries of genes from maize and soybean whose functions are quickly being elucidated. In the future, the focus will be on altering the expression of these endogenous genes to modify properties of the crop. This will not involve the use of “foreign” genes. These intra species transfers will pose very little risk of generating unintended effects such as new toxins or allergens and should be regulated accordingly.

Today I have reviewed some of the technologies currently being used for crop improvement. I have discussed how new traits are integrated into elite germplasm to result in commercial products that create value to the food production system. I have described some of the factors that impact if, and when, a product developer would choose to employ some of these new technologies. One of the key factors that impact this decision is the regulatory environment within which the plant breeder must operate. The use of new biotechnologies will be facilitated by clear, predictable and harmonized regulatory processes that are complementary to product development process and timelines.

Thank you.

An Illinois corn and soybean farmer's experiences with genetically modified crops

Wentworth, S.

President, Foundation EARTH

I come from a long line of Macon county Illinois farmers who have adopted emerging technologies over the last 130 years to improve their farms. In the 1870's my great-great grandfathers saw how John Deere's steel moldboard plow could enhance the productivity of sticky Illinois prairie soils. They soon realized Cyrus McCormick's reaper would allow them to harvest more grain in a day. In 1870, 53% of the US population were farmers. By the turn of the century, my great-grandfather used the power of steam engine to run a threshing machine. At the turn of century, 37.5% of all US workers were farmers; by 1920 that percentage had dropped to 27%. When A.E. Staley Company of Decatur Illinois first started processing soybeans in October 1922 it profoundly changed my grandfathers' crop rotation and all of US agriculture. Soybeans were no longer a hay crop or a nitrogen source to be plowed under, but a cash crop. With the use of hybrid seed corn starting in the 1930s, Macon county farmers saw a tremendous increase in yield. During the same decade gas powered tractors were used for the first time to till the soil.

In 1950, the year I was born, and two years after my father started farming, 11.6% of the working population of this country were farmers. During my Dad's farming career, he saw our farm's productivity zoom through the use of soil testing, greater use of commercial fertilizers, herbicides to control yield robbing weeds, insecticides to prevent disastrous insect infestations, advances in electronics that allowed more accurate planting, spraying and harvesting operations. By 1970, only 3% of the US work force were farmers.

Since 1974, the year I started farming, we have gone from moldboard plowing to soil conserving minimum tillage and No-till. Back then our big tractor was a state of the art John Deere 4430 with 125 horsepower, now our large tractor is a Case 9370 with 365 horsepower. The John Deere 4400 combine we used in the early 1970's would harvest 4,000 bushels of corn in a big day. With our current John Deere 9600 it is not uncommon to shell 20,000 to 22,000 bushels of corn a day. The machine that is used the most on our farm is the computer. We are currently on our fifth computer. Unlike combines that do wear out, computers are obsolete shortly after you walk out of the store. In the past it took eight to ten pounds per acre of herbicides to get satisfactory weed control. Now, as little as four ounces per acre of the new generation herbicides will give a farmer a weed free field. When I started farming I would spread the same analysis of fertilizer across an entire field.

For the last few years I have been using variable rate fertilization technology coupled with Global Positioning to apply phosphate and potash in the most economically optimum ration for that exact location in the field. My combine is now equipped with a yield monitor and a GPS unit so I know what every square meter of a field yields. Now the challenge is to interpret the data, and determine what causes the differences between the lowest yielding areas and the highest areas. Precision farming combined with the advances in biotechnology promises to further speed the incredible rate of change in production agriculture.

Advances in technology have allowed me and the rest of American agriculture to become more productive and environmentally sustainable at the same time. Currently, less than 1.5% of working Americans are farmers. I believe biotechnology holds a promise for even more improvement, but should not be viewed as some kind of a silver bullet solving all of agriculture's problems. Each of technological advances I have described over the last 130 years have created both new opportunities and management challenges for the American grain farmer. None of these advances were problem free or easy. Likewise, the use of transgenic crops is extremely promising, but it would be naive for anyone to believe there are not tough issues to be dealt with.

Roundup Ready soybeans have been probably the most visible of the early transgenic crops. 1998 is the third year I have grown *Roundup Ready* soybeans. The rapid acceptance of this technology has been nothing short of astonishing. Representatives of some of the leading seed companies tell me the market share of *Roundup Ready* has grown from zero in 1995 to over 60% for the 98 growing season. They expect in 1999 over 75% of all soybeans they sell in this country to be *Roundup Ready*.

If we look at the economics involved, it is easy to understand why this new technology has gained such a rapid acceptance among farmers. On my conventional soybeans, before planting I apply a pre-emerge grass herbicide followed in June by postemerge broadleaf chemical at a cost of $38 per acre for the chemicals plus two applications at $4.50 each per acre for total expense of $47 per acre. With *Roundup Ready* soybeans I spend $13 per acre for the *Roundup* and $4.50 per acre for a single application. Monsanto collects a technology fee of $5.00 per 50 pounds of *Roundup Ready* seed used. This amounts to $5.00 to $7.50 per acre depending on seed size and planting rates. Overall, I am saving $22 to $24.50 per acre, which on our farm's 1,300 acres of soybeans is a $28,000 to $31,000 cost reduction. *Roundup* kills a wider spectrum of weeds while at the same time being gentler on the growing beans. It is obvious why *Roundup Ready* in central Illinois soybeans have gone from nothing to over 60% market share in four years.

There are numbers of management issues about *Roundup Ready* soybeans that farmers need to address. If any conventional beans are planted in a *Roundup Ready* field or sprayer operator treats the wrong field all of those soybeans will die. If the winds are too strong or out of the wrong direction a neighbor's corn, or worse, his wife's tomatoes will be destroyed. This year, for the first time, Dekalb is offering *Roundup Ready* corn. If a farmer plants any of these fields to *Roundup Ready* soybeans in 1999, any volunteer corn growing in his soybean field would also be resistant to *Roundup*. If farmers kept using *Roundup* on both their corn and soybeans for years, in theory, it would be possible for a weed to develop a resistance to the chemical. The Monsanto people say the chances of that happening are almost nil.

There has been much debate in farm circles this past fall and winter if *Roundup Ready* soybeans yield as well as conventional beans. From the data I have seen and my own experiences, I am convinced that the best *Roundup Ready* soybeans will yield with any conventional variety. Seed companies are working feverishly to get the *Roundup Ready* characteristic into their best genetic package.

For years farmers planted soybeans saved from the previous crop for seed. This practice is called using "bin run seed". Over the last 25 years farmers have increasingly purchased new seed every spring to insure themselves of the best genetics and the highest quality. Today, when a farmer buys a bag of *Roundup Ready* soybean seed he pays the $5 technology fee that goes to Monsanto and he signs a contract that he will not keep any production back for seed. If he is caught in violation of this agreement he can be fined over $1,000 per acre. Most producers understand that the tech fee allows Monsanto to recover their previous research costs, give a return to the stockholders, and provide research dollars for the development of future transgenic crops. But, it is a new and difficult mind set for a few farmers that those soybeans he just harvested cannot be kept for seed.

The European corn borer does more economic damage than any other insect to corn, with losses and control costs exceeding $1 billion annually. The decision on whether or not to grow *Bt* corn is not as clear cut as it is with *Roundup Ready* soybeans. A central Illinois farmer knows with a great deal of certainty what the weed pressure will be in his soybean fields, but the reliability of predicting the level of European corn borer infestation before planting is not very consistent. The severity of the previous winter; corn borer diseases present; a farmer's and his neighbor's tillage; the timing, velocity, and direction of summer and fall winds; and number and strength of summer thunderstorms all impact corn borer survival. Farmers in the Western Corn Belt usually have greater corn borer economic losses and on a more regular basis. Integrated pest management (IPM) offers some defense against European corn borer, but is not practiced to any great degree. IPM requires skillful scouting multiple times in extremely uncomfortable conditions and is very time consuming. Even if economic levels of corn borer are detected, the treatment insecticides are expensive and not always very effective. There also can be health and environmental concerns. Beneficial insects will be reduced. *Bt* corn basically offers a corn farmer an insurance policy against moderate to severe infestations of European corn borer. *Bt*, *Bacillus thuringiensis*, is a naturally-occurring soilborne bacterium. *Bt* produces crystal-like proteins that kill certain insects when ingested. Plant geneticists create *Bt* corn by inserting selected exotic DNA into the corn plant's own DNA. This is called an "event". The EPA has registered four unique events for commercial use: 176 (Novartis Seeds and Mycogen Seeds), BT11 (Northrup King/Novartis Seeds), MON810 (Monsanto), DBT418 (DEKALB Genetics Corp.), and recently CBH351 (AgrEvo). Event 176 hybrids produce *Bt* protein only in green tissues and pollen, whereas BT11, MON810, and CBH351 produce *Bt* protein throughout the plant. While all the events do control corn borer larvae, but to different degrees.

Bt is a defensive characteristic. Putting *Bt* into a corn with mediocre genetics is not going to make that variety great. Giving me the latest set of Calloway Big Berthas would not make me a better golfer than Tiger Woods with golf clubs from the 1950's. Also some corn varieties, such as Pioneer 3489 or Garst 8481IT, can tolerate more corn borer injury than others. As a farmer, I need to decide if the extra $14 to $15 per acre I will spend for *Bt* corn is justified. This spring, I planted my first *Bt* corn on about 5% of my acres using five different varieties. Later this month, the yield monitor on my combine will show me how these *Bt* corns performed against my favorite non-*Bt* hybrids in 1998. Corn Borer intensity this summer was the lightest it has been this decade. I will be surprised if *Bt* corn shows a positive economic return for 1998. But that will be no guarantee of *Bt* being advantageous or not in 1999. The University of Illinois research shows *Bt* corn will probably pay for itself or seven years out of ten in my area of East Central Illinois, eight out of ten years in Northwest Illinois, but only two out of every ten years in deep Southern Illinois. My best guess is that as more companies start putting *Bt* into their highest yielding genetics the market share of *Bt* corn will grow dramatically.

The concern of farmers, seed companies, universities, and others is that European corn borer will develop resistance to *Bt* if growers start planting whole farms to corn with this technology. To prevent this farmers are being advised to plant *Bt* only on acres where there is risk of severe infestations. Also, corn farmers are instructed not to plant any single field to more than 75% of *Bt* varieties. Planting a 25% refuge of non-*Bt* corn will allow susceptible European corn borers to mate with potentially *Bt* resistant corn borers. If not managed properly, *Bt* corn will be a short-lived benefit to corn farmers. Future transgenic crops, like rootworm resistant corn, which Monsanto has recently developed, will not be released unless farmers show good stewardship of biotechnology products such as *Bt* corn.

Farmers are intently following the rapid structural changes of the companies selling them inputs. They are asking themselves, what does it mean to us for Monsanto to purchase Asgrow, Dekalb and Holden seed companies and then to be purchased themselves by American Home Products. DuPont now owns 20% of Pioneer? Is it good or bad? Only time will tell. What is obvious, is that biotechnology is the primary catalyst for these recent acquisitions. The first generation of biotech traits have been input-related. What is truly exciting for me as a farmer is the prospect of biotechnology increasing the value of my corn and soybeans. Maybe because of biotechnology I will be growing a different commodity. Crops will be genetically engineered to replace materials that now come from petroleum such as chemicals, fuel, and plastics. Transgenic crops have great potential in animal health, neutraceuticals, and pharmaceuticals.

For generations American farmers have adopted to changing technologies. The rate of change facing farmers today is truly incredible. Biotechnology is another tool for farmers to utilize to bring abundance to America and to the rest of the world.

Strategic regulation of agricultural biotechnology products in the United States

Schechtman, M.G.

Animal and Plant Health Inspection Service (APHIS), US Department of Agriculture

The development of agricultural biotechnology in the USA is moving at a rapid pace. This is not an accident. The magnitude of the potential impacts of biotechnological innovation on agriculture was recognized early. Officials recognized the vast potential for the technology - to increase the volume, quantity, reliability, and nutritional quality of the food supply, and perhaps to help address some existing environmental problems as well.

The potential value of biotechnological innovation to agriculture was recognized early and continues to be studied. In 1987, then-Senator and now Vice-President Gore stated: "The most lasting impact of biotechnology on the food supply may come not from something going wrong but from all going right."

Government interest in the overall role of biotechnology was reflected in the early 1990's, when the former Congressional Office of Technology Assessment conducted a series of studies on biotechnology and its many applications, from both commercial and technical perspectives.

Early on in the development of agricultural biotechnological innovation in the USA, officials recognized not only this vast potential, but also the need to create an overall structure to ensure the safe development of the products of biotechnology in the USA. While the decade of the 1970's was one of laboratory experimentation only, the 1980's brought the recognition that genetically engineered organisms were going to require testing and then use in the open environment.

Through the 1970's and the 1980's, there was a significant public debate about the appropriate structure for a regulatory system that would address all real safety concerns without stifling the technology. We have always been cognizant of the danger of creating a regulatory system based on fear, rather than on science, one that would sacrifice the very real potential benefits of the new technology without getting any greater assurance of safety in return. We believe that we have avoided this danger.

I will be presenting a general talk about the overall regulation of biotechnology products in the USA, in part in a conceptual way. I will also discuss how various traditional regulatory mechanisms that apply to these products as well work in the USA.

Now as the debate in the USA unfolded, the decade of the 1970's saw the development of a system of guidelines for safe laboratory research with recombinant DNA organisms. Discussions initiated by the White House in the early 1980's resulted in the publication in 1986 of the Federal Government's plan for regulating additional uses of biotechnology products outside the laboratory, referred to as the Coordinated Framework. The approach adopted in that document deliberately builds upon existing laws and institutions, and adapts the long history and relevant expertise to the safety issues raised by biotechnology products.

The approach was founded on a careful scientific analysis, which indicated that the sorts of safety issues raised by these products are no different in kind from those with which we are already familiar from other products in the same industrial sectors. The magnitude of individual concerns may, however, vary from product to product.

For regulators in the USA, this means, in practical terms, that products are captured for review on the basis of a credible risk trigger (e.g., a plant pest risk in the case of environmental issues for new plant varieties) rather than on the basis of the manufacturing technique. This has allowed us to focus governmental risk analysis and oversight resources in areas where they might usefully be invested, and avoid wasting them on products that are not truly novel.

Under the Coordinated Framework, responsibility for regulating products in different industrial sectors is assigned to those regulatory authorities with a history of responsibility and up-to-date experience in those sectors. This approach allows us to take into account risk factors that are specifically associated with the types of uses to which products in the sectors are put.

Oversight responsibility for these products in the USA is divided principally among three agencies, and these agencies use laws already in existence as the legal basis for these roles.

These are: the Animal and Plant Health Inspection Service, APHIS, of the Department of Agriculture, with respect to protecting American agriculture; the Environmental Protection Agency, with respect to the safe use of microbes and pesticidal and herbicidal substances in the environment; and the Food and Drug Administration with respect to ensuring the safety and labeling of drugs and of the nation's food supply, excluding meat and poultry.

APHIS, in particular, is lead agency in regulating the safe testing and commercialization of new plant varieties. The relevant APHIS regulations first went into effect in 1987. You have probably heard about the details of the regulatory processes at these agencies past symposia of this type or at other meetings, so I will not talk in detail about them. Let me simply indicate that USDA oversees the safe testing, under controlled conditions, of genetically modified plants, and also their importation or interstate movement. These actions require authorization from APHIS, essentially a certification by the agency that the tests will be conducted in a safe manner. APHIS can also reach decisions that in essence deregulate individual organisms from the agency's perspective, when an applicant petitions the agency with enough information for us to determine that the organism poses no plant pest risk, and is as safe to use in agriculture as traditional varieties. I will not have time to speak here about the detailed regulatory processes under which we operate, nor those of EPA or FDA, but I would be happy to answer any questions later that you might have.

I am, however, going to speak about general features that underlie the regulatory processes in these three agencies and, more broadly, that enable the integration of these products into agricultural use and into existing, well-established agricultural institutions in the USA.

First, though, let me give you some idea of the rate at which activities and progress has been made in agricultural biotechnology in the USA. I will provide just a few statistics to demonstrate the continued expansion of field testing of new crop varieties, the number and diversity of products that have reached the marketplace, and the extent to which these products are now being commercially grown in the USA.

As of July 31, 1998, we have evaluated and authorized over 4,100 field trials of new agricultural products at over 18,270 field sites in 43 of our 50 States and one territory. These trials have involved 52 different plant species as diverse as sugar cane, poplar trees, soybeans, turfgrasses, rice, and sunflowers. Derivatives of all of the major crops in the USA, with the exception of one, wheat, have been extensively tested and developed using the new technologies. That is, corn, soybeans, potatoes, tomatoes, cotton, and tobacco have had a large number of trials, and only wheat has lagged behind, for technical reasons. Corn, i.e., maize, is the leading crop in terms of field trials.

Thirty-five different products in ten crop species have completed APHIS reviews determining that they no longer require special oversight by the agency. Most of these have completed all of the applicable regulatory requirements from the other agencies, EPA and FDA, as well. Some but not all of these have entered commercial production, and many have entered traditional breeding programs to have the new traits crossed via conventional means into additional useful genetic backgrounds.

The acreage planted with new genetically engineered crop varieties in the USA has increased from very small acreages in 1995 to 3.6 million acres in 1996, to 20.1 million acres in 1997, and further significant increases have also been seen in 1998. The numbers that have been talked about this year are in the 50-60 million acre range.

There is another way to view progress as well - by considering what underlies those statistics - that is, examining the evolution of regulatory and government structures designed to facilitate those numerical developments while continuing to ensure safety and involve the public. This, therefore, brings me to the key topic of strategic regulation.

Strategic regulations are regulations, developed and applied in a strategic manner, so as to provide a framework or process for actions that lead to consistent and planned results.

What are some of the regulatory features that we have recognized as key to the development of our approach?

1. Regulations should have identifiable, science-based triggers that are consistent, easily understood, and transparent.
2. Regulations should be effective and responsive as well as flexible and dynamic.
3. Regulations must meet domestic and international needs.
4. Let me highlight just a few features important in our consideration of these factors.

In terms of identifiable, science-based triggers, we have focused in providing clear standards and clear, identifiable risk triggers. It must be clear what is regulated and what is not, and the bases on which decisions on regulated organisms are made. We provide clear guidance to applicants on what kinds of information we require. The best science available must be used to address whatever uncertainties may exist.

Obviously, setting standards for risk are national decisions, or in some instances there may be regional or State standards instead. As regards allowable risk, at the risk of belaboring the obvious, our system recognizes that a standard of zero absolute risk is not an appropriate context for looking at biological systems. We recognize the need to look at significant impacts and set a baseline, which is provided by the known effects of the unmodified parental organism and the context in which it is used. Thus the concept of "zero risk" is not applicable, and does not give a way of truly evaluating whether there is a significant impact. It is also not achievable for any engineered OR non-engineered organism.

If one considers pesticidal substances, for example, it is clear enough that they are designed to have environmental impacts. No pesticidal substance is going to be without non-target effects. Conventional pesticides clearly have significant effects on beneficial insects. In the USA, we have recognized that the decision whether to permit the use of particular plant-pesticidal substances in genetically modified plants (or to allow the application of conventional pesticides) must be taken in the context not only of any impacts identified, but also in the context of other options that are currently being used by farmers. The replacement of a conventional pesticide may in some instances yield lesser non-target impacts, even if those impacts are not zero. The standard employed by the Environmental Protection Agency for all pesticides is "no unreasonable adverse effect" to the human environment.

Similarly, we know that the implementation of "zero tolerances," to address the proven risks of a toxin or a carcinogen, becomes more and more difficult and costly, and more detached from an actual risk, as time goes on and ever more sensitive analytical methods are developed to detect the material that one is trying to exclude. This is even more true when trying to address issues which may be more hypothetical, as is the case with many issues associated with GMOs.

Next in this item is transparency. Transparency not only removes regulatory uncertainty, but also is critical for safe technology utilization and technology development. It provides a clear window for those who have concerns about the biotechnology activities being regulated to observe and evaluate government actions. Much of the current favorable climate regarding agricultural biotechnology in the USA we attribute to the fact that the actions of our regulatory agencies, including the development of new regulations, are open for public scrutiny and public comment. We provide enormous amounts of information via Email and hard copy about our regulatory actions, and hard copies of submissions from applicants, to the public.

Transparency serves another useful function, that of eliminating hidden barriers to research in the public or private sectors, or barriers to movement of new technologies into established commercial channels when safe and useful products are developed.

Flexibility and adaptability have been built into US regulatory policy for the products of agricultural biotechnology. APHIS regulations, for example, which originally went into effect in 1987, have been modified a number of times including significant modifications in 1989, 1993, and 1997, so as to keep pace with the accelerating growth of knowledge. In these adjustments, APHIS has, among other things, provided new regulatory options to facilitate the safe testing of potential new products, and put in place systems to facilitate the commercial use of products determined to be safe. This is just one example of a very important feature the US regulatory system for biotech products embodies: an ability to adjust based on increasing experience.

Regulations need to be effective - that is, implementable by the government, with standards that can be met by industry - and responsive to the needs of the public and changes in our scientific understanding.

On the topic of meeting domestic needs, we have recognized the importance of consulting with our local State authorities, who have intimate knowledge of local environmental conditions, prior to decisions for field trials or deregulation of new crop varieties. Our efforts have been rewarded, as nearly all the biotechnology-specific legal structures adopted at first by some individual States have been discontinued. Seven States at one time or other had regulations to address concerns regarding testing of biotechnology-derived products. Only one State still does so.

What have we learned in that process? What have States wanted to know? The States, first off, wanted to know what new varieties were entering their borders or being field tested. Again, this is a matter of reasonable local interest and responsibilities. States enforce local quarantine measures for particular pest problems. What did States want to have the opportunity to do? To provide their input on decisions made at the Federal level. We have specifically asked States if they want to provide any input on petitions to deregulate organisms under the APHIS system. It has been our role at the Federal level to see that any State concerns are addressed prior to a field trial going forward or a new product being determined to have nonregulated status or when any significant Federal action on them is taken.

The topic of international needs is one I could spend additional hours on. Rather than do that, let me just indicate that we are very active in this area, and that we work bilaterally, regionally, and multilaterally (in fora we are comfortable with and in fora where we are more disadvantaged but need to be present) to achieve our main aim of harmonization - compatible regulatory approaches that will enable the flow of safe products between countries.

We have worked, and continue to work, with countries to exchange information and identify commonalities in our oversight systems so that we can build confidence in each other's review processes and extend existing arrangements that have covered traditional products to new agronomically enhanced ones. We strive to provide an "early warning system", on a technical and informal basis, for issues that may arise when particular products nearing commercialization need to be considered outside national contexts. Critically, we work to assure that scientific principles are used for evaluation of organisms, a cornerstone of our national and international policies for review of agronomically enhanced crop varieties.

For strategic regulations in biotechnology, consideration of risk versus safety is of paramount importance. Our commitment to this is no less than the commitment of regulators anywhere else in the world. The aim, of course, is to ensure that new products are as safe as their traditional counterparts. We also recognize that other considerations are important as well:

- ability of products to be utilized
- safe technology transfer
- economic competitiveness
- international harmonization
- global needs and acceptance.

We believe that all of these features have been carefully considered in the development of strategic regulations in the USA for agricultural biotechnology products.

Let me talk for a moment about a few key aspects of these other features that are relevant in this meeting. First, I think it is important to mention that product utilization has implications for farmers and for downstream users of products as well as ultimate consumers. Products determined to meet our standards of safety for use in agriculture or the environment or as food, must still be able to make it through the complex set of steps leading to the final products that consumers want to buy and can afford.

In the USA, not all of the regulatory structures that apply to biotechnology-derived products are Federally based. Some traditional standardization systems are administered by the Federal government, some at a more local level. They may not even be mandatory, even though it is in everyone's interest to ensure that the public good that these systems attempt to address is maintained. Cooperative approaches

involving government at various levels and the private sector have enabled the maintenance of high standards.

For example, in the USA, there is a system of Variety Registration for both genetically modified and traditional varieties. It is entirely voluntary and is administered at the Federal level. Not all companies register their new varieties, and some may simply use other means of intellectual protection for their new innovations. For seed companies, putting their name on a new variety of seed is intended to convey to their customers that the seed meets the standards that they have come to expect.

Variety registration in the USA does not, as in many other places in the world, require a determination that a new variety is in some way "better" than pre-existing varieties. The US approach is that such decisions are best left to the marketplace, and our experience with new varieties is that those decisions are very carefully weighed by farmers.

Similarly, the USA has a voluntary system of seed certification, which is administered via both a private organization that collects fees for testing, the Association of Official Seed Certifying Agencies (AOSCA) as well as State Crop Improvement Associations. Companies may or may not elect to have seed officially certified, but again their name on the label is often seen as a guarantee for their customers that the required standard of seed quality, in terms of contamination by off-types and weed seed, is met or exceeded. AOSCA sees to it, however, that all US seed that enters international commerce meets any OECD Seed Schemes to which we belong.

So it has not been our approach that all issues require mandatory regulatory action, and that all actions must take place at the Federal level. Utilizing a variety of mechanisms has worked well for us.

In the USA, our regulatory agencies cooperate well to achieve coordinated and balanced regulation of new agricultural biotechnology products. We have, at various times, had formal consultation mechanisms in place for resolution of policy issues. As issues have been resolved, these mechanisms have been replaced by informal and collegial consultations. We have, in some instances, overlapping areas of authority, but it is generally clear who has the lead on which issues, and we cooperate where there are known overlaps. USDA and EPA, for example, have arrangements that allow scientists in each agency to view confidential business information received on relevant submissions by the other agency on plants engineered to have new pesticidal properties.

All of the agencies are cognizant of the fact that the organisms in question are living plants, and as such have relatively fixed schedules of planting and harvest. We try hard to reach decisions in a timely manner and have fixed review timelines so that the developers of new varieties can have some reasonable expectations as to their future commercial timetable, and farmers are able to make their decisions about seed purchase and planting. However, we will not speed up our regulatory decisions if to do that would compromise our review requirements or safety.

For the final piece of the picture in the USA, what other mechanisms do we have that have encouraged the safe development of biotechnology in the USA? We have no industrial policy *per se* to influence the level and composition of our nation's output in this sector, but there is a special emphasis in the USA in supporting fundamental scientific research, and biotechnology has been taken note of in this emphasis. USDA sponsors both in-house and university agricultural biotechnology research. About 10% of its research budget is directed toward biotechnology, and one percent of all USDA biotechnology research funds are directed toward risk assessment research.

There are also well-established mechanisms to enable transfer of innovations from the public sector, or the academic sector, to the private sector. These are mechanisms that apply, again, not only to the transfer of useful innovations in agricultural biotechnology, but to transfer of other new technologies as well. These are mechanisms that allow government scientists to work as closely as necessary with private firms to help the companies commercialize technology based on the scientists' research. Under these mechanisms, companies can gain the first right to exclusive licenses on patented inventions made under the agreement. Licensing fees and royalties can be shared between the research scientists and the government agency for which they work. These arrangements allow for the kinds of interactions under which innovative technology can develop and flourish.

In the end, however, in order for products that have gone through the R and D phases and that have made it to commercialization to become viable in the marketplace, they must meet with public acceptance. Much of the debate that seems to be taking place in various countries around the world at present took place in the USA over the past several years, and now it seems that products meet with a substantial amount of acceptance from farmers and from the public.

As more and more products with new traits that directly benefit the consumer become available, we expect that these will contribute further to increased consumer familiarity with the new technologies, and acceptance of them.

The atmosphere of openness and the transparency of our actions have allowed many citizens to become more familiar, more knowledgeable, and more comfortable with the new products entering our marketplace today. The wider adoption of clear and transparent, farsighted science-based procedures worldwide can bring about the same effects outside the USA as well.

The process in Canada for regulating plants with novel traits (PNTs) from field testing to commercialization

Watson, G.

Variety Section, Canadian Food Inspection Agency, Nepean, Ontario, Canada

All assessments/authorizations in Canada for Plants with Novel Traits (PNTs) are conducted by the Canadian Food Inspection Agency (CFIA) with the exception of food safety assessments conducted by Health Canada.

The CFIA was created in April 1997. It includes employees across Canada from four different federal departments: Food Production and Inspection Branch of Agriculture and Agri-Food Canada, Health Canada, Industry Canada, and Fisheries and Oceans Canada.

Its role is to monitor health, safety and quality of Canada's animals, plants, fish and food products, fraud prevention, environmental risk assessments and sale of inputs (seed, fertilizer, feed). The Plant Biotechnology Office (PBO) (which is responsible for conducting environmental assessments) is located in the CFIA.

Federal framework for biotechnology

The Plant Biotechnology Office works within the Canadian Federal Framework for Biotechnology Regulation which was established in 1992.

The Federal Framework embraces the following goals:

- Maintain Canada's high standards for the protection of worker's health, the general public and the environment.
- Use existing legislation and regulatory institutions to clarify responsibilities and avoid duplication.
- Continue to develop clear guidelines for evaluating products of biotechnology which are in harmony with national priorities and international standards.
- Provide for a sound scientific database on which to assess risk and evaluate products.
- Ensure both the development and enforcement of Canadian biotechnology regulations are open and include consultation; and
- Contribute to the prosperity and well-being of Canadians by fostering a favorable climate for investment, development, innovation and adoption of sustainable Canadian biotechnology products and processes.

The step-wise approach to release into the environment

The regulatory oversight of PNT development can be divided into five steps;

- Import into Canada through a permitting procedure under the Plant Protection Act and Regulations.
- Contained research in a laboratory facility or greenhouse, covered by Medical Research Council (MRC) guidelines.
- Confined field trials authorized under the Seeds Act and Regulations by the PBO of CFIA.
- Unconfined release into the environment authorized under the Seeds Act and Regulations by the PBO of CFIA.
- Variety Registration granted under the Seeds Act and Regulations by the Variety Registration Office (VRO) of CFIA.

The product-based approach

In Canada, PNTs are regulated on the basis of the characteristics of the product, not the specific process by which the product was made. The primary trigger of the regulatory process is the novelty of the plant species, its characteristics (traits) and use, in the Canadian context.

Therefore products of traditional breeding or mutagenesis, as well as the products of recombinant DNA technology may be considered novel and regulated under the Seeds Act.

For example: Herbicide resistance may be achieved through mutation breeding, selection of naturally occurring variants or by the use of recombinant DNA technology. In any case the important aspect in terms of the environmental interactions is the trait, herbicide resistance.

Safety-based model

The safety-based model for regulation of PNTs is based on the concepts of familiarity and substantial equivalence. Familiarity applies to the species, the trait, and cultivation practices. Substantial equivalence refers to potential alteration of environmental interactions and considers the following criteria:

- Potential to become a weed of agriculture
- Potential to be invasive of natural habitats
- Potential gene flow to weedy relatives that may become weedy or invasive
- Potential to become a plant pest
- Potential impact on non-target species
- Potential impact on biodiversity.

Risk management is applied to confined field testing by the imposition of terms and conditions to the field tests, e.g., confinement especially reproductive isolation, site monitoring, post harvest land use restrictions. For unconfined release any identified risk is minimized by placing specific conditions on areas of production and use.

Summary of Biotechnology Regulations

Canada has been regulating PNTs since 1988. From 1988-1996 confined trials were conducted using guidelines only. In December 1996, Regulations were promulgated under the Canada Seeds Act which reaffirmed the legal basis for regulating PNTs. The Regulations provide the general information requirements for conducting environmental risk assessments for both confined and unconfined releases. Specific information requirements are found in our Regulatory Directives.

Exemptions

- PNTs that are grown in containment are exempt from the Regulations.
- Varieties which are derived from PNTs and are deemed to be substantially equivalent to those PNTs which have been authorized for unconfined release are also exempt from regulatory scrutiny.

Once a PNT has been granted an unconfined release, if the proponent or owner becomes aware of new information regarding a risk to the environment, including risk to human health, the PBO Office must be notified immediately. At that time:

- the conditions of the release could be changed,
- additional conditions could be imposed, or
- the release could be cancelled and conditions imposed to minimize the risk.

Normally, the environmental risk assessment is only part of the overall regulatory activity for a PNT. If a product has potential for livestock feed use, it must receive approval under the Feeds Act or if it is for human food use it must be approved under the Health Canada Food and Drugs Act.

Since 1988 the PBO has authorized confined trials of the following agricultural and horticultural crops: wheat, tomato, tobacco, soybean, potato, flax, corn, canola, alfalfa, grapes, sugar beets, poplar trees, strawberries, peas, Japanese trefoil, mustard, oats, barley, broccoli, cherry.

Since 1988 companies and institutions have been conducting trials in Canada on crops with the following traits: Novel herbicide tolerance, male sterility/restoration, insect resistance, nutritional change, virus resistance, stress resistance, fungal tolerance, pharmaceutical, genetic research, generation of mutants.

The first unconfined field release occurred in Canada in 1995 with the authorization of a glufosinate ammonium tolerant (Liberty) canola from AgrEvo. Since then there have been 29 more.

Variety registration

The next step after a variety has been granted unconfined environmental release is variety registration. The Variety Registration system has three mandates to ensure:

- agronomically inferior or unadapted varieties are excluded from the Canadian marketplace,
- new varieties meet current requirements for resistance to economically important diseases, and
- high quality products for processors and for consumers.

In Canada varieties of 30 major crops are subject to registration prior to being sold commercially. Registration is based on merit and has been in place since 1923 (75 years). Varieties are entered into several years of trials administered by recognized recommending committees. There are several types of registration: permanent, interim, regional, interim regional and contract.

- Permanent
  - Can be sold anywhere in Canada.
  - Majority of varieties registered have this type of registration.
  - No restrictions on the production of seed or commodity crop.
- Interim
  - All the rights and privileges of permanent, but have expiry date.
  - Used where additional data required on certain traits (e.g., production of grain for market acceptability tests).
  - Minimum of one year of experimental data required.
  - Maximum life of five years for this category.
- Regional
  - May only be sold in specified regions of Canada.
  - Has potential to cause harm to industry if grown in other areas; e.g. seed/grain distinguishability, or where variety may be detrimental to human or animal health or to the environment.
- Interim Regional
  - May be sold only in certain specified regions.
  - Has expiry date including maximum life of five years.
- Contract
  - Where delivery of the resulting commodity into traditional commodity channels could cause adverse effects to those channels.
  - Must be grown under closed loop contract system, e.g., high erucic acid rapeseed would cause harm to canola.
  - Applicant must have complete quality control system in place.

For plants with novel traits developed by genetic engineering or by traditional plant breeding using transgenic parents, the applicant must:

a) submit a notarized affidavit with the application, indicating that molecular tests conducted on seed of the variety entered in variety registration trials and of the legal reference sample, confirm that the variety contains the correct genetic makeup, and
b) provide the detailed laboratory protocols used to conduct the molecular tests.

Fees

As of December 1, 1997 the PBO and VRO offices for their assessments and registrations started charging fees (partial cost recovery).

Commercialization of PNTs in Canada

First PNTs commercialized in Canada was AgrEvo's Liberty Link canola in 1995.

1998

Soybeans: - 2.1 Million total acres in Ontario
- approximately 150,000 acres (7%) herbicide tolerant (Ht)
- 14 out of 180 registered soybean varieties are (Ht)
- could be up to 400,000 acres of Ht varieties in 1999.

Canola: - 13.4 Million total acres in Western Canada
- 50% of acreage was Roundup Ready and Liberty Link - (rDNA)
- additional 10% was Pursuit - (mutagenic)
- 22 out of 130 *B. napus* varieties registered are Ht
- 2 out of 31 *B. rapa* varieties registered are Ht.

Corn: - 3 Million acres of corn grown in Canada
- 2.2 Million acres (70%) of this is grown in Ontario
- 248 varieties recommended for growing in Ontario
- of these 20 are European corn borer (ECB) resistant
- 7 Liberty Link (Glufosinate tolerant)
- 1 Pursuit tolerant
- 500-600,000 acres of ECB resistant corn (16-20%)
- 1999: – 1/3 of Canadian acreage will be ECB resistant with up to 300,000 acres Ht.

Potatoes:
- 3 varieties with interim registrations out of 145 potato varieties have Colorado potato beetle (CPB) resistance
- approximately 5,000 commercial acres in Canada (40,000 in North America).

Future directions/Trends in Canada

Shift in types of products being tested

Technological developments in the plant biotechnology industry are driving changes in the both types of traits being targeted and the number of plant species being manipulated for eventual commercialization.

As the market becomes saturated with first generation technologies in the major agricultural crops, technology companies are introducing these traits into secondary crops.

The types of PNTs currently under development or in commercialization may be broken down into two broad groups: first generation or input-related plants, and second generation or output-related products.

First generation (input-related) PNTs:

- Herbicide tolerance,
- *Bacillus thuringiensis* (*Bt*)-based insect tolerance,
- Stress tolerance.

Second generation (output-related) PNTs:

- Compositional changes, modified nutritional qualities, modified oil,
- „Nutraceutical" plants engineered to produce compounds that have alleged health enhancing effects,
- Pharmaceutical production.

Products that have been commercialized in Canada to date are primarily either herbicide tolerant or (*Bt*-based) insect resistant. At the level of confined field trials those submissions involving herbicide tolerance as the primary breeding objective have been declining (Figure 1) while those submissions involving compositional changes, insect and disease resistance, and stress tolerance have been steadily increasing as a percentage of confined submissions.

Many second generation PNTs such as pharmaceutical plants, phytoremediators, and plants with modified biochemical compositions raise concerns for production and disposition conditions which will be required to ensure isolation from the traditional commodity crops.

Harmonization efforts between Canada and the USA

In mid-July 1998 Animal and Plant Health Inspection Service (APHIS) of USDA, CFIA and Health Canada met to compare and harmonize where possible, the molecular characterization components of the regulatory review process for transgenic plants.

Agreement was reached on common information requirements with respect to most aspects of the molecular characterization.

The results of this meeting, and other activities, may lead to mutual acceptance of assessments in the future. In the near term, the continued exchange of information between the CFIA, Health Canada and USDA-APHIS further enhances the understanding of the three agencies; respective regulatory systems and requirements, and should expedite the review process.

The CFIA is responsible for the regulation of importation, environmental release and feed use of plants with novel traits which include, but is not limited to, transgenic plants. Health Canada has jurisdiction over novel foods, including food products derived from transgenic plants. In the USA, APHIS is responsible for the regulation of importation, interstate movement, and environmental release of transgenic plants that contain plant pest components, but regulatory authority for food and feed use of plants lies with the Food and Drug Administration (FDA).

Canadian biotech strategy

A Canadian Biotechnology Advisory Committee (CBAC) will be formed:

- Independent expert panel, chair and nominating panel to be announced shortly,
  members of panel to be nominated through a public process,
  and committee membership decision to follow.
- A forum to voice views and participate in a dialogue.
- To advise Ministers on ethical, social, economic, scientific, regulatory, environmental and health aspects of biotechnology,
  will not arbitrate regulatory decisions.

Major review of variety registration system

The CFIA is initiating a review of the variety registration system this fiscal year (1998-99).

With biotechnology developments continuing to grow rapidly in Canada and internationally, a registration system is required that keeps Canada competitive with other countries from a market access perspective without losing sight of our domestic needs. This means developing a system with some stability for the next five to ten years.

CFIA website

The Canadian biotechnology documents are available on our CFIA website. It includes the Seeds Act and Regulations, Regulatory Directives, Crop Biologies, Decision Documents.

The PBO website is:
http://www.cfia-acia.agr.ca/english/plant/pbo/home_e.html
http://www.cfia-acia.agr.ca/français/plant/pbo/home_f.html

The variety registration documents are also on the CFIA website. It includes List of Varieties Registered in Canada, Procedures for the Registration of Crop Varieties in Canada, Variety Registration Application Form, List of Varieties with Novel Traits and Their Progeny Registered under the Canada Seeds Act and Regulations.

The VRO website is:
http://www.cfia-acia.agr.ca./english/plant/variety/home_e.html
http://www.cfia-acia.agr.ca./français/plant/variety/home_f.html

Authorization of herbicides complementary to herbicide resistant crops

Hohgardt, K.[1)], Schiemann, J.[2)], Zwerger, P.[3)]

[1)] Fachgruppe Chemische Mittelprüfung,
[2)] Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit,
[3)] Institut für Unkrautforschung,
Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Messeweg 11-12, 38104 Braunschweig, Germany

Introduction

There will be a considerable increase of genetically modified (transgenic) crops in the near future, and their commercial cultivation rises the question of how to deal with the evaluation and authorization of plant protection products. When evaluating and authorizing plant protection products for use on genetically modified crops a distinction has to be made between complementary herbicides and other plant protection products. A herbicide is called complementary when a crop plant became resistant to this herbicide by genetical modification.

The evaluation and authorization of plant protection products is laid down in EU Directive 91/414/EEC (set into force in Germany by the Plant Protection Act). Without an authorization the commercial use and the application of a plant protection product is not possible. The market for plant protection products in Germany and their export in 1995 is shown in Table 1. The data represent the results of the German notification procedure for 1995 (Schmidt, 1998).

Table 1 Market for plant protection products in Germany and their export for 1995

Active substance group	German market		Export	
	Tons	%	Tons	%
Herbicides, incl. safener	16,065	46.5	33,722	38.0
Fungicides	9,652	28.0	29,808	33.6
Insecticides, acaricides incl. synergists	4,925	14.3	16,558	18.7
Growth regulators, sprout inhibitors	2,435	7.1	4,424	5.0
Others	1,454	4.2	4,127	4.7
Sum	34,531	100.0	88,639	100.0

A survey is given of how the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA; = Federal Biological Research Centre for Agriculture and Forestry) proceeds with the evaluation and authorization of plant protection products to be used in cultures with transgenic plants.

Legal basis

The procedure for the evaluation and authorization of active substances and plant protection products is laid down in EU Directive 91/414/EEC. This directive was amended several times and further regulations and guidance documents were published since 1991. Table 2 gives an overview of the areas where an evaluation takes place.

Table 2 Evaluation areas for the authorization of active substances and plant protection products

Area	Active substance (as) and/or plant protection product (ppp)	
Identity	as	ppp
Physical, chemical and technical properties	as	ppp
Data on application	-	ppp
Further information	as	ppp
Analytical methods	as	ppp
Efficacy data	-	ppp
Toxicological and metabolism studies	as	ppp
Residues in or on treated products, food and feed	as	ppp
Fate and behaviour in the environment	as	ppp
Ecotoxicological studies	as	ppp
Classification and labelling	as	ppp

For the use of herbicides complementary to herbicide resistant plants special emphasis should be given to data on application and residues in or on treated products, food and feed. In Table 3 special data requirements are summarized.

Table 3 Special data requirements for the authorization of herbicides complementary to herbicide resistant crops

Data on application	Residues in or on treated products, food and feed
Field of use envisaged	Metabolism, distribution and expression of residue in plants
Effects on harmful organisms	Metabolism, distribution and expression of residue in livestock
Details of intended use	Residue trials
Application rate	Livestock feeding studies
Concentration of active substance in material used	Effects of industrial processing and/or household preparation
Method of application	Residues in succeeding crops
Number and timing of applications and duration of protection	Proposed maximum residue levels (MRLs) and residue definition
Necessary waiting periods or other precautions to avoid phytotoxic effects on succeeding crops	Proposed pre-harvest intervals for envisaged uses, or withholding periods or storage periods, in the case of post-harvest uses
Proposed instruction of use	Estimation of the potential and actual exposure through diet and other means
	Summary and evaluation of residue behaviour

The evaluation of plant protection products in Germany is based on the German Plant Protection Act (PflSchG) of 15 September 1986 as amended on 14 May 1998 (Bundesgesetzblatt I S. 971, 1527, 3512), its § 15 being the most relevant for a product's authorization. For the deliberate release of genetically modified organisms (GMO) and their placing on the market the German Genetechnology Act (GenTG) of 24 June 1994 (Bundesgesetzblatt I S. 1416) is applied. Detailed instructions on genetechnological procedures are given by the "Gentechnik-Verfahrensverordnung" (GenTVfV) as amended on 10 December 1997 (Bundesgesetzblatt I S. 2884), making sure that the applications submitted reveal details on the introduced new genes and characteristics of the GMO and, independent from the risk assessment, make a proposal for their labelling.

Plant protection products for use on transgenic plants are subject to the Plant Protection Act. They are only permitted for applications mentioned in the instructions for their use. In addition, the Act on Food

and Commodities has to be observed. The "Ordinance on Maximum Residue Limits" which is based on this act sets maximum limits for residues of plant protection substances which must not be surpassed when the foods are marketed.

Non-complementary plant protection products

The authorization of a plant protection product requires a thorough evaluation of the studies and documents submitted to the BBA. For certain plant species there often is a huge number of registered varieties from which usually four to six undergo evaluation in the frame of the plant protection product authorization. When new varieties are introduced in the market once a plant protection product has been authorized this usually does not mean an extension or repeat of the evaluation. Whenever required labelling instructions are given for sensitive varieties ("positive list") or other special characteristics.

When the intention is to apply a non-complementary plant protection product on genetically modified crops the scientific and technological state of the art does not require a new process of authorization and evaluation, provided the plant protection product was already approved for the plant in question. In such a case the assumption is that the experience gathered during the practical application of the approved product on a plant is sufficient for judging the plant's possible range of reactions to the product.

Usually the new traits of transgenic crops have thoroughly been analysed under the aspects of molecular genetics and molecular biology. As long as the results do not give any reasons to assume possible effects on the metabolism of known agents from plant protection products no further action is required with a view to the approval of the product. In the case of a well-founded assumption of a threat to human beings and to the environment it is always possible to demand special requirements for the application and to give instructions for a special label on the product pack. This equally applies to non-transgenic and transgenic crops.

Complementary plant protection products

In case of transgenic crops which are resistant to certain substances in plant protection products the introduced genes have to be evaluated according to the Genetechnology Act, and for plant protection products containing these substances another evaluation according to the Plant Protection Act is required, based on the new mechanism of resistance which was introduced, and on the new situation as to metabolism and residues in the plant when the product is applied. In addition, complementary resistance may entail changes in application, e.g., a change of the timing, or the development stages at which the product is applied to the plant, or of the quantity to be applied.

When evaluating complementary plant protection products one should consider that different mechanisms may result in different metabolites and residues. Among other mechanisms of resistance, the following are introduced into crops by genetechnological methods:

- resistance against glufosinate by introduction of an N-acetylation enzyme, and
- resistance against glyphosate by introduction of a glyphosate-oxidoreductase or by introduction of a glyphosate-tolerant enolpyruvylshikimate phosphate synthase.

So, in case of complementary resistance introduced by genetechnological methods, plant protection products should be approved for defined mechanisms, and their applications should be clearly identified. For example, glufosinate resistant crops are additionally marked "glufosinate-resistant crop" and "resistance mechanism: inactivation by means of enzymatic N-acetylation".

This is a necessity in so far as labelling of the GMO placed in the market in accordance with the Genetechnology Act does not automatically reveal this differentiation of mechanisms. The Federal Plant Varietal Office will not arrange for the varieties to be individually labelled for resistance mechanisms; neither the labelling will mention a plant protection agent or a trade brand of the plant protection product.

The only identification of the field of application as mentioned above is not sufficient, the resistance gene in many genetically modified plants being contained as a mere marker. The resistance gene was introduced into these plants along with other genes of interest, it serves to select intended traits. Herbicide resistance not being the purpose of the manipulation was ignored in the breeding process. Nevertheless there would be no legal objections to the application of the complementary plant protection product. To avoid misuse the definition of the range of application includes another restriction which

expressively refers to the relevant complementary product. For glufosinate resistance, the restriction is: "Only to be applied to species and/or varieties additionally marked Liberty Link". The company marketing "Liberty" is responsible for making sure, in cooperation with breeders, that "Liberty Link" is issued only when there is an adequate compatibility of the plant variety or seeds lot.

At the moment, two active substances are under evaluation: the use of glufosinate in herbicide resistant maize, winter oilseed rape and sugar beets in Germany, and of glyphosate in herbicide resistant fodder beets in Denmark. EU import tolerances for cotton seed, maize, oilseed rape seed, soybeans and sugar beets to cover the use in the USA are discussed. According to the latest information available the use of glyphosate in herbicide resistant maize, oilseed rape, soybeans and sugar beets is under evaluation in France. In Germany an authorization is recently granted for the herbicide LIBERTY (glufosinate) in herbicide resistant maize. We expect an application form to grant an authorization for glyphosate in resistant plants in the near future.

Complementary herbicides for selection

Combining different transgenes with a herbicide resistance marker can be reasonable from the breeder's view since this would mean a facilitation of the selection of characteristics linked with the herbicide resistance during the breeding process. A practical example for this, which can be used beyond the breeding process for the production of seeds, is the combination of male sterility with glufosinate resistance. In this case the herbicide does not serve for killing weeds but for the selection of desired plants. Again, this application is subject to the Plant Protection Act. To give a sound legal basis to breeders and seeds producers for the application of herbicides to select plants by means of a suitable selection marker, the definition of the field of application is changed into a new objective: "Selection of desired plants for breeding purposes and seeds production".

"Maximum Residue Levels" for transgenic plants

When non-complementary plant protection products are applied to crop plants after their treatment by genetechnological methods the metabolism of these crop plants usually will not change in comparison to the application on plants which have not undergone such a treatment. As a consequence, in such cases the state of the scientific and technological art does not require another evaluation, also with regard to maximum residue levels.

Due to new mechanisms of herbicide decomposition or tolerance of the agent, the use of complementary herbicides may lead to a new situation as to the metabolism or residues of the agent. Factors to be taken into account are, e.g.,

- that due to the new resistance the amount of herbicide to be applied will be possibly increased in comparison to the application upon non-transgenic lines,
- a modified absorption and distribution within the plant, and thus the residue accumulation within the consumable parts of the plant.

Here, the Plant Protection Act and the Act on Food and Commodities demand an evaluation by the BBA, performed in coordination with the Federal Institute for Health Protection of Consumers and Veterinary Medicine, to check whether in individual cases a revised definition of residues and a determination of maximum residue levels are required.

Genetically modified plants producing active substances

When a crop is genetically modified in a way that the crop itself produces an active substance, (e.g. the endotoxin of *Bacillus thuringiensis*) the plant protects itself through this genetic modification. In Germany such a plant is not considered as a plant protection product. There is no necessity to grant an authorization for this plant.

Nevertheless, it is possible that for the given active substance applied as plant protection product a Maximum Residue Limit was set on an EU level or on a national level, respectively. In this case a new evaluation of the residue behaviour and an assessment of the new residue situation are necessary to answer the following questions:

- Is there any risk for consumers associated with the introduced residues?
- How can these residues be differentiated from those applied via a plant protection product?

Conclusions

Before applying herbicides complementary to herbicide resistant crops they have to undergo a specific evaluation process to authorize them for their use. This authorization will be connected with restriction and conditions dealing with the specific requirements for the use in herbicide resistant crops.

Different organizations and institutions in Germany as well as in the EU are involved in the process of granting the deliberate release of genetically modified organisms into the environment and the process of granting an authorization for plant protection products. There is a need for the exchange of information between the experts involved in the different regulatory frames. An extensive communication has been realized within the BBA. In the future it would be helpful to improve the harmonization of the evaluation processes in the frame of the two Directives 90/220/EEC and 91/414/EEC.

References

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Decision support systems for safety assessment of agri-biotech applications

Tzotzos, G.T.

Head, Biodiversity Unit, United Nations Industrial Development Organization, Vienna, Austria

Abstract

Electronic decision support systems provide new capabilities to facilitate the evaluation of risks arising from the environmental application of genetically modified organisms (GMOs). The development of such systems is rendered feasible by advancements in information technology and the explosion of theoretical and empirical data related environmental applications of biotechnology. Decision support systems for biological risk assessment are of particular relevance to developing countries where the capacity and resources to exercise regulatory oversight is severely limited.

The paper describes the basic architecture of a pilot system currently under development by the United Nations Industrial Development Organization (UNIDO).

The use of artificial intelligence (AI) technology has found wide fields of application into health, safety and environmental risk analysis. However, the applicability of AI technology in the assessment and management of risks associated with the environmental use of organisms and products derived by means of recombinant technologies is of limited value at best. The reason for this is the complexity of the interactions of genetically modified organisms (GMOs) with the environment into which they have been released as well as the scarcity of relevant data.

Environmental release of GMOs does not submit to the rigor of quantitative risk assessment and, instead, scientific knowledge and qualitative judgement is invoked to anticipate potential risks to human, animal health and the ecosystem. Such qualitative risk assessment is normally conducted by review panels basing their judgement on:

- empirical knowledge related to GMOs and the environment of their release,
- situational knowledge about test conditions and objectives,
- theoretical knowledge related to ecological relationships,
- normative knowledge about policies and acceptance criteria.

However, this practice has some inherent limitations which have to do with the difficulty of codifying such knowledge into a form that can be reapplied to future cases, with the necessary refinements and modifications. These limitations are exacerbated in the case of most developing countries where the capacity and resources to exercise regulatory oversight through expert panels is severely limited.

Recent innovations in information technology have made possible the development of “knowledge assistant” systems (KAs), in which a broad range of information from disparate sources can be captured and applied to risk assessment of biotechnology applications. Such systems rely on knowledge rather than data and differ in distinct ways from AI systems.

Table Comparison of expert system and knowledge assistant technology

Expert system	**Knowledge assistant**
Consultation based	Transaction based
System initiative	User initiative
Self-contained	Fully coupled
Goal directed	Data driven
Narrow scope	Broad scope
Static knowledge	Cumulative knowledge

The application of KA systems in biotechnology risk assessment can provide "road maps" enabling the sequential ordering of information and database interrogation at each stage of a review procedure. KA systems rely on the effective user interaction and are, therefore, capable to function with incomplete sets of information, requesting the user to supply additional information, if necessary.

The development of risk assessment decision support systems can provide an invaluable tool to preserve, disseminate and interpret available data and information regarding specific organism/transgene/ environment combinations. They can significantly enhance familiarity with environmental introductions of GMOs and provide information support to regulatory authorities, researchers and biosafety officers in public institutions and commercial enterprises. This is particularly relevant in the case of developing countries for the reasons mentioned above.

The development of such decision support systems by the Biosafety Information Network and Advisory Service (BINAS) of UNIDO has been prompted by extensive interaction with regulatory authorities, academic institutions and biotechnology enterprises in the developing world over the past 15 years and by the phenomenal opportunities offered by recent advances in information technology.

The BINAS model system provides a road map for safety issues related to the biology of initially three major crop plants, namely, rice, potato and oilseed rape and on commonly "engineered" traits, such as herbicide tolerance, virus resistance mediated by viral coat protein genes and insect resistance through the expression of *Bacillus thuringiensis* toxin gene. The system describes:

1. The physiological properties and function of the unmodified organism.
2. The centre of origin or diversity and geographic distribution of the species and its near relatives.
3. The breeding system of the species with special reference on the tendency to outcross and rates of dispersal as a function of distance (information for the crop species in question is available, primarily in terms of requirements for isolation distances in the production of certified seed).
4. The existence of weed problems of the species and its close relatives, in regions in which this has been reported.
5. Differences between the genetically modified plant and its unmodified parent.

The above information is integrated into risk assessment flowcharts permitting interactive queries proceeding through the various steps of risk assessment. Query outputs are used as inputs to subsequent queries leading typically to:

- Identification of specific hazards and referral to risk management procedures related to the hazard(s) in question.
- Identification of additional research/testing needs or risk management procedures compensating data/information gaps.
- Exit from the risk assessment scheme and referral to regulatory advise.

Flowcharts on Ecosystem Effects Assessment provide general guidance on evolutionary and ecological issues resulting from the possible interaction of the GMO with non-target ecosystems. Good developmental practices for GMO containment and/or control of the GMO are presented in detail.

Although flowcharts are constructed much like decision trees, their outputs point to specific actions rather than to qualifications like "safe" or "not safe".

The system consists of stand-alone modules interconnected through relational databases and will be accessible on-line once it has undergone adequate validation. Annex I describes a module dealing with the assessment of the genetic and agro-ecological risks from biotechnologically derived herbicide resistant crops (BD-HRC). The latter takes into account factors related to agronomic management.

A major part of the database content originates from the Biotechnology Consensus Documents of the Organization of Economic Cooperation and Development (OECD).

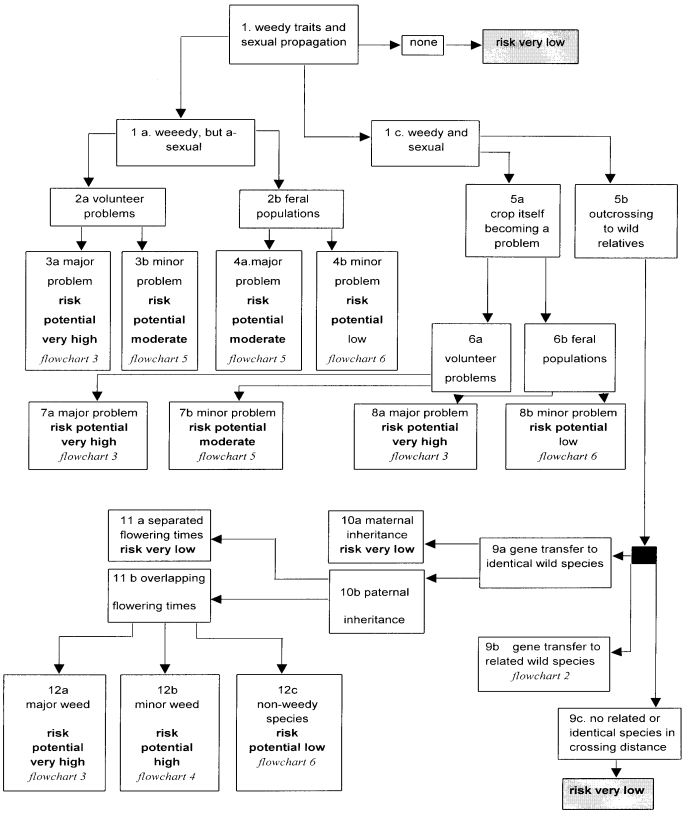

Figure Draft risk analysis herbicide resistant GMOs – Flowchart: Dispersal and weediness

Decision tree for the assessment of the genetic and agro-ecological risks arising from transgenic herbicide resistant crops. Developed for UNIDO by Ton Rotteveel[a)] and Jonathan Gressel[b)]: [a)]Plant Protection Service, 6700 HC Wageningen, The Netherlands, and [b)]Plant Sciences, Weizmann Institute of Science, Rehovot, 76100, Israel

Introduction to the decision tree

The decision tree is presented as a system of keys, and operates as a ranking system. The evaluation system is composed of two parts. In key 1 the hazards imposed by the weediness and dispersal of the gene are ranked in relation to the inherent biological characteristics of the crop and the related weed species. If the hazard is found to be very low or no reasonable hazard can be conceived, no further examination of risk is needed or possible. When one has an unknown risk due to lack of relevant information on the possibility of introgression with wild relatives, it is assumed that there is a moderate risk, and one is directed to key 4. In all other cases one is directed to another key, 2 till 8, which all basically ask the same questions about the agricultural systems.

The answers, e.g. risk categories vary also with the initial input of hazard category. They also vary as a function of herbicide use and modes of action and metabolism. There are five potential risk categories to be examined within agriculture (very low, low, moderate, high, and very high) within agriculture and two outside of agriculture (feral low and feral high) in seven extra keys (2-8). The system is analogous to the one developed for evaluation of the risks of evolution of pesticide resistance. The system ranks risks but does not quantify them, and it should be realized that the keys offer insights on broad, possibly somewhat overlapping risk categories.

Risk categories should be interpreted as follows

- Risk very low: all risk factors indicate a low chance on problems evolving. In key 1 (hazards with inherent risk) risk very low will be reached as an end-station if the biological factors all indicate low risk.
- Risk low: most factors in the low risk category indicate low risk, but a few may indicate a higher risk.
- Risk moderate: in this category the picture is not very clear, about a same number of modifiers change risk in opposite directions.
- Risk high: in risk high most risk determining factors increase risk, some indicate a lowering of risk.
- Risk very high: in this category all inherent and agricultural factors contribute to an increase in risk.

The first part of the tree (key 1, hazards imposed by biological factors) is meant for use by biologists together with weed scientists, as it needs knowledge of breeding systems of plants and their agro-ecology in order to be able to correctly interpret and answer the questions. The second part of the key system (all keys but key 1) is for use by agronomists/weed scientists who are best qualified to judge all factors of the agricultural system, and hence selection pressure. Co-operation among the experts is in fact essential for reaching meaningful conclusions.

Janus face of biotechnology and biosafety in Central and Eastern European Countries

Balázs, E.

Agricultural Biotechnology Center, Gödöllö, Hungary

The development of biotechnology and its regulatory systems like Janus faces both ways. The biotechnological research and regulations are showing high heterogeneity. On the one hand, there are centers of excellence in research, and on the other hand, there are countries where almost a complete lack of such research or centers could be found. This duality has deep historical roots. During the socialist/communist regimes in this region the state of development and the economic situation of these countries were very different and this was also manifested in their scientific activity. Biotechnology developed in rather different ways and times in these countries. Some countries initiated a biotechnology programme as early as in the 70s, while there are countries which are launching biotech programmes only today. The development of science, especially biotechnology was greatly hampered in those countries, which have recently gained their independence. This is also true for the former Yugoslavia where independent states are now being re-established. One can realise the wide differences in the level of science and technology among the countries. It is also evident that certain countries are quite active in biotechnology in spite of their inbalanced economy. They make great efforts to support at least selected fields of the high-tech biology/agriculture. Poland, the Czech Republic, Hungary, the Russian Federation and Slovenia could be classified as leading countries in biotechnology. It is also clear that different priorities in biotechnology exist in this region. In some countries the classical biotechnology is still the main target, while there are some centers of excellence where recombinant DNA technology is as advanced as in the developed countries. It is also noteworthy that the activity of the various international organisations has already exerted a positive impact on the development of research and education in biotechnology. Participation in the ICGEB collaborative research and training programmes is one of the most faithful missions for this region. In general, it is also evident that the reconstruction of the industry and agriculture in this region, the change in the economical system, i.e. from the central planning to the market oriented economy left the science and technology out of consideration. The support of science and education is hardly envisaged in these countries, and this is one of the main reasons why the trained and especially the well-trained scientists of the region leave their countries and try to find jobs in North-America or Western Europe. This braindrain causes a serious problem for these countries. The low salary in the science and technology sector and the poor reputation of science are not favourable for the development of biotechnology. Lack of the venture capital in this region does not allow the establishment of the biotech companies.

The technology from the most advanced countries usually remains in the advanced part of the world, and less developed technologies are transferred to the Central and Eastern European countries. The relatively cheap labour in this region helps to develop sectors of agriculture/industry that rely on high amounts of labour. The above mentioned centers of excellence in biotechnological research are competing on the international level with other European and American laboratories. These centers of excellence in Poland are located in a triangle, namely Warsaw, Poznan and Crakow. In the Czech Republic these centers are in Prague, Ceske Budojevice and Olomouc. Hungary also has two major biotechnology research bases, one in Szeged, the Biological Research Center, while the other the Agricultural Biotechnological Center at Gödöllö, inaugurated in 1990. Biotechnology research centers both in Slovenia and Bulgaria are located in their capitals, Ljubljana and Sofia, respectively. In the Russian Federation several internationally recognised centers are existing, mostly in Moscow and Puschino.

In line with the programme of this conference I list some of the major areas in plant biotechnology in these countries.

In Bulgaria the main projects are: studies of the role of phytohormones in somatic embryogenesis, the genetic background of N_2 fixation, the transformation and regeneration of vegetables and alfalfa, genetic engineering for virus resistance of tobacco and vegetables, the expression of genes encoding *Cercospora* resistance in wild and cultivated rice, and the DNA polymorphisms and the genetic variability of barley plants obtained from tissue culture.

In the Czech Republic the following topics have priority: Regulation of plant morphogenesis, DNA repair in plants, molecular and cell biology of gametophytic and embryogenic pollen development, metabolism and mode of action of auxins and cytokinins in plants, molecular characterisation of plant genomes and plant virus primary structures including individual chromosomes. Their genetic engineering efforts mainly focus on potato for improving quality.

In Hungary, already in the 1970s plant biology became one of the leading programmes through establishment of the Szeged Biological Center, and their activities have stimulated biotechnological research in other institutes and universities both in Hungary and abroad. Recognition of biotechnology research of scientific community gave a booster for further development, manifested in the establishment of the Agricultural Biotechnology Center at Gödöllö. The main research areas in biotechnology are highlighted by the work of molecular structure of maize intramitochondrial plasmids, the first demonstration of foreign gene in alfalfa, which lead to the gene mapping programme of this crop. This pioneering work is followed by different well recognised programmes of the Hungarian scientific community, namely: the light-regulated gene expression in plants; the study and identification of key molecular events related to the somatic embryogenesis; the hormonal activated cell division and stress responses in the reinitiation of the ontogenic programme in cultured alfalfa cells, which includes the analysis of the role of the genes in the cell cycle. In the applied science area one of the most outstanding results came from the development of the highly efficient transformation and regeneration system of maize. Molecular marker-assisted breeding has been initiated also especially in cereal breeding. Intensive work on developing new transformation technologies was initiated as early as in the mid 1980s, including the development of a PC-directed fully automatised biolistic device (Genebooster™). Several crops including tobacco, potato, vegetables, corn, and alfalfa are being used for introducing agriculturally important new traits. The first transgenic plants are ready for registration. One of the major targets in this transgenic technology is to establish an environmentally friendly agriculture, so priorities are given to produce insect, virus and fungus resistant plants. Molecular biology of the important pathogen (*Fusarium*) is also one of the Hungarian competitive research areas. To improve the biocontrol ability of *Trichoderma* fungi, genetic engineering has been performed and resulted in a new genetically modified microbe with high chitinase activity. In summary the plant molecular biology became an integrated component of plant breeding and modern environmental biology.

Poland was among the first countries of Central and Eastern Europe which made great and successful attempts to leave the socialist block. This country has a deep tradition of biological/agricultural research and has early realised the significance of improving biotechnological research and development. The main fields of interest with respect to agricultural biotechnology are production of disease resistant plants, vaccines and fodder additives. The following research programmes are representing the strength of the Polish biotechnological research: RFLP mapping of potato; breeding for quality and low temperature tolerance of different crops; pathogen-derived resistance of potato to PLRV and PVY, development of artificial plant chromosomes; nuclear genes of mitochondrial biogenesis of yeast; regulation of heme and hemoprotein biosynthesis in yeast; genetic engineering of protein secretion in *Trichoderma* sp.; nucleotide analysis of the yeast genome; the role of hormones in gene expression of the insect pest; genetic transformation of lupin to improve quality and disease resistance; chemical and enzymatic synthesis of oligonucleotides; synthesis of antiviral compounds and investigations on their structure-activity relationships; organisation, structure and expression of plant cell cycle genes; molecular genetics of plant-microbe interactions with special regard to nitrogen fixation in legumes/*Rhizobium* and lupin/*Bradyrhizobium lupini* systems - cloning of developmentally regulated nodule specific genes and studies on nodule-specific promoters; biosynthesis of vitamin B_{12} by propionibacteria; lactic acid fermentation; structural modifications of plasma membranes of microorganisms, cryopreservation of microorganisms; artificial seed production and *in vitro* somatic embryogenesis of cucumber; antibacterial substances produced by *Lactobacillus* and *Propionibacterium* species; microbiological denitrification of vegetable juices. The broad spectrum of this research clearly demonstrates that the Polish scientists are one of the main driving forces in the R and A development of this region.

Scientists in the Russian Federation are covering almost all aspects of plant molecular biology and biotechnology, however, it is very difficult to evaluate their real research results and their strength for two main reasons:

- Most of the senior scientists have left the country and are working either in North America or Western Europe;
- The recent economical crisis left the science in a complete turmoil.

In Slovenia a governmental programme has been adopted on the strategy of the agricultural development. For this reason, in the field of biotechnology the following priorities have been formulated: cellular metabolites; fermentation technology and engineering; animal biotechnology; plant biotechnology; environmental biotechnology; food biotechnology; recombinant DNA and hybridoma technology in biotechnology; recombinant DNA technology in medicine for diagnostic purposes.

Slovenian scientists are very active in the fermentation industry. This includes the study and cloning of genes from *E. coli, Streptomyces* sp. and lower eukaryotes to obtain biologically active peptides. A new initiative is also to promote science in Slovenia in the field of biotechnology. This means that an infrastructural center, named Planta, has been founded together with the industry (KRKA, Novosmesto and Semenarna). This also shows a new pathway in this region where industry directly supports biotechnological fundamental and applied research.

In the region the international organisations, like ICGEB, UNESCO and FAO have an important catalytic role for re-establishing the R and A co-operation among the former socialist countries, which had been completely ceased when they gained their independence. The best example of this catalytic activity could be attributed to the ICGEB activities. This global organisation is supporting collaborative research between ICGEB of the network of affiliated centers in the field of biotechnology and genetic engineering. During the period from 1988-1996, 130 grants for a total of approximately 7,2 million USD were issued to ICGEB-affiliated centers of which 34 grants went to the Central and Eastern European countries. The scope of the grants varied from country to country. For example, in Bulgaria it was primarily plant biology, while in Hungary it was plant biology, agricultural biotechnology, and biocomputing. This support on collaborative research - as the grants are evaluated on the peer review basis - is supporting only the centers of excellence of this region and helps them to restore their scientific capacity. ICGEB also helps the development of the networks in the field of biotechnology of the region.

In 1995 when UNESCO-BAC established the Biotechnology Education Training Centers all over the world, the European regional BETCEN has been inaugurated in the Agricultural Biotechnology Center at Gödöllö, Hungary. During this short period of time more than 15 young scientists obtained up to one-year education through research in the field of plant molecular biology and biotechnology. The higher education for young scientists is supported by the organisation of regular workshops and summer courses supported in these countries by different international organisations, like ICRO, UNESCO-BAC, FEMS, FEBS, EBS, FAO, ICGEB, OECD, NATO.

As the biotechnological research developed rather differently in this region, the regulatory issues are following the same pattern. There are only three countries at present, where legally binding documents are existing. In 1996 the Russian Federation adopted a Law on Genetic Engineering, while in March 1998 the Hungarian Parliament adopted an Act on Genetechnology. Both laws are in the implementation phase. In Hungary where the law will be in force from 1 January 1999, ministerial acts are in preparation for establishing the Genetechnology Committee and the details of the process for obtaining permission for contained use and deliberate releases of GMOs. A databank is also being established. This will provide whole transparency of the GMO-related activities. In the other countries there either is not any envisaged plan for establishing regulation and formulation of the law, or there is progress on different levels. This is the case in the Czech Republic, Bulgaria, and Slovenia where the formulation of the regulatory frameworks is in advanced stages. Some of the countries are waiting till the CBD Biosafety Protocol will be adopted. To help on the regulatory issues with the support of UNEP regional co-operation on the safe use of biotechnology and its regulation was initiated in 1995 at Keszthely, Hungary, where delegates from the region agreed on the co-operation in the regulatory area. Their yearly meeting promotes the information exchange and the development of the national regulatory frameworks. Very recently within the UNEP/GEF pilot project on establishing National Biosafety Framework four countries applications have been approved and supported. This includes Poland, the Russian Federation, Bulgaria and Hungary. This National Biosafety Framework programme includes a review and assessment of existing biosafety legislation, guidelines, sectorial manuals and institutional and administrative measures. The programme will develop national, bilateral and multilateral co-operative

projects in research and development and application of biotechnology, with special emphasis on risk assessment and risk management. An important part of the programme is a continuous survey of current releases of living modified organisms and commercial products. Under the framework programme, workshops will be organised regularly. These will cover the benefits of the use of biotechnology in the context of sustainable development, and the current situation as regards biosafety. Particular attention will be given to risk assessment and risk management. In the workshops ethical issues and environmental questions will also be debated.

In establishing the national biosafety framework, the industry, the Scientific Community and consumer and environmental organisations will have the opportunity to express their views on the use of this technology. The major tasks of this programme are to ensure the introduction of the use of the latest applications of biotechnology for sustainable development in an environmentally safe manner, and to promote the understanding of this important technology by the public.

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Market-stage precautions: Managing regulatory disharmonies for transgenic crops in Europe

Levidow, L., Carr, S., Wield, D.

Centre for Technology Strategy, Open University, Milton Keynes MK7 6AA, UK

Transgenic crops have generated political conflicts among European countries, as well as across the Atlantic. Commercial authorization has been obtained more readily in the USA than in the European Union, where applicants face long regulatory delays, requests for more information, and/or pressures for market-stage precautions. It has been difficult to achieve regulatory harmonization, scientific consensus and public confidence for safety claims. The commercial stage has stimulated further debate over the predictability and acceptability of potential undesirable effects.

After gaining EU market approval, moreover, transgenic products have faced national bans and consumer boycotts. Some opponents link genetically modified (GM) food with socio-economic-environmental harm from cultivating the crop. Consumer choice becomes a means to "vote" for or against particular agricultural models.

How can the European conflicts be understood? According to biotechnology proponents, some European regulators have raised "non-risk" issues and have politicized risk assessment. According to biotechnology critics, safety regulation has failed to apply the precautionary principle. As an alternative perspective, there are contending accounts of "precaution" and "science-based regulation". Whenever safety judgements come under public scrutiny, scientific facts "are nothing but answers to questions that could just as well have been asked differently - products of rules for gathering and omitting" (Beck, 1992).

This essay analyses European market-stage precautions on many levels: as risk-assessment research which continues after market approval; as a compromise for managing regulatory disharmonies; and as a public accountability for the implicit politics of risk assessment.

What harmonization?

For the European Community in general, regulatory harmonization has been intended to complement the "internal market", by eliminating trade barriers and thus allowing products to circulate freely. Safety approval was to be achieved through a "mutual acceptance of data" among countries (e.g. Pelkmans, 1987). Rather than establish a new bureaucracy, European integration would facilitate new expert networks, develop European forms of knowledge and thus provide authority for Europe-wide policy (Delors, 1992; Barry, 1993). An expert-based harmonization would *define* the new Europe as well as integrate it.

In that vein, the EC Deliberate Release Directive 90/220 was enacted to "establish harmonized procedures and criteria" for Europe-wide market approval of genetically modified products (EEC, 1990). When judging safety, however, member states have given different interpretations to key statutory terms, e.g. the "adverse effects" which must be prevented, and the "step-by-step" principle for gaining safety information. The national Competent Authorities have often resorted to voting on product approval, in lieu of any other means to resolve the disputes (Levidow et al. 1996, 1997a).

When the harmonization scenario ran into such obstacles, criticism was directed at the original legislation for imposing excessive precaution, or at some regulators for lacking adequate expertise - rather than at the original technocratic ideal. The regulatory conflicts led to pleas that risk assessment be "based on science", and complaints that it was being politicized - as if a scientific basis could be value-free. To overcome the impasse, the European Commission sought advice from its own scientific committees, firstly regarding an insecticidal maize (SCP, 1996; EC, 1997a).

After the BSE scandal erupted in early 1996, the EC authorities and their expert advisors were suspected (perhaps unfairly) of having colluded with UK malpractices. Consequently, all the scientific committees were reconstituted in several ways. They were transferred to the Directorate-General for Consumer Policy DGXXIV from the DGs which held statutory authority for safety regulation; committee members were appointed as individual experts rather than as national representatives; and risk management was

separated from risk assessment, so that committees would advise only on the latter. These changes aimed to keep scientific advice above politics, presuming that such expertise would remain neutral.

For transgenic crops under Directive 90/220, the DGXXIV-based Scientific Committee on Plants has regularly given advice, in response to the international disharmonies and the wider public debate. In dispute are the following issues: how to assign the burden of evidence for risk or for safety, and with what criteria for meaningful evidence; how to set a normative baseline for judging the acceptability of potential undesirable effects; and how to judge the scientific basis for predicting such effects (Figure 1).

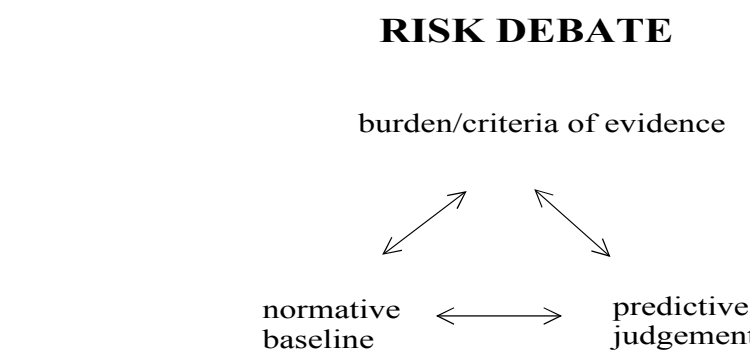

Figure 1 Risk debate

The normative baseline has high stakes because biotechnology has become a test case for contending accounts of environmental precaution and sustainable agriculture. In practice, market approvals have been granted on the basis that any undesirable effects would be no worse than those caused by present agricultural practices (and often on the implicit assumption that the transgenic products offer an environmental improvement). For example, the prospect of herbicide-tolerant weeds or insecticide-resistant insects were deemed acceptable rather than be assessed as "adverse effects". Along with environmental NGOs, however, some European governments have argued that such effects could jeopardize future options for crop protection and thus for sustainable agriculture.

Objections to market approval have been labelled as "political", but the same can be said about support for such approval. Risk/safety assessment always rests upon a normative baseline, which in turn involves a broader Technology Assessment about the types of agricultural systems for which products are designed. Presumed benefits implicitly influence the definition of environmental harm, thus facilitating safety judgements (Levidow et al. 1997a).

As the risk debate continues into the market stage, further precautions are being implemented or planned. "The precautionary principle is being extended beyond Directive 90/220, creating uncertainty for us about what to do, about what more precautions to take. What is required remains a grey area", according to a company regulatory officer (Interview, 24 November 1997). Market-stage precautions involve four elements: cause-effect models of undesirable effects; cultivation protocols designed to avoid these effects; monitoring large-scale use; and basic/ecological research to inform predictability judgements (Figure 2). Linkages among the four elements are analysed below in two cases, insect-protected maize and herbicide-tolerant oilseed rape.

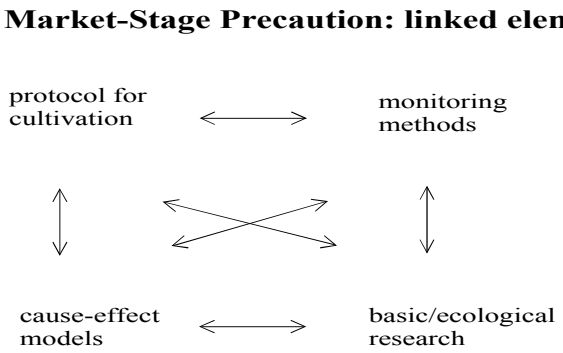

Figure 2 Market-stage precautions

Insect-protected maize

Since the 1980s biotechnology companies have been inserting toxin genes from the naturally occurring microbial pesticide *Bacillus thuringiensis* into other microbes or crops. There has been concern that long-term exposure to such insecticidal crops could intensify selection pressure for resistant insects; if so, then this effect would shorten the useful lifespan of the product. Insect resistance might also reduce the future utility of naturally occurring microbial *Bt*. Of course biotechnology companies have a self-interest in prolonging the commercial life of their insecticidal crops, but this may conflict with an interest in recouping their investment quickly, especially as rival companies market similar products.

Europe-wide market approval was granted to the first such product, a Ciba/Novartis *Bt* maize, on the basis that reduced efficacy of the insecticidal agent would be acceptable. According to the European Commission, the generation of insect resistance "cannot be considered an adverse environmental effect, as existing agricultural means of controlling such resistant species of insects will still be available" (EC, 1997a; cf. SCP, 1996). This normative argument came from France, as the national rapporteur which had advocated approval. Implicitly, the normative baseline was the present use of chemical insecticides.

Nevertheless insect resistance management (IRM) has become quasi-mandatory for cultivating insecticidal crops in Europe. Under the Directive 90/220 procedure, the relevant companies have undertaken to implement IRM plans for their products (e.g. as noted in the recital of EC, 1998). Under the procedures for adding new plant varieties to the National List, moreover, monitoring has been required for various environmental effects, including insecticidal efficacy, insect resistance to *Bt*, harm to non-target insects, etc. (France, 1998; Spain, 1998). The latter risk became a more prominent issue after lab tests indicated that *Bt* could harm carnivorous predators of insect pests; if this happened in the field, then farmers would lose a means to control *Bt*-resistant pests (Hilbeck et al. 1998). In France the monitoring is to be evaluated by the new, broadly-based advisory committee of the Environment Ministry (Joly and Roy, 1998).

The basic IRM plan is called a "high-dose/refugia" strategy. *Bt* crops are designed to produce a sufficiently high dose to kill all resistant insects, while nearby refugia of non-*Bt* crops allow susceptible insects to survive and interbreed with any resistant ones. Meanwhile fields are to be monitored for any insect resistance. However, all these aspects involve scientific uncertainties and methodological difficulties.

Monitoring methods

As regards monitoring, resistance genes may have spread considerably in the population by the time any resistant insects are detected. According to the prevalent model, homozygously-resistant individuals can survive the high dose, while heterozygously-resistant individuals have higher resistance than the fully susceptible ones. Heterozygously-resistant insects may survive, e.g. if the insects learn to avoid high-

dose *Bt* crops and/or if *Bt* levels decline at the end of the growing season, as in the Ciba/Novartis maize (EC, 1997a).

The most simple monitoring method would be farmers looking for any crop damage higher than expected, or for any surviving insects, which would then be tested in the laboratory. However, some member states regard such a method as inadequate and have demanded an "active" systematic monitoring. Entomologists have developed methods to sample the insect population for any increase in heterozygously-resistant insects, for example, with a "discriminating-dose assay" to test survival at relatively low doses.

A more sophisticated method has been devised by entomologists at INRA-Paris, along lines similar to US entomologists (Andow and Alstad, 1998). After four generations in the field, insect samples would be tested in the laboratory at various doses. After eight generations in the field, new samples would be interbred over several generations and tested at progressively higher doses. The inbreeding would concentrate any resistance alleles in the F2 generation, thus simulating development of homozygously-resistant insects. With these standard laboratory tests for *Bt* susceptibility, baseline levels could be measured and then compared over time and across environments. Testing would be done more frequently for the Mediterranean cornborer than for the European cornborer (DGXI EGBtIRM, 1998a,b).

Cultivation protocol

As a cultivation protocol, farmers would grow refugia of non-*Bt* plants to provide a nearby supply of susceptible insects. Any resistance alleles are dispersed to heterozygous ones in the next generation, thus avoiding the persistence or development of homozygously-resistant progeny. Such protocols are outlined in growers' manuals in the USA (e.g. Novartis, 1998; Monsanto, 1996).

The IRM plans there have undergone debate and change. In 1997 new scientific evidence was published on multiple-*Bt* resistance and the potential speed of resistance development. Because *Bt* is naturally occurring in the environment, insect populations contain a proportion of individuals with resistance to more than one toxin. Exposure to high doses of *Bt* selects for these tolerant individuals and favours their reproduction while the susceptibles decline (Tabashnik, 1997a,b; Gould et al. 1997).

According to the US EPA's advisors, therefore, the original guidelines may be inadequate to delay insect resistance, especially for the first Ciba/Novartis maize, so they recommended more stringent conditions. Originally the US EPA recommended that refugia cover at least 4% of the cultivated area, or 20% if the crop is sprayed with chemical insecticide. For subsequent *Bt* maize authorizations in mid-1998, the EPA imposed mandatory refugia, "structured" in specific patterns, and much larger than before: 20-30%, or 40% if sprayed with chemical insecticide (US EPA-OPPTS, 1998a,b; see examples in UCS, 1998; Novartis Seeds, 1998). The new requirement illustrates how a cultivation protocol is dynamically linked with changes in cause-effect models, scientific knowledge and normative judgements; indeed, the scientific questions are recast.

For its *Bt* maize cultivated in Europe in 1998, Novartis originally planned to follow the early US EPA guidelines but then promoted the more stringent ones through a financial incentive: "Growers who buy a significant amount of *Bt* seed receive substantial savings if at least 20% of their order includes non-*Bt* hybrids. With this programme, Novartis Seeds is offering to share IRM stewardship responsibilities with its customers" (pers. comm., Novartis, 15 October 1998). Little *Bt* maize has been cultivated in 1998 in France, so the protocol may be fulfilled anyway by nearby non-*Bt* crops. The refugia design depends upon assumptions about the distance travelled by insects to feed and breed, so biotechnology companies have contracted entomologists to study these behaviours.

All these plans have been scrutinized by the European Commission under pressure from some Directorates-General, EU member states and environmental NGOs. When evaluating the proposal for Monsanto to commercialize its *Bt* maize, the EU's Scientific Committee on Plants regarded the IRM plan as "adequate to delay resistance", while implying that such an effect would anyway be an agricultural problem rather than environmental harm (SCP, 1998a). In sum, commitments were made to prevent and monitor undesirable effects whose acceptability was in dispute.

Herbicide-tolerant oilseed rape

For herbicide-tolerant oilseed rape, controversy has continued on the potential spread of herbicide-tolerance genes to other rape crops or to weedy relatives. Inadvertent hybridization could generate persistent herbicide-tolerant weeds which would jeopardize the efficacy of the herbicide. According to critics, such scenarios could adversely affect overall herbicide usage or could preclude options which are environmentally preferable.

Under Directive 90/220, such products have gained approval on the basis that the inadvertent generation of herbicide-tolerant weeds would be acceptable. When the PGS glufosinate-tolerant oilseed rape gained approval for seed production, the European Commission declared "that any spread or transfer of the herbicide-tolerance gene could be controlled by using existing management strategies" (EC, 1996). That normative argument came from the UK, as the national rapporteur which had advocated market approval. A similar argument came from the official advisors in France when that country advocated full commercial authorization for the same product (EC, 1997b).

European countries continue to disagree over whether glufosinate-tolerant weeds would be an "adverse effect" under Directive 90/220, or merely an "agricultural problem", relevant only to pesticide regulation (EEC, 1991). By contrast to Europe, the US statutory framework clearly provided no basis for regulating such effects, and herbicide-tolerant oilseed rape has been simply deregulated there.

Risk-assessment research

As regards the predictability of undesirable effects, scientific models have identified uncertainties which warrant further research. These models emphasize hybrid fitness/fertility - as well as reproductive links - between the crop, volunteers and feral populations (e.g. Van Raamsdonk and Schouten, 1997).

For herbicide-tolerant oilseed rape, a plausible effect would be survival of the seeds into a following crop on the same farm, thus precluding the use of the corresponding herbicide to remove herbicide-tolerant volunteers. Few critics regard this scenario as environmental harm. More complex scenarios have gained plausibility from additional evidence, as cited below.

Transgene flow has considerable potential. Some viable pollen travels up to 2 km (Timmons et al. 1996). Volunteer rape has a constant flux with feral rape outside the field, via a flow of pollen and of seeds (Squire et al. 1996).

Interspecies hybridization has been more thoroughly tested in field experiments studying the initial hybrid and then back-crosses with the weed, since their viability is crucial for environmental persistence of the herbicide-tolerance trait. Fertile, weed-like plants were found after just two generations of crosses between *B. napus* and *B. campestris/rapa* in Denmark (Mikkelsen et al. 1996; Jørgensen, 1996). In back-cross experiments with *R. raphanistrum* in France, highly fertile progeny were found after the fourth generation, though there was lower transmission of the herbicide-tolerance transgene (Chèvre et al. 1997a,b). Those hybridization studies went beyond earlier ones which had tested back-crosses mainly with the crop, rather than with the weed (e.g. Scheffler and Dale, 1994). According to a UK field survey, however, only 7% of *B. rapa* populations had adequate proximity to yield hybrids with oilseed rape, and only minimal hybridization occurred there (Scott and Wilkinson, 1998).

In the *B. rapa* back-crosses cited above, transgenic herbicide tolerance conferred no metabolic cost (Snow and Jørgensen, 1998) - by contrast to naturally occurring herbicide tolerance. The latter effect had been cited to predict that transgenic herbicide tolerance too would confer a selective disadvantage in the wider environment. Yet it appears to be selectively neutral and thus would more plausible persist.

There has been concern about inadvertent "stacking" of herbicide-tolerance genes because each company will commercialize oilseed rape varieties tolerant to its own herbicide. When transgenic oilseed rape with tolerance to three different herbicides were cultivated in close proximity, some progeny had multiple tolerance (Reboud et al. 1998).

Quite different conditions and different scientific questions led to the above results, so debate continues over their predictive significance for commercial cultivation. There remain methodological issues about how field studies can meaningfully simulate agricultural conditions (Jørgensen et al. 1996; Sweet et al. 1997). Nevertheless the empirical results indicate that herbicide-tolerance could readily spread to the

wider environment, persist in wild populations and return to subsequent crops; multiple tolerance could develop as well. Given that such effects are plausible, their environmental significance has been evaluated according to different normative baselines in national debates and regulatory procedures.

Further controversy and controls

In France transgenic crops have faced mounting protest. Environmental NGOs have advocated a moratorium on the commercial use of all transgenic crops; many French scientists signed a similar petition. INRA staff criticize herbicide-tolerant oilseed rape as a threat to weed-control methods and to organic agriculture. During 1997 the French Environment Ministry gained more influence over the safety regulation and established a more broadly-based advisory committee, which emphasized environmental uncertainties to be resolved.

In November 1997 the French government announced that it would not approve any herbicide-tolerant oilseed rape, thus blocking EU-wide market approval of products which it had originally advocated (e.g. EC, 1997b). One reason was the prospect that the herbicide-tolerance trait could spread to wild relatives, even though earlier the French authorities had regarded such an effect as *not* environmental harm. In July 1998 the government extended the ban for two years, pending further studies.

In the UK, opposition intensified during 1997-98, with widespread demands for a moratorium on commercial use. For oilseed rape, the debate focused on two related scenarios: the inadvertent generation of herbicide-tolerant weeds, and the destructive effects of broad-spectrum herbicides on wildlife habitats in or near fields (e.g. JNCC, 1997). The government was already funding large-scale trials to research both issues (Sweet et al. 1997).

Given that herbicide-tolerant weeds could jeopardize the efficacy of the product, the UK agricultural supply industry has been preparing management guidelines. These aim mainly to prevent the spread of herbicide-tolerant volunteers and pollen; preventive measures include labelling, segregation of seeds, spatial separation of crops, monitoring, etc. Moreover, “Failure to comply will result in sanctions...” by supply companies against such farmers (BSPB, 1998). However, these guidelines lack public credibility, partly because biotechnology companies have violated the terms of their consents for R&D trial releases (EC, 1997; ENDS, 1998).

Under various pressures, the UK Agriculture Ministry held a consultation exercise on herbicide-tolerant crops. Its consultation paper classified many environmental concerns as “agricultural problems” or as “disadvantages” rather than as environmental harm (MAFF, 1997). As before, herbicide-tolerant weeds were officially deemed “environmental harm” only if they harm private property, e.g. if they spread uncontrollably to other farms or leave farmers without any effective herbicides (Levidow et al. 1997b; ACRE, 1998).

In response, the Agriculture Ministry received 300 comments, some of which challenged the UK’s official regulatory boundaries. Even some companies suggested that the definition of adverse effects should be broadened: “As the potential disadvantage to agriculture... is the survival of tolerant volunteers, outcrossing changes to herbicide usage and biodiversity, this in itself is a potential environmental impact which should be addressed under Part IV of Annex II to Directive 90/220” (AgrEvo, 1997). During 1998 the Environment and Agriculture Ministries continued to avoid responsibility for undesirable effects which were downplayed in the original risk assessment of herbicide-tolerant oilseed rape. A game of “pass the parcel” was underway (ACRE, 1997; ENDS, 1998).

The impasse was somewhat overcome in October 1998, when the two relevant Ministers gave joint statements announcing further precautionary measures: a managed development of GM crops, whereby the first plantings (in autumn 1999) would be strictly limited and monitored for ecological effects; a scientific review of pesticides to compare the likely impact on biodiversity, e.g. from the likely usage on herbicide-tolerant crops; and an environmental stakeholders’ forum to discuss all these issues (DETR, 1998). Regulators would evaluate the “indirect effects” of herbicide usage on wildlife habitats; if such effects caused more harm than present herbicide usage, then the marketing consent for the crop would be withdrawn (interview, DETR, 13 November 1998). Through the forum, moreover, critics would be invited to evaluate the scientific criteria for anticipating and monitoring undesirable effects.

Given that some member states objected to commercialization, the issues were considered by the DGXXIV-based Scientific Committee on Plants. It acknowledged that gene transfer to wild *Brassica* relatives "is a new issue in Europe" but doubted that these would infest crops; rather, any herbicide-tolerant weeds would be the oilseed rape itself, "which could be controlled in subsequent crops by conventional agricultural methods". Although not regarding this problem as an adverse environmental effect, the committee recommended that commercial use should follow an agreed code of practice for the particular crop, as well as a monitoring programme with an agreed design and implementation plan (SCP, 1998b,c). In consultation with the German Competent Authority, AgrEvo undertook to monitor commercial use for outcrossing and volunteer management, through "an intensive product stewardship with farmers" (pers. comm., 30 November 1998; cited in Dreyer and Gill, 1998). In sum, commitments were made to prevent and monitor undesirable effects whose acceptability was in dispute.

Conclusion: managing disharmonies

Market-stage precautions can be understood as managing regulatory disharmonies, both within and among European countries, as well as managing any risks. These measures dynamically link four elements (see Figure 2): any change or challenge to one element can destabilize the others. In these ways, market-stage precautions recast key regulatory terms:

- "Adverse effects": Originally, market approval was to provide a definitive judgement regarding the "adverse effects" which must be prevented by law. Some plausible, undesirable effects were officially classified as merely "agronomic problems" and thereby deemed irrelevant to risk regulation. Now "adverse effects" are implicitly broadened, e.g. to encompass herbicide-tolerant weeds and insecticide-tolerant pests.
- "Familiarity": In the early risk debate, some undesirable effects were called "familiar" problems, as if they were thereby predictable and/or acceptable. Now "gaining familiarity" is understood as a task for continuing research, alongside management efforts to prevent or delay the effects themselves.
- "Stepwise procedure": Originally market approval was envisaged as the ultimate step, so that GM crops could be cultivated without any further conditions. Now commercial use is being planned and managed as a larger-scale experimental stage, accommodating broader accounts of "adverse effects". In a double-edged logic, commercial use is justified as acceptable because of the extra precautions, and justified as necessary to test any future risks.
- "Risk assessment/management": For EU-level expertise, the European Commission sought to obtain a purely scientific risk assessment, kept separate from the risk-management decisions made by regulators. Yet that official distinction is readily blurred in practice: when expert committees give risk-assessment advice, this rests upon a particular risk-management framework, e.g. in defining "adverse effects" and in promoting market-stage precautions.
- "Precautionary principle": There has been broad agreement that this should be "applied" but disagreement about its meaning. Now the "principle" is being defined anew in practice, case by case. The wider risk debate is being further translated into testable uncertainties, e.g. by tightening the normative baseline for unacceptable effects, and by tightening the burden of evidence to demonstrate their implausibility (see again Figure 1).

Market-stage precautions also strengthen public accountability for the value-laden aspects of risk assessment. Public debate continues to scrutinize how scientific questions are asked, as particular uncertainties are either investigated or omitted (Beck, 1992). Experts remain under pressure to justify the scientific basis of risk-assessment research, cause-effect models and safety claims.

As initially conceived, regulatory harmonization was intended to achieve a mutual recognition of risk assessments, so that safety judgements would eliminate trade barriers and products would freely circulate as normal commodities. That scenario is in crisis for transgenic crops in Europe. If regulatory harmonization is to be achieved, then it will have a quite different meaning. It could mean putting products on trial, democratizing expertise, and further debating the implicit politics of risk assessment. In these ways, market-stage precautions provide greater accountability for the commercial use of transgenic crops.

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Evaluation of commercial scale usage of virus-resistant transgenic plants

Tepfer, M. (Moderator)
Laboratoire de Biologie Cellulaire, INRA-Versailles, 78026 Versailles Cedex, France

Scope of the workshop

This workshop covered the present state of knowledge relevant to the major potential risks associated with virus-resistant plants expressing viral sequences. The first paper, by James White, was an overview of currently authorized field cultivation of virus-resistant transgenic plants in the USA. Allen Miller presented an overview of potential risks associated with luteovirus sequences, including risks associated with recombination or with heterologous encapsidation. Marc Fuchs's paper showed that gene flow from cultivated transgenic squash could lead to creation of virus-resistant wild squash. It has long been known that when plants are infected by more than one virus, there can be either interference, no interaction, or synergy (disease worsening). So it was reasonable to expect, as was demonstrated in the presentation of Peter Palukaitis, that a similar range of interactions, including interference and synergy, could be observed in transgenic plants infected with a single virus. The papers of Richard Allison and Rachid Aaziz et al. were both concerned with the potential for recombination in plants expressing viral sequences to lead to new viral genomes. The former paper showed that indeed recombination can be observed when experimental conditions lead to high selection pressure in favour of recombinants, and that this can lead to viruses with novel biological properties. The latter paper presented strategies for detecting recombination under conditions of low selection pressure, closer to what might be expected in the field. The last paper, by Philip Dale, showed that infection with cauliflower mosaic virus (CaMV) can lead to silencing of transgenes including CaMV sequences, which would be unwanted when for instance a CaMV promoter is used for obtaining characters other than CaMV resistance. The potential risks discussed could be grouped according to the nature and potential extent of their effects. When their effects are short-term, and are not expected to go appreciably beyond the infected plants, the potential risks are essentially agronomic (synergy, heterologous encapsidation, gene silencing). In contrast, plant-to-plant gene flow and plant-virus recombination, if they affect the biological properties of either wild plant or virus populations, could lead to more durable effects that would have an ecological impact.

Rapporteur's comments

There was something of a gap between the intended scope of this workshop, which if possible should have centered on questions specifically pertinent to commercial scale, and the current objectives of work in this field, as reflected in the papers presented. Research is still primarily focused on identification/evaluation of potential hazards, and on studies of the underlying mechanisms. It is clear that this is essential research to carry out, since only when the mechanisms of both resistance and of potential hazards are understood can genes be designed that retain their beneficial characteristics, while eliminating the sources of potential hazard, or attenuating their effects. From several papers, there was a sense that perhaps in the future greater attention should be paid to the consequences of potential risks, rather than to mechanisms. For instance, in cases where transgene flow to wild relatives will occur, will this confer a selective advantage on the wild species? If infecting viruses incorporate transgene sequences by recombination, will this lead to pathogens that cause more harm than currently existing ones? This will require not only characterization of the biological properties of the plants and viruses that result from these processes but also a clear determination of the appropriate baseline. In the case of recombination, there is general agreement that the correct baseline against which to compare recombination in virus-infected plants expressing viral sequences is the situation in non-transgenic plants infected with two or more viruses. Regarding gene flow, when equivalent naturally occurring resistance genes occur, the consequence of their transfer to wild species is the obvious baseline situation for movement of a transgene to a wild relative. It is far less clear what the appropriate baseline would be in cases where the transgene would confer a completely novel resistance to the wild species.

At some point, risk assessment of virus-resistant plants will no longer be necessary. What is not yet clear is where in the following overlapping processes one can consider that risk assessment is completed: risk assessment → risk/benefit analysis → commercial release. This problem can also be stated as how to make the distinction between "want to know" and "need to know" questions. Several years after the first commercial releases in China and the USA, it may seem paradoxical to ask this question, but in the face of greater uncertainty in Europe than elsewhere concerning genetically modified plants, it would be of interest to try to develop a broad international consensus on the question.

An overview on cultivation of virus-resistant crops in the United States

White, J.L.

Plant Protection and Quarantine, Animal and Plant Health Inspection Service (APHIS), US Department of Agriculture, 4700 River Road, Riverdale, MD 20737-1237, USA

Abstract

Over 400 field tests of transgenic virus-resistant plants have been performed in the USA without any reported adverse environmental effects. After receiving all Federal approvals, transgenic squash and papaya plants are being grown in 1998 on a commercial scale on thousands of acres.

Field testing of virus resistant plants started in the USA in 1986 with a test of tomato mosaic virus resistant tomatoes by Monsanto. Since then more than 4,100 field tests of GMOs have been authorized by USDA, APHIS and approximately 10% of these field tests have had virus resistance as a phenotype. Table 1 lists the plants and the phenotypes of virus resistant plants that have been field-tested since June of 1997. In this table, the combination of phenotypes listed accurately reflect the presence of multiple resistance phenotypes to several viruses in a single plant. It does not reflect the stacking of additional phenotypes. For example, some virus resistant potatoes have in addition to virus resistance: Verticillium resistance, Coleopteran resistance, bruise resistance, and altered carbohydrate metabolism. Some recent tests include barley yellow dwarf virus (BYDV) resistant oats (first authorized test of transgenic oats in the USA) and series of tests on geminivirus resistant tomatoes. Several new resistance genes have been tested including: the first tests of nonfunctional movement protein of raspberry dwarf virus in raspberry (also the first test of transgenic raspberries). Also, the first test of the N gene for resistance to tobacco mosaic virus (TMV) in transgenic tobacco plant to address if the poor agronomic performance of N gene introduced into burley tobacco cultivars by plant breeding is a result of the N gene per se or of linked genes that are also introduced during sexual crosses. Other novel genes include a double stranded specific ribonuclease from *Schizosaccharomyces pombe* and a protein kinase from mouse as sources of resistance. Many of the genes/donors listed as CBI are from companies.

Table 1 Field tests of virus resistant transgenic plants authorized by USDA-APHIS since June 1997[1)]

Crop	Phenotype	Gene	Donor
Beet	BNYVV resistant	Coat protein	Beet necrotic yellow vein
Cucumber	CMV, PRSV, WMV2 & ZYMV resistant	CBI[2)]	CBI
Grape	CBI	CBI	CBI
Grape	Nepovirus resistant	CBI	CBI
Melon	CMV resistant	Coat protein	Cucumber mosaic
Melon	CMV, WMV2 & ZYMV resistant	Coat protein Coat protein Coat protein	ZYMV WMV2 CMV
Melon	CMV, PRSV, WMV2 & ZYMV resistant	CBI	CBI
Melon	CMV, PRSV, SqMV[3)] & WMV2	CBI	CBI
Melon	CMV, WMV2 & ZYMV resistant	Coat protein Coat protein Coat protein	Cucumber mosaic Watermelon mosaic virus 2 Zucchini yellow mosaic
Melon	CMV, PRSV, SqMV, WMV2 & ZYMV resistant	CBI	CBI
Melon	CMV & PRSV resistant	CBI	CBI
Melon	WMV2	CBI	CBI
Melon	WMV2 resistant	Coat protein	Watermelon mosaic virus 2
Melon	ZYMV resistant	Coat protein	Zucchini yellow mosaic
Oat	BYDV resistant	ORF 1 & 2	Barley yellow dwarf

Crop	Phenotype	Gene	Donor
Papaya	PRSV resistant	Replicase	Papaya ringspot
Papaya	PRSV resistant	Coat protein	Papaya ringspot
Pea	PEMV resistant	Coat protein	Pea enation mosaic
Pea	BLRV[3], BYMV[3], PEMV, PSbMV[3] & PeSV[3] resistant	Double stranded ribonuclease	*Schizosaccharomyces pombe*
Pea	BYMV & PSbMV resistant	Coat protein	Bean yellow mosaic
Peanut	TSWV resistant	Nucleocapsid	Tomato spotted wilt
Pepper	CMV resistant	CBI	CBI
Potato	PVY resistant	Replicase	Potato virus Y
Potato	TRV resistant	Coat protein	Tobacco rattle
Potato	TRV resistant	Pseudoubiquitin	Potato
Potato	TRV resistant	Double stranded ribonuclease	*Schizosaccharomyces pombe*
Potato	PLRV, PVY & TRV resistant	Double stranded ribonuclease	Human
Potato	PLRV resistant	Replicase	Potato leaf roll
Potato	PLRV, PVY & TRV resistant	Protein kinase	Mouse
Potato	PLRV, PVY & TRV resistant	Pseudoubiquitin	Potato
Potato	PVY resistant	Nuclear inclusion protein b	Potato virus Y
Potato	PVY resistant	Double stranded ribonuclease	*Schizosaccharomyces pombe*
Potato	PVY resistant	Coat protein	Bean yellow mosaic
Potato	PVY resistant	Coat protein	Potato virus Y
Potato	PVY resistant	Genome-linked protein	Potato virus Y
Potato	PVY & PLRV resistant	Replicase Coat protein	Potato leaf roll Potato virus Y
Raspberry	RBDV resistant	Nonfunctional movement protein	Raspberry dwarf
Soybean	SbMV resistant	Coat protein	Soybean mosaic
Squash	CMV, PRSV, WMV2 & ZYMV resistant	CBI	CBI
Tobacco	PVY, TVMV & TEV[3] resistant	Coat protein	Tobacco vein mottling
Tobacco	PVY resistant	Coat protein	Potato virus Y
Tobacco	TMV	N gene	Tobacco
Tobacco	TSWV resistant	Nucleocapsid	Tomato spotted wilt
Tomato	BCTV[3] resistant	CBI	CBI
Tomato	CMV resistant	Coat protein	Cucumber mosaic
Tomato	CMV resistant	Replicase	CBI
Tomato	CMV resistant	CBI	CBI
Tomato	Geminivirus resistant	CBI	CBI
Tomato	Geminivirus resistant	Rep protein	CBI
Tomato	PVY resistant	Coat protein	Potato virus Y
Tomato	ToMoV resistant	CBI	CBI
Tomato	TYLCV[3] resistant	CBI	CBI
Watermelon	WMV2 & ZYMV resistant	CBI	CBI
Wheat	WSMV resistant	Coat protein	Wheat streak mosaic
Wheat	BYDV resistant	Coat protein	Barley yellow dwarf
Wheat	BYDV & WSMV resistant	Double stranded ribonuclease	*Schizosaccharomyces pombe*

[1] Does not include multi-year field tests authorized before June 1, 1997;
[2] CBI = confidential business information. A single notation of CBI does not necessarily mean that a single gene was used to encode the phenotype;
[3] Virus abbreviations: SqMV = squash mosaic, BLRV = bean leafroll, BYMV = bean yellow mosaic, PSbMV = pea seed-borne mosaic, PeSV = pea streak, TEV = tobacco etch, BCTV = beet curly top, TYLCV = tomato yellow leaf curl.

What is APHIS' experience with over 400 field tests of virus resistant plants? Challenge inoculations are permitted for most field tests. In general, nothing unexpected or unusual has happened. Often, transgenic plants that were resistant during greenhouse testing, did not show resistance in the field sufficient for commercial use, but this was not unexpected. We have no evidence of novel interactions that we lead on to conclude that recombination, transcapsidation, or synergy has occurred at this stage of testing.

Three virus resistant plants have been approved for commercial use in the USA, zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus 2 (WMV2) resistant squash (ZW20), another squash containing cucumber mosaic virus (CMV), ZYMV, and WMV2 resistance (CZW3), and papaya ringspot virus (PRSV) resistant papayas. Two products are pending approval (potato leaf roll virus (PLRV) resistant and potato virus Y (PVY) resistant potatoes, both of these events also contain Colorado potato beetle resistance). Table 2 summarizes selected genetic elements that have been engineered into these lines. All the resistance transgenes have been derived from coat proteins except PLRV that used ORF 1 and 2 that are believed to encode the helicase and replicase functions. All genes were derived from aphid transmissible strains that are widely prevalent in the USA. For the potyviruses, all the CP genes were modified in some way to contain additional amino acid(s) and initiation codon, cap sequences, and untranslated regions. The transgenes in all cases were driven by caulimovirus promoters.

Table 2 Summary of selected genetic elements in deregulated viral resistant plants in the USA

Plant	Company	Gene(s)	Donor	Promoter	Other
ZYMV & WMV2 resistant SQUASH ZW20	Asgrow (Seminis) 92-204-01p	Coat proteins	WMV2 ZYMV	both 35S CaMV	> All CPs from aphid transmissible strains > ZYMV & WMV2 transgenes contain untranslated region and initiation codon from CMV
CMV, ZYMV & WMV2 resistant SQUASH CZW3	Asgrow (Seminis) 95-352-01p	Coat proteins	CMV WMV2 ZYMV	all 35S CaMV	> All CPs from aphid transmissible strains > ZYMV & WMV2 transgenes contain untranslated region and initiation codon from CMV
PRSV resistant PAPAYA 2 events	Cornell U. & Univ. of Hawaii 96-051-01p	Coat protein	PRSV	35S CaMV	> Transgene contains untranslated region and initiation codon from CMV > CP from chemically mutagenized viral strain
PLRV & CPB[1)] resistant POTATO[2)] 3 events	Monsanto 97-204-01p	ORF 1 & 2	PLRV	35S FMV	> From aphid transmissible strain
PVY & CPB[1)] resistant POTATO[2)] 4 events	Monsanto 97-339-01p	Coat protein	PVY	35S FMV	> CP from aphid transmissible strain > CP contains additional methionine

[1)] CPB = Colorado potato beetle; [2)] Pending approval

Table 3 summarizes the data submitted that APHIS requires of the potential issues that have been raised in using viral derived transgenes and for characterizing transgenic plants.

- Transgenes were inherited in Mendelian fashion as a single locus (excepting male sterile transgenic potatoes).
- DNA genomic analysis either by Southerns or PCR demonstrated that a complete viral derived gene was present.
- The transgene CP levels were significantly less than CP levels in infected nontransgenic plants except in PVY-resistant potatoes where no CP could be detected by Western analysis. The PLRV helicase protein (ORF 1) could not be detected.

- With ZW20 squash, transgene CP levels were increased when the plants were inoculated with PRSV, although the levels were generally lower than those found in inoculated nontransgenic plants. In contrast, CZW3 squash showed no increase in transgene CP levels were detected when the plants were inoculated with PRSV. No viruses except PRSV infect papayas in the USA and thus data requirements were adjusted to reflect this situation.
- Synergy was not seen when any of the transgenic plants were inoculated with widely prevalent viruses that naturally infect the plants.
- Transgene RNA levels were from less than comparable RNA from infected nontransgenic plants.

Table 3 Summary of data submitted that pertain to issues regarding use of viral derived transgenes

	ZW20 squash	**CZW3 squash**	**PRSV papaya**	**PLRV potato**	**PVY potato**
Mendelian inheritance	Single gene	Single gene	Single gene	Not applicable	Not applicable
DNA analysis for viral transgene	Complete cassette by Southern analysis	Complete cassette by Southern analysis	Complete cassette by Southern analysis	Complete CP by PCR analysis	Complete CP by PCR analysis
Transgene protein level	~ 500 fold less than CP levels in infected control plants	~100 fold less than CP levels in infected control plants	89 to 93% less than CP levels in infected control plants	No protein detected	No protein detected
Transgene RNA level	Not determined	~100 fold less than RNA levels in infected control plants as total vRNA	~100 fold less than RNA levels in infected control plants as total vRNA	Levels from 5 to 10 times lower than RNA levels in infected control plants as total RNA	Levels from 2 to 50 times lower than RNA levels in infected control plants as total RNA
Full length transgene RNA detected	Not determined	Not determined	Not determined	Yes, by Northerns	Analysis underway
Transcapsidation	CP transgene level increased when inoculated with PRSV	No change in CP levels when inoculated by PRSV	Not applicable - no other virus infects papayas in the USA	Not applicable	No increase in CP levels when infected by PVA
Other comments			Susceptible to PRSV strains exotic to USA	PLRV symptoms seen on small per cent of inoculated transgenic plants	

I would like to note some unique properties of these five products. With the PLRV resistant potatoes, the transgene was driven by figwort mosaic virus promoter that drives transgene expression to mesophyll tissues where the luteoviruses do not replicate to a significant degree. To address the question whether expression of the viral replicase in tissues where the virus does not replicate increases the frequency of recombination or recombinant virus, APHIS held a public meeting (a summary of the conclusions can be found at http://www.aphis.usda.gov/biotech/virus/virussum.html). At the same time, Allen Miller et al. (1997) published a paper that raised issues regarding luteoviral transgenes and satellite RNAs, host RNA-dependent RNA polymerase, and amplification of host RNAs. To summarize their conclusions, the scientists felt that most of these issues posed no significant risk. They did believe that the expression of viral replicase in mesophyll tissues might raise the rate of recombination but not the likelihood of a recombinant virus arising.

With the PLRV resistant potatoes, some plants develop leaf roll symptoms under field conditions. This is apparently not caused by development of resistance breaking strain but probably weak expressing of the transgene under certain environmental conditions or certain plants. The PLRV titers in these symptomatic transgenic plants are extremely low. Monsanto has stated that these symptomatic plants do

not show net necrosis, which is the symptom that reduces the price paid for the tubers and that is the major concern of the farmers and focus of this engineering.

Although it is outside the scope of this presentation, I would like to briefly address the issue of increased weediness in pest resistant plants. For all the virus-resistant (VR) phenotypes, traditional resistance or tolerance genes have been identified in the recipient plant's gene pool. In one case, ZW20 squash, phenotypically identical resistant cultivars were already marketed in the USA. APHIS concluded the three deregulated transgenic plants would likely to be no more weedy than phenotypically identical plants developed by traditional breeding. Our conclusion is consistent with Ecological Society of America's (Tiedje et al. l989) statement, "We contend that transgenic organisms should be evaluated and regulated according to their biological properties (phenotypes), rather than according to the genetic techniques used to produce them". Discussing the experience derived from traditional breeding, the article states, „This vast and often anecdotal record provides useful information on the effects of moving domesticated species from one location to another, and on moving genes via hybridization to create new strains. This record provides useful information for the evaluation of genetic alterations similar to those that might have been produced by traditional means, and such alterations are likely to pose few ecological problems". The US National Academy of Sciences (1987) and National Research Council (1989) reached similar conclusions.

Although the ZW20 squash were approved for use in late 1994, the first large commercial use of the cultivars bred from the original transformation event occurred this summer. Concurrently, large-scale plantings of the VR papayas are underway in Hawaii. However, large-scale for these plants are only a few thousand acres. It is anticipated that the first truly large-scale releases of VR plants will not occur until Monsanto's VR potatoes are commercialized early in 1999. APHIS does not believe that there is adequate scientific justification to require post-commercialization monitoring of these VR plants.

Conclusions

Over 400 field tests of transgenic virus-resistant plants have been performed in the USA without any reported adverse environmental effects. After receiving all Federal approvals, transgenic squash and papaya plants are being grown in 1998 on a commercial scale on thousands of acres.

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Is gene flow a serious environmental safety issue for virus-resistant transgenic squash?

Fuchs, M.*, Gonsalves, D.

Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA;
*INRA, Laboratoire de Pathologie Végétale, 28 rue de Herrlisheim, 68021 Colmar, France

Abstract

We analyzed the movement of coat protein (CP) genes from the commercial transgenic squash line CZW-3 into its free-living relative *Cucurbita texana*. Transgenic squash line CZW-3 expresses the CP genes of cucumber mosaic cucumovirus (CMV), zucchini yellow mosaic potyvirus (ZYMV), and watermelon mosaic virus 2 potyvirus (WMV 2), as well as the neomycin phosphotransferase (NPT II) gene, and is highly resistant to these three viruses. We focused our study on the outcomes of an initial hybridization between transgenic squash CZW-3 and *C. texana*, and designed field experiments to evaluate the rate of introgression and the relative fitness of *C. texana* x CZW-3 hybrids that acquired the transgenes. Our results showed that introgression readily occurred from transgenic F1 hybrids and their progeny into *C. texana*. Under conditions of high disease pressure, hybrids that acquired the CP genes exhibited increased fitness over *C. texana* by growing more vigorously, displaying resistance to CMV, ZYMV, and WMV 2, and producing more mature fruits and more viable seeds. In contrast, under conditions of low disease pressure, hybrids that expressed the three CP genes did not appear to have any selective advantage over *C. texana* or their non-transgenic counterparts. Given the low incidence of viruses in natural populations of *C. texana*, our data suggest that free-living *C. texana* that acquire CP transgenes for resistance to CMV, ZYMV, and WMV 2 through gene flow are unlikely to become a threat to the environment as an invasive and noxious weed pest.

Introduction

Gene flow has been raised as one of the major environmental safety issues for the release of transgenic crops, including virus-resistant transgenic crops (Dale, 1992; Hancock et al. 1996; Kareiva et al. 1994; Rissler and Mellon, 1996; Snow and Palma, 1997; Tepfer, 1993). Recently, movement of transgenes conferring herbicide tolerance has been well documented in *Brassica* species (Mikkelsen et al. 1996; Chèvre et al. 1997), however, there is no report yet in the case of virus-resistant transgenic crops.

So far, the majority of transgenic crops developed for virus resistance result from the application of the pathogen-derived resistance strategy (Sanford and Johnston, 1985). This strategy to engineer virus resistance is based on the expression of gene constructs derived from the viral genome itself, in particular the coat protein (CP) gene, in transgenic plants. Therefore, if virus-resistant transgenic crops are grown in proximity to cross compatible free-living relatives, virus-derived gene constructs can be transferred through pollen flow, and wild x crop hybrids can develop. Hybrids that acquire the CP transgenes can subsequently exhibit virus resistance. If virus resistance provides a selective advantage, genetically modified free-living crop relatives can emerge with enhanced fitness and increased weediness. Such altered free-living species can eventually become invasive and more noxious weed pests.

We addressed the issue of gene flow by monitoring the transfer of CP genes from commercial transgenic squash line CZW-3 into the free-living squash *Cucurbita texana*. Since gene flow has been described between cultivated non-transgenic squash cultivars and *C. texana* (Kirkpatrick and Wilson, 1988; Wilson, 1990), gene flow will most likely also occur with transgenic squash, including line CZW-3. Therefore, we focused our study on the outcomes of an initial hybridization between transgenic squash line CZW-3 and *C. texana*, and designed field experiments to evaluate the rate of introgression and the relative fitness of hybrids that acquired the CP transgenes in order to determine the impact of the release of virus-resistant transgenic squash into the environment.

Materials and Methods

Transgenic squash line CZW-3 expressing the CP genes of CMV, ZYMV, and WMV 2, as well as the neomycin phosphotransferase (NPT II) gene, was used as donor of transgenic pollen. Transgenic line

CZW-3 was obtained by *Agrobacterium tumefaciens*-mediated transformation with plasmid pPRBN-CMV73/ZYMV74/WMBN22 (Tricoli et al. 1995). Expression of each CP gene was driven by the cauliflower mosaic virus (CaMV) 35S promoter, part of the intergenic region of RNA 3 of CMV used as leader sequence, and the nopaline synthase or the CaMV 35S terminator sequences (Tricoli et al. 1995). Plants of transgenic line CZW-3 are highly resistant to single and mixed infections by CMV, ZYMV, and WMV 2 (Tricoli et al. 1995). Transgenic line CZW-3 was cleared in 1996 by APHIS-USDA for commercial release in the USA (APHIS-USDA, 1998).

The free-living squash species, *Cucurbita texana*, commonly known as Texas gourd, was used as receptor of transgenic pollen. *C. texana* is a geographically restricted wild growing squash species found in some Southcentral States in the USA (Decker, 1988; Oliver et al. 1983; Wilson, 1990). It has been described as a localized weed problem in cotton and soybean fields (Andres, 1995; Weidemann and Templeton, 1988). *C. texana* is susceptible to CMV, ZYMV, and WMV 2.

F1 hybrids (= *C. texana* x CZW-3), and two back cross progenies, were obtained by hand pollination in the greenhouse with *C. texana* as pistillate parent.

Test plants were exposed in the field to conditions of low or high disease pressure. Low disease pressure was achieved by transplanting healthy plants at locations with extreme isolation in order to reduce the incidence of aphid-vectored viruses. High disease pressure was achieved by transplanting a few *C. texana* (20% of the total number of plants tested in a field) which were mechanically inoculated with either CMV, ZYMV, or WMV 2 prior to the transplanting. Infected *C. texana* served as virus source for secondary aphid-vectored spread. Dispersal of transgenic pollen was vectored by wind and indigenous populations of bees.

Movement of the three CP and the NPT II transgenes was assessed by analysis of the expression of the NPT II gene in germinating seeds by enzyme-linked immunosorbent assay (ELISA) using commercial g-globulins (Fuchs and Gonsalves, 1997a,b). This approach was feasible because the CP and NPT II genes were co-engineered within the T-DNA borders of the binary vector used for transformation (Tricoli et al. 1995). Also, we found a nearly perfect correlation between resistance to CMV, ZYMV, and WMV 2, and expression of the NPT II gene, in transgenic line CZW-3 and any of its progenies.

Results

Introgression studies

Field experiments were designated to assess the rate of introgression of the CP genes with transgenic F1 hybrids set in the center of replicated plantings of *C. texana*. Thus, the only source of transgenic pollen was from the F1 hybrids. ELISA for NPT II proved to be a fast, sensitive, and reliable technique to conveniently screen a large number of seeds, and to determine the frequency of self pollination (ELISA negative) and introgression (ELISA positive).

Our results showed that introgression of the CP and NPT II genes readily occurred from transgenic F1 hybrids, as demonstrated by the expression of the NPT II protein in some germinating *C. texana* seeds (Fuchs and Gonsalves, 1997b). The rate of introgression increased with overlapping flowering patterns of transgenic F1 hybrids and *C. texana*, and with a high ratio of transgenic F1 hybrids over *C. texana*. Importantly, introgression readily occurred over three consecutive generations of *C. texana* under conditions of low disease pressure. In contrast, introgression was severely inhibited under conditions of high disease pressure. As expected, *C. texana* that acquired the CP genes exhibited resistance to CMV, ZYMV, and WMV 2 (Fuchs and Gonsalves, 1997a).

Relative fitness comparisons

Since introgression of the CP transgenes translated into virus resistance, would virus resistance provide a selective advantage to *C. texana*? Field experiments were designated to directly compare the relative fitness of *C. texana*, transgenic squash CZW-3 and three generations of *C. texana* x CZW-3 hybrids (F1, BC1, and BC2). Our results showed that selection pressure markedly affected the performance of the hybrids (Fuchs and Gonsalves 1997a, b). Under conditions of high disease pressure, hybrids that expressed the three CP genes exhibited increased fitness over *C. texana* and non-transgenic hybrid segregants by growing more vigorously, displaying resistance to CMV, ZYMV, and WMV 2, and

producing more mature fruits and more viable seeds. In contrast, under conditions of low disease pressure, hybrids that expressed the three CP genes did not appear to have any selective advantage over *C. texana* or their non-transgenic counterparts.

Conclusions and Discussion

Gene flow is an important environmental safety issue in the case of transgenic squash because two virus-resistant transgenic lines have been deregulated in the USA. Transgenic line ZW-20 which is resistant to ZYMV and WMV 2 has been cleared in 1992, and transgenic line CZW-3 which is resistant to CMV, ZYMV, and WMV 2 received exemption status in 1996 (APHIS-USDA, 1998). Gene flow is also a critical environmental safety issue because Central, North, and South Americas are centers of origin and diversity for squash species (Wilson, 1990).

Gene flow has probably occurred between cultivated squash and wild relatives for a very long period of time since domestication of squash has been traced back in North America to 100 centuries ago (Nee, 1990; Smith, 1997). Thus, there has been ample time for exchange of genetic information between cultivated and wild squash species. For this reason, we focused our risk assessment study not only on the occurrence of introgression but also on its outcomes to determine the environmental impact of *C. texana* that acquire CP transgenes.

Our field experiments showed that the CP genes of CMV, ZYMV and WMV 2 readily moved from transgenic squash CZW-3 into *C. texana* upon hybridization and introgression. Movement of the CP genes provided resistance against these three viruses and a selective advantage to *C. texana* under conditions of high, but not low, disease pressure. These data suggest that virus-resistant *C. texana* might have a competitive advantage only when CMV, ZYMV and WMV 2 are endemic to natural habitats of *C. texana*, and if the incidence of these three viruses is high in natural populations of *C. texana*. Interestingly, preliminary surveys indicate that *C. texana* are not readily infected by viruses in their natural ecosystems. Thus, free-living *C. texana* resistant to CMV, ZYMV, and WMV 2 are unlikely to become a significant threat to the environment as invasive and eventually more noxious weed pests.

Although our data indicated that virus-resistant transgenic squash are likely to have limited to no detectable environmental impact beyond natural background level, will this be a general trend, or will a different situation occur with other virus-resistant transgenic crops which have cross compatible wild relatives? Will virus-resistant transgenic perennial crops, which have cross compatible wild relatives, and are grown for longer periods of time than annual crops like squash, have to be put under closer scrutiny? Limited information is available on the occurrence of viruses and their effect on the dynamics and evolution of populations of wild plant species. Therefore, it will be important to evaluate on a case by case basis the potential impact of virus-resistant transgenic crops that are destined to commercialization.

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Risk assessment of virus resistant transgenic plants

Allison, R.F.[1)], Schneider, W.L.[2)], Deng, M.[1)]

[1)] Department of Botany and Plant Pathology, Plant Biology Building, Michigan State University, East Lansing, MI 48824-1312, USA;
[2)] The Samuel Roberts Noble Foundation, Inc., PO Box 2180, Ardmore, OK 73402, USA

Abstract

RNA recombination is an active natural viral evolutionary process. Experimental results demonstrate clearly that in virus resistant transgenic plants (VRTPs) the viral transgene is available for RNA recombination to replicating viruses. While smaller transgenes may be less likely to be involved in recombination events, it is not apparent that transgene recombination can be prevented. Since the transgene is constitutively transcribed, opportunities for recombination events are likely greater than in natural mixed infections. Therefore, as VRTPs are released to the field, they should be monitored for development of new plant viruses.

Introduction

Plants can be genetically modified to be resistant to plant viruses. This is accomplished by introducing a segment of virus genome to the plant chromosome. Transcription, and in some cases translation, of the viral segment provides resistance to the virus from which the segment was derived. With limited sources of natural virus resistance genes that can be introduced by traditional breeding techniques, genetic engineering for virus resistance will undoubtedly reduce crop losses to viruses and contribute significantly to increased food production.

But all techniques come with associated risks and it is prudent to evaluate potential problems prior to universal acceptance. In the case of virus resistant transgenic plants several areas of concern have been identified. These include the flow of transgenes through pollen, the consequences of transencapsidation and the possibility that the viral transgene may become incorporated into another virus through RNA recombination. We have addressed RNA recombination in virus resistant transgenic plants (VRTPs).

Viral transgenes can be involved in RNA recombination

Our initial experiments were to determine if a viral transgene was physically available to a replicating virus for recombination. Two Bromoviruses were chosen for these studies, cowpea chlorotic mottle virus (CCMV) and brome mosaic virus (BMV). In these closely related tripartite viruses, RNAs 1 and 2 are sufficient for replication while both the capsid and movement genes of RNA 3 are required for systemic movement. To address the potential involvement of a viral transgene in RNA recombination events, *N. benthamiana* was transformed with the 3' two thirds of the capsid gene and the complete 3' untranslated region (UTR) (Greene and Allison, 1994). This small transgene did not provide resistance to CCMV infections. These plants were challenged with a CCMV deletion mutant which lacked the 3' one third of the capsid gene. As a result of this deletion, the mutant was incapable of systemic infection. Since systemic movement was dependent on a restored capsid gene, systemic infection signaled a successful recombination event.

Three percent of the transgenic plants inoculated became systemically infected. Each infection was passaged to a non-transgenic plant from which recombinant virus was isolated. Analysis of the recovered virus revealed the marker mutations that distinguished the nucleotide sequence of the transgene from wild type (WT) CCMV. Numerous nucleotide sequence variations were observed in these recombinant viruses which apparently resulted from recombination events. When transferred to cowpeas, several recombinants produced unique foliar symptoms but when inoculated simultaneously, none successfully competed with WT CCMV. Similar recombination events have been reported in several other viral systems (Lommel and Xiong, 1991; Gal et al. 1992; Schoelz and Wintermantel, 1993).

Currently, similar experiments are exploring the possibility that RNA recombination may also involve the polymerase gene of CCMV RNA 2. A CCMV deletion mutant lacking 307 nucleotides near the 3' terminus of the polymerase gene (Traynor et al. 1991) was used to inoculate transgenic *N. benthamiana.* The transgene included the 3' two thirds of CCMV RNA2. While the deletion mutant was replication

competent, the virus titer was significantly lower than WT and the infection progressed more slowly. Since the deletion mutant migrated more rapidly than WT RNA 2 in agarose gel electrophoresis, recombinants were easily detected. To date, recombinants restoring this deletion have been recovered from 12% of the inoculated plants. Perhaps not unexpectedly, the polymerase gene, too, is active in recombination events.

To determine if recombination could involve two different viruses, transgenic *N. benthamiana* plants transcribing the 3' two thirds of the CCMV capsid gene and the associated 3' UTR were challenged as described above with a BMV deletion mutant which lacked 26 nucleotides of the capsid gene. This BMV mutation was sufficient to inhibit systemic infections. Again 3% of the transgenic plants became systemically infected which suggested BMV/CCMV recombinants. In the experiments described above, recombinant viruses were passed to non-transgenic plants prior to sequence analysis. However, in these experiments cDNA clones were prepared directly from virion RNA. Analysis of these clones revealed an array of recombinants some reflecting as many as six recombinant events within a single cDNA clone. But none of these clones contained open reading frames (ORFs) capable of encoding a functional capsid protein.

Analysis of viable recombinants was possible only following passage of the infection to non-transgenic plants. Apparently passage to a new host served as a bottleneck where strong selection pressure discriminated among the recombinant RNAs and only the most functional genomic RNAs perpetuated the infection. The cDNA derived from these passaged infections contained recombinant capsid genes with complete ORFs. Of the functional recombinants derived, one had an expanded host range which included *N. benthamiana,* barley and cowpeas.

Exclusion of the 3' UTR from the transgene reduces recovery of recombinants

In all of the Bromovirus recombination experiments described above, the transgene included the complete 3' untranslated region of the virus. In an effort to determine if the length of the transgene encourages RNA recombination, several transformants were made which contained less than full length 3' UTRs (Greene and Allison, 1996). When challenged with the same CCMV capsid gene deletion mutant used in our initial study which lacked the 3' third of the capsid gene, no systemic infection was detected. This suggests that: 1) shorter transgenes, 2) transgenes lacking the stability afforded by the natural 3' UTR or 3) transgenes lacking the replication signals present in this segment are less likely to recombine than transgenes with the complete 3' UTR.

Our recombination experiments have involved two closely related Bromoviruses, BMV and CCMV. By observing virus recombination with transgenes derived from the same virus or its close relative and using transgenes which included complete 3' UTRs, some of the recovered recombinants may have formed by an unexpected means.

The viral replication initiation site is located within the 3' UTR of these viruses and is included in the transgenes that supported recombination. If a transgene is recognized by the viral replication complex, a complementary copy of the transgenic transcript may be formed. This would result in both a positive and negative sense copy of the transgene and both would be available for RNA recombination. Our preliminary data indicates that a complementary copy of the transcript is generated during infection but it is present at a much lower concentration than the plus sense transgenic transcript. With the minus sense copy of the transgene present in our system, we are unable to discern whether recombination occurs during plus or minus strand synthesis.

Our experiments, and those of others, clearly demonstrate that a virus is capable of recombining with itself. The most significant question is whether the transgene responsible for resistance to one virus can recombine with another virus which may challenge the transgenic plant. In the field, plants are challenged by viruses that are pathogenic and non-pathogenic to that particular plant species. Protoplast experiments indicate that many viruses which are considered non-pathogens of a particular species may undergo replication in initially infected cells. Consequently, they too have an opportunity for recombination with the constitutively transcribed viral transgene. In this scenario there is a strong selection pressure for the formation of a systemic pathogen. Therefore, if a viral transgene contains a motif which can be usefully incorporated into a challenging virus, recombination provides the mechanism of incorporation.

Similarities in nucleotide sequences among different viruses suggest that RNA recombination has played a significant role in the evolution of these pathogens. Opportunities for recombination must have occurred during mixed infections when a replicating virus was exposed to the RNA of another virus. While mixed infections are frequently detected in the field, the presence of two viruses in the same plant or even the same cell does not necessarily mean that the two viruses had unlimited recombination opportunities. In fact simultaneous replication of two or more viruses within the same plant cell may be rather rare. A likely scenario is that when a virus is introduced to a cell it undergoes replication. While some of the genomic RNA proceeds to adjacent cells to continue the infection process, viral RNA remaining in infected cells is stabilized and protected by encapsidation. In this form it is not available for recombination. Thus unless two viruses were simultaneously introduced to the same cell, recombination opportunities may be limited compared to those afforded to a virus by VRTPs. Since transgenes are constitutively transcribed, they are continually available in each plant cell for recombination with any challenging virus.

We have attempted to produce mixed infections in *N. benthamiana* by simultaneously inoculating with both CCMV and BMV. Approximately one third of these plants became systemically infected by both viruses. In contrast, mixed infections were established in 80% of the plants when the inoculation of the second virus was delayed by two weeks. This suggests that mixed infections are more easily established if the second infection is introduced following the establishment of the first infection. While recombinants were recovered from plants where mixed infections were established by simultaneous inoculation, no recombinant was detected from plants where mixed infections were established by delayed inoculation. Consequently, recombination opportunities in mixed infections may be significantly less than recombination opportunities provided by plants transcribing viral transgenes. If this is true, virus resistant transgenic plants could have a significant effect on the evolution of plant viruses.

Conclusions

Although laboratory experiments have convincingly demonstrated that a viral transgene may recombine with a replicating virus, it is difficult to predict the consequences of such recombination events in the field. Strong selection pressure has developed existing WT viruses which demonstrate a coordinated expression of their genes, a refined interaction with their host, and RNA, protein and virion structures that resist host degradation. However, natural evolutionary processes periodically produce new viruses. In the case of VRTPs it is impossible to experimentally test all possible recombination events, thus as VRTPs are released to the field, we should be alert and carefully monitor these and adjacent fields for unusual plant viruses.

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Strategies for detection of recombination in virus-infected plants expressing a viral transgene

Aaziz, R.[1)], Salánki, K.[2)], Balázs, E.[2)], Jacquemond, M.[3)], Tepfer, M.[1)]

[1)] Laboratoire de Biologie Cellulaire, INRA, 78026 Versailles cedex, France;
[2)] Agricultural Biotechnology Center, Institute for Plant Sciences, 2101 Gödöllö, Hungary;
[3)] Station de Pathologie Végétale, INRA-Domaine St.-Maurice, BP 94, 84143 Montfavet cedex, France

Abstract

RNA recombination in virus-infected transgenic plants expressing viral sequences could lead to potential risks due to generation and spread of viruses with novel properties. In an attempt to assess these risks, we have developed molecular and biological strategies to detect potential recombinant viruses. In these studies, we have used transgenic tobacco plants expressing a gene encoding the cucumber mosaic cucumovirus (CMV) coat protein, which have been infected with a related cucumovirus. The molecular and biological detection techniques developed are currently being used to compare the frequency of appearance of recombinant viruses in transgenic plants with that in doubly-infected non-transgenic ones. We hope that this will allow us to answer certain biosafety questions relative to future commercial release of transgenic virus-resistant plants.

Introduction

Most transgenic plants protected against viruses express a viral coat protein (CP) gene. It is increasingly clear that interactions occur between products of the viral CP transgene and an infecting virus. Three types of potential risks due to these interactions are generally cited (Tepfer, 1993): synergism (Pruss et al. 1997), heteroencapsidation (Lecoq et al. 1993) and recombination (Greene and Allison, 1994). Of these, recombination is generally considered to be of greatest concern, since it can lead to irreversible changes in viral genomes, whereas synergism and heteroencapsidation are phenomena that are expected to be reversible, and would thus not be expected to have lasting effects.

RNA recombination is a general phenomenon in the "RNA world" (Lai, 1992; Simon and Bujarski, 1994). Several studies have pointed out that RNA recombination has played, and continues to play, a key role in increasing variability and thus driving evolution of RNA viruses (for review see Roossinck, 1997). Viral RNA dependent RNA polymerase (RdRp), which lacks proofreading functions, introduces frequent errors in daughter RNA molecules. It has thus been proposed that RNA recombination would also play an important role in the repair of viral RNA segments, since sequence exchange would make it possible to eliminate regions bearing mutations (Domingo and Holland, 1997; Carpenter and Simon, 1996).

To account for RNA recombination, three models have been proposed: breakage-ligation, breakage-induced template switching and replicase-driven template switching (Nagy and Simon, 1997). Although none of them has been formally demonstrated, the third model is the most generally accepted. According to this model, once the viral RdRp has initiated synthesis of a nascent RNA strand, it can switch to another template strand and continue elongation of a chimeric nascent RNA (Figure 1).

Three types of RNA recombination can be distinguished: homologous recombination occurring between two related RNA molecules at corresponding homologous sites, aberrant homologous recombination occurring between two related RNA molecules at non-corresponding sites, and nonhomologous recombination occurring between unrelated RNA molecules (Lai, 1992). An alternative classification, based on both the recombination mechanism and the nature of the recombinants generated, was proposed recently (Nagy and Simon, 1997).

Numerous experimental systems have been developed for studying RNA recombination. For instance, studies have been carried out with bacteriophages, including Qß (Palasingam and Shaklee, 1992) and 06 (Onodera et al. 1993); with animal viruses, including coronaviruses (Makino et al. 1986), alphaviruses (Weiss and Schlesinger, 1991), orthomyxoviruses (Bergmann et al. 1992), polioviruses (Jarvis and Kirkgaard, 1992), and nodaviruses (Li and Ball, 1993); and with plant viruses, including bromoviruses (Bujarski and Kaesberg, 1986; Allison et al. 1990), carmoviruses (Cascone et al. 1990), tobamoviruses

(Beck and Dawson, 1990), alfalfa mosaic virus (Van der Kuyl et al. 1991), tombusviruses (White and Morris, 1994), and potyviruses (Gal et al. 1998).

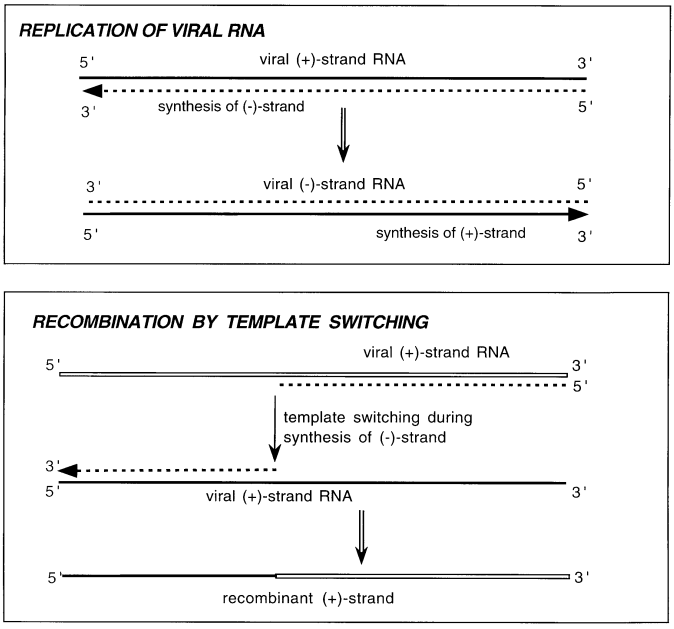

Figure 1 Schematic representation of viral RNA replication and recombination by the template switching model. Plus-strand and minus-strand viral RNAs are represented by solid and dashed lines and arrows, respectively.

As increasing numbers of plant RNA virus genomes have been sequenced, their analysis has revealed genetic features that could best be attributed to RNA recombination events between viruses that occurred naturally during their co-evolution. Many cases have been described, in viruses including tobraviruses (Goulden et al. 1991), potyviruses (Cervera et al. 1993; Revers et al. 1996), luteoviruses (Gibbs and Cooper, 1995), and nepoviruses (Le Gall et al. 1995). Several cases of insertion of host plant sequences in viral genomes have also been described (Mayo and Jolly, 1991; Masuta et al. 1992). Generally speaking, these results confirm the importance of recombination in virus evolution.

Recombination between the RNA transcribed from a viral transgene and the genome of an incoming virus has been observed under laboratory conditions. To date, three experimental systems have been described: cauliflower mosaic caulimovirus (CaMV) in *Brassica napus* (Gal et al. 1992) or in *N. bige lovii* (Schoelz and Wintermantel, 1993), and cowpea chlorotic mottle bromovirus (CCMV) in *N. benthamiana* (Greene and Allison, 1994). It is worth noting that in these three studies, recombinants were obtained under conditions of strong selection pressure; i.e., only recombinant viruses that move systemically could be detected. In a fourth example, recombinant viruses generated by recombination between CaMV and viral sequences transcribed from a transgene in *N. bigelovii* have been obtained under conditions of moderate selection pressure (Wintermantel and Schoelz, 1996).

Since these results clearly show that recombination can indeed occur in plants expressing viral transgenes, the potential impact of recombination merits thorough evaluation. In risk assessment studies, risk is often broken down into two elements (risk = hazard x frequency). In an evaluation of potential risk associated with recombination, we have considered that the element of "hazard" corresponds to the possibility that recombination will lead to viruses with novel properties that could be more deleterious

than the parental strains. In order to investigate this possibility, we have created artificial recombinant cucumoviruses, and then tested their biological properties (Salánki et al. 1997; Jacquemond et al. 1997).

The evaluation of the risk impact of the frequency of recombination is more delicate, since here the baseline corresponds to the naturally occurring frequency of recombination in the absence of transgenic plants, i.e. the frequency of recombination in non-transgenic plants infected by two viruses with normal, wild-type viability. Thus, in order to determine if the frequency of recombination in plants expressing a viral transgene can be considered to present elements of risk, we must compare the frequency of recombination in two situations:

1. co-infection of non-transgenic plants with two related viruses,
2. infection of plants expressing a CP transgene with a single virus.

Since in the former situation there is normally little or no selection pressure in favour of recombinants, it is essential to study also the second situation under conditions of equally low selection pressure. Due to the predicted rarity of RNA recombination events, we have developed molecular and biological strategies to detect potential recombinant viruses under low selection pressure, as described below. Molecular detection was based on specific amplification of recombinants by reverse transcription (RT) of viral RNA to produce cDNA, followed by polymerase chain reaction (PCR) amplification with sequence-specific primers (RT-PCR). Biological detection was based on differences in host range of the viruses studied.

These studies were carried out with members of the cucumovirus group. The genome of cucumoviruses is composed of three plus-sense single-stranded RNAs. RNAs 1 and 2 encode proteins involved in viral RNA replication. RNA 3 is bicistronic, and encodes movement protein (MP) and coat protein (CP). Cucumber mosaic virus (CMV), the type-member of this group, has an exceptionally broad host range, infecting more than 800 plant species, including both dicots and monocots (Palukaitis et al. 1992). CMV is also of interest because it has a considerable impact on agriculture worldwide. A second cucumovirus tomato aspermy virus (TAV) can be distinguished from CMV by its sequence (approximately 60-70% identity), and also by differences in host range. In particular, all CMV strains infect cucurbits systemically, whereas infection of these plant species by TAV is restricted to the inoculated leaf.

Results and discussion

In this work, we have used the following viruses: R-CMV, which was isolated in France from *Ranunculus* (Jacquemond and Lot, 1981), and P-TAV, which was isolated from pepper in Hungary (Salánki et al. 1994). We have focused our attention exclusively on RNA 3 recombination, since this RNA molecule contains the CP gene, which is used frequently in plant virus resistance transgenes, and also because RNA 3 is not essential for viral RNA replication.

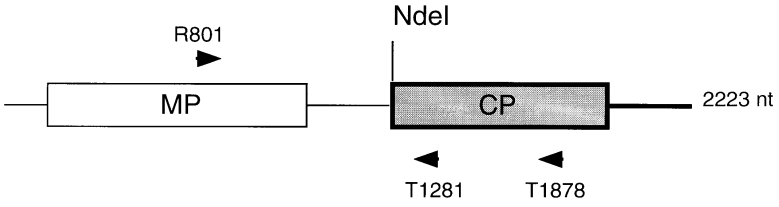

Figure 2 Schematic representation of the artificial recombinant R1R2RT3 cucumovirus. Only RNA3 is shown (not to scale). CMV sequences are indicated by thin lines and an open box; TAV sequences by thick lines and a shaded box. The NdeI site introduced at the initiator ATG of the CP gene of the parental RNA 3 clones, which was used to create the RT3 recombinant, is shown. The relative positions of oligonucleotides used in RT-PCR experiments are indicated by short arrows.

The first step was to determine the sensitivity of the methods used, i.e. to determine the threshold of detection of recombinant molecules in the presence of excess parental RNA. For this, in addition to the above parental viruses, we have used an artificial recombinant cucumovirus, called R1R2RT3 (Salánki et al. 1997). This virus is composed of RNAs 1 and 2 of R-CMV and a chimeric RNA 3, which contains the 5' non-coding region (NCR), the MP gene and the intergenic region of R-CMV and the CP gene and 3' NCR of P-TAV (Figure 2). The most sensitive molecular detection techniques currently used are based on RT-PCR. Dilution experiments of R1R2RT3 viral RNA (referred to below as RT3) in viral RNA of R-CMV or P-TAV were carried out, followed by RT-PCR detection of recombinant molecules with RT3 specific primers.

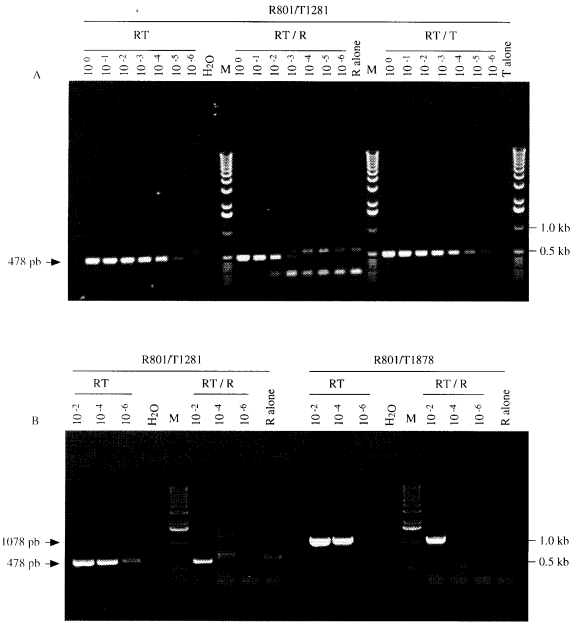

Figure 3 Threshold of detection by RT-PCR of R1R2RT3 viral RNA diluted in R-CMV or P-TAV viral RNA. One µg of purified R1 R2RT3 viral RNA was diluted in water ten-fold successively to reach a final dilution of 10^{-6}, either in water alone (RT), or in the presence of 1 µg of R-CMV (RT/R) or PTAV (RT/T) viral RNA. RT-PCR was carried out with the R1R2RT3-specific primers R801/T1281 (top panel and lower left part of bottom panel) or with R801/T1878 (bottom-right). H20 is a negative control RT-PCR with water. The expected position of bands is indicated by arrows on the left, and molecular size markers are shown on the right: M (Gibco BRL 1 kb ladder). The reverse-transcription step was carried out at 42°C (A) or 60°C (B) with either MuMLV (Gibco BRL) or AMV (Boehringer Mannheim) reverse transcriptases, respectively, followed by 30 PCR cycles (94°C 30 sec, 52°C 45 sec, 72°C 45-60 sec) with 0.5 units of Taq polymerase (Gibco BRL) in a Perkin Elmer PTC-100 apparatus.

As shown in Figure 3A, amounts of RT3 below the picogram level could be detected with the RT3-specific primer couple R801/T1281 (primers are designated by R or T, for R-CMV or PTAV, followed by the nucleotide position on RNA 3). In the presence of a 10^6-fold excess of TAV, RT3 still remains detectable below the picogram level. By contrast, in the presence of CMV, the sensitivity did not go beyond a 10^4 dilution, due to non-specific recognition of the 3' primer T1281 on R-CMV during the reverse transcription step. The presence of total tobacco RNA did not affect the observed threshold of detection (not shown). Sensitivity depends on the 3' primer used in the reverse transcription step, since

with primers R801/T1878, detection was not possible below the nanogram level (data not shown). In an attempt to enhance sensitivity, and to eliminate non-specific cDNA synthesis, we have carried out the reverse transcription step under more stringent conditions (at 60°C). This allowed us to improve the specificity of the 3' primer T1878 to the picogram level, but the interference in the presence of R-CMV RNA could not be eliminated (Figure 3B).

As an alternative to molecular techniques for detection of recombinant viruses, we have also developed a biological screen, based on differences in host range, to detect potential events of recombination between the infecting virus and viral sequences expressed from the transgene. As mentioned previously, R-CMV is systemic on cucumber and other cucurbits, whereas P-TAV infection of cucumber is subliminal, since the virus is unable to move from initially infected cells (Salánki et al. 1997). The same authors showed that the RT3 recombinant virus, which differs from R-CMV only by having the P-TAV CP gene and 3' NCR, is able to invade the inoculated leaf on cucumber, but does not move systemically in this species. This defect in systemic movement is specific to cucurbits, since RT3 moves systemically, like the parental strains, in all other species tested (Salánki et al. 1997). When plants expressing a CP-CMV transgene are infected with RT3, recombination between the mRNA of the CP-CMV transgene and the corresponding viral RNA could result in the creation of a virus that had recovered the CP gene from CMV. This new recombinant would be predicted to be systemic on cucurbit plants, whereas RT3 is not. By inoculating cucumber seedlings with dilutions of R-CMV in RT3, we are currently determining the threshold of detection of potential recombinants that would result from transfer of the CP gene from the mRNA to the viral genome.

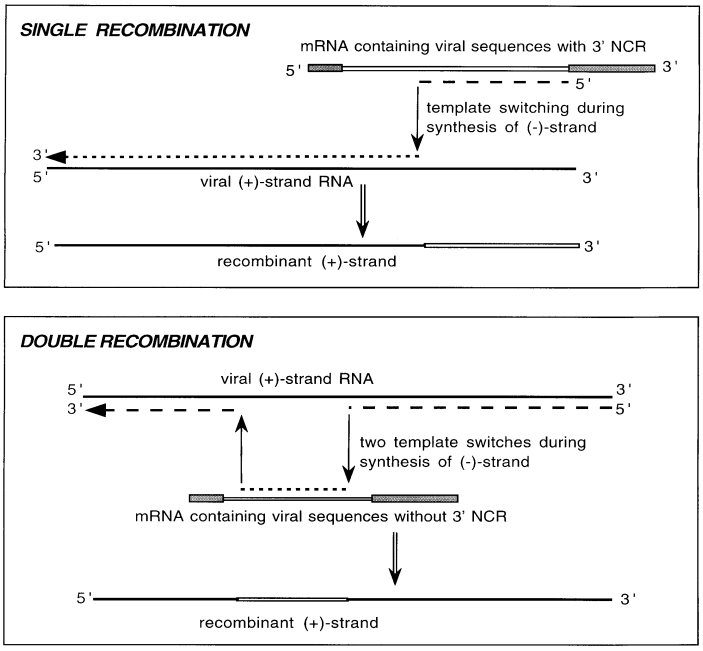

Figure 4 Schematic representation of single and double recombination during the synthesis of minus-strand viral RNA in transgenic plants expressing viral sequences, according to a template switching model. In the single recombination model, the viral replicase initiates (-)-strand synthesis on the cellular mRNA, which in this case includes the viral 3' NCR. A single template switch to a viral RNA allows completion of (-)-strand synthesis, which then serves as template for normal (+)-strand synthesis. In the double recombination model, the viral replicase cannot initiate on the cellular mRNA, which lacks the 3' NCR. In this case, after initiation on the viral template (+)-sense RNA, two template switches during (-)-strand synthesis are necessary for synthesis of a recombinant (-)-strand viral RNA.

In RNA viruses, the 3' NCR is the recognition site for initiation of the synthesis of the minus strand by the viral RdRp. In the *Bromoviridae* family, the 3' NCR (-200 nucleotides) is conserved between different RNA molecules of the same strain virus. Greene and Allison (1996) have shown that recombination between transgenic and viral RNAs was reduced to undetectable levels when they deleted the 3' NCR from the CP-CCMV transgene. In order to evaluate the importance of the 3' NCR in RNA recombination in the cucumovirus model, we shall use two types of transgenic tobacco plants expressing an R-CMV CP gene: CCP5 and CpR6, which do and do not include the 3' NCR, respectively. As shown in Figure 4, in the case of plants expressing a CP transgene including the 3' NCR, such as CCP5, a single recombination event between the viral sequences in the transgene-derived mRNA would be sufficient to create a viable recombinant RNA 3, whereas in CpR6 plants, which express a CP gene lacking the 3' NCR, double recombination would be required.

As shown in Figure 5, in order to enhance the probability of appearance of potential recombinants, we are carrying out successive passages of viral R1R2RT3 on transgenic tobacco lines CCP5 and CpR6. Following each passage, viral RNA will be purified, and the presence of potential recombinants investigated by both RT-PCR and back-inoculation of cucumbers. In parallel experiments, nontransgenic plants will be inoculated with both P-TAV and R-CMV, in order to determine the baseline level of recombination in doubly infected plants. We do not know at this time if the frequency of recombination is high enough for it to be detected under these experimental conditions. If we do not detect recombinants, this will allow us to conclude that their prevalence is below the experimental threshold of detection.

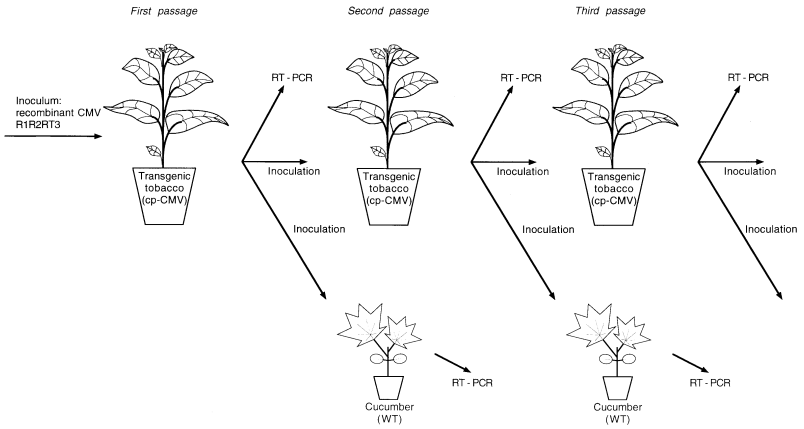

Figure 5 Experimental protocol for the detection, under low selection pressure, of recombinant viruses in transgenic plants expressing a CMV coat protein gene. The recombinant virus R1R2RT3 is passaged on transgenic tobacco plants expressing a CMV coat protein gene either with or without the 3' NCR. At each passage, viral RNA is purified, and either used for RT-PCR analysis, back-inoculation of non-transgenic cucumber plants, and inoculation of transgenic tobacco plants.

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Synergy of virus accumulation and pathology in transgenic plants expressing viral sequences

Palukaitis, P.

Virology Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Abstract

Synergy, the interaction of two viruses to produce enhanced disease, readily occurs when some combinations of viruses infect the same plants. An assessment of 12 transgenic tobacco lines for synergy with four viruses, which interact synergistically with each other, showed that synergy occurred in six combinations. However, only two of these involved transgenic lines showing resistance to the target virus, and the synergy consisted of a slight or moderate increase in disease symptoms with no increase in virus accumulation. Unexpectedly, in six combinations non-target virus interference was also observed in transgenic lines; two of these lines also showed resistance to the homologous target virus. Thus, pyramiding viral transgenes not only will reduce the risks of synergy, but also can increase the breadth of the resistance against non-target viruses, reducing the number of viral transgenes required to obtain effective resistance to the viral pathogens of a particular crop.

Introduction

The concept of pathogen-derived resistance (PDR) (reviewed by Lomonossoff, 1995; Prins and Goldbach, 1996) developed from models that sought to explain cross-protection (rev. by Hamilton, 1980; Palukaitis and Zaitlin, 1984; Fulton, 1986; Ponz and Bruening, 1986), a phenomenon in which infection by one virus interfered with subsequent infection by a related virus. While cross-protection has been used successfully in some cases, concerns about back mutation of attenuated viruses to a severe variant, or escape of the attenuated viruses into non-target crops in which the attenuated viruses might be more pathogenic have limited the application of cross-protection (Hamilton, 1980; Palukaitis and Zaitlin, 1984; Fulton, 1986; Palukaitis, 1991). The advent of plant transformation and regeneration technologies allowed parts of a virus to be expressed in transgenic plants, rather than the whole virus, which reduced the risks identified via the use of infectious viruses. Subsequently, different viral transgenes have been used to engender resistance to the homologous viral pathogen. This strategy has been used successfully with defective or intact genes encoding coat proteins, replication proteins and movement proteins (rev. by Lomonossoff, 1995; Prins and Goldbach, 1996; Palukaitis and Zaitlin, 1997). However, information generated on the biology of plant viruses and their various interactions has led to the identification of possible risks associated with the use of transgenic plants expressing viral sequences (Hull, 1990; de Zoeten, 1991; Palukaitis, 1991; Tepfer, 1993). These range from natural processes such as pollen or seed transmission, heterologous encapsidation, recombination, and synergy, to more fanciful human-plant virus interactions such as allergies, gout, and even infection! The increasing assumptions of negative interactions and disregard for the published literature, unless it serves to substantiate the negative interactions, has led to a climate of extreme concern about the widespread use of transgenic plants expressing plant viral sequences (Hull, 1990; de Zoeten, 1991; Kling, 1996; Mikkelsen et al. 1996; Miller et al. 1997; Tepfer and Balázs, 1997). This has resulted in the need to examine the various identifiable risks and establish their likelihood of occurrence, as well as to test approaches that should mitigate or eliminate the risks (Palukaitis, 1991; Miller et al. 1997; Tepfer and Balázs, 1997). In other chapters of this book, some of the other risks identified with the use of transgenic plants expressing viral sequences will be examined, while in this chapter, the effects of viral synergy will be considered.

Synergy is the interaction of two agents to produce an enhanced effect. A number of plant viruses react with each other synergistically (rev. by Bennett, 1956; Kassanis, 1963). Since infection of plants by multiple viruses is rather common (Kassanis 1963; Falk and Bruening, 1994), there are many opportunities for synergistic interaction leading to enhanced pathogenicity in infected field crops. In addition, in some cases, it has been observed that natural resistance to infection by one virus may be affected by co-infection with an unrelated virus (Anderson et al. 1996; Palukaitis and Kaplan, 1997). This form of synergy involves a potyvirus as one of the virus pairs. Recent data have established that the HC-Pro gene (encoding a protein that functions as a helper component for aphid transmission, a proteinase, and a factor enhancing virus accumulation) is the genetic determinant of viral synergy

between potyviruses and other viruses (Shi et al. 1997; Pruss et al. 1997). The protein encoded by the HC-Pro gene may be a transient inhibitor of plant gene expression (Wang and Maule, 1995; Aranda et al. 1996; Kasschau et al. 1997) resulting in the inhibition of host responses against virus infection, leading to enhanced virus accumulation and pathogenicity and/or the breakage of conventional resistance to the normally restricted virus. While transgenic plants expressing various potyviral genes have been demonstrated to engender resistance to the homologous (target) virus, the HC-Pro gene has not been demonstrated to engender resistance (Lomonossoff, 1995; Prins and Goldbach, 1996). This is probably because the mechanism of PDR in many cases is via homology-dependent gene silencing and HC-Pro has been shown to prevent gene silencing, when expressed as a transgene (Carrington et al. 1998), and also to depress silenced transgenes, when expressed from a virus vector (Baulcombe, 1998). Thus, transgenic plants expressing the potyvirus HC-Pro gene should not be used as a source of PDR.

Since synergy is known to occur between other virus pairs where neither virus is a potyvirus (Garces-Orejuela and Pound, 1957; Close, 1964), it is clear that other viral genes may affect host responses leading to a synergistic interaction. Thus, it is important to assess such transgenic plants for viral synergy, to determine the level of risk associated with the widespread use of such plants. Specifically, transgenic plants expressing four of the five genes of cucumber mosaic virus (CMV), three of the five genes of tobacco mosaic virus (TMV) and the polymerase (NIb) gene of potato virus Y (PVY) were assessed for synergy with TMV, CMV, PVY and potato virus X (PVX).

Results

Parameters of synergy between CMV, PVX, PVY and TMV

To examine whether synergy occurred between one virus and a transgene derived from a second virus, it was first necessary to examine the nature and extent of the synergy between the corresponding viruses. Because the transgenes to be analyzed were all expressed in tobacco, this host was tested for synergy involving pairs of viruses. For those combinations in which synergy of both virus accumulation and pathology were observed, the synergy in pathology preceded the increase in virus titre.

CMV showed synergy in tobacco with PVX and TMV, as well as with the potyvirus, PVY (Table 1). Synergy was exemplified by both enhanced pathology as well as an increase in the levels of CMV accumulation. The latter increase was similar, whether the co-inoculated virus was a potyvirus (PVY), a potexvirus (PVX), or a tobamovirus (TMV).

PVX also showed synergy in tobacco with CMV, PVY and TMV, and the level of PVX increased 8-16 times over that observed in plants infected by PVX alone (Table 1). The infection of tobacco by PVX was temperature sensitive, with no systemic infection at 32 ^{o}C. The synergistic viruses CMV, PVX and TMV did not affect the temperature sensitivity of PVX in tobacco (data not presented).

PVY and TMV were able to induce synergy with CMV and PVX, but in each case only the level of accumulation of the latter viruses showed an increase (Table 1). When co-inoculated, PVY and TMV showed synergy of pathology, but no increase in the levels of accumulation of either virus was observed (Table 1).

Detection of synergy in transgenic tobacco

The various transgenic tobacco plants were infected with CMV, PVX, PVY, or TMV and the plants were assessed for symptoms and virus accumulation. Most combinations of transgenic plants and viruses tested did not show synergy either in pathology or virus accumulation, although in six combinations, synergy was observed (Table 2). However, in four of these combinations synergy was observed in transgenic lines expressing functional viral genes rather than dysfunctional genes, and these same transgenes did not exhibit resistance to the homologous virus. In the remaining two transgenic lines, which exhibited resistance to CMV and PVY, respectively, there was an increase in pathology, but not virus accumulation, following infection by PVY and TMV, respectively (Table 2). In the former instance, the pathology of PVY on tobacco changed from a very mild vein-clearing to a mild (Line R2.2) or pronounced (Line R2.5) vein-clearing, while in the latter case, the pathology of TMV increased from severe mosaic to very severe mosaic (data not shown). This demonstrates that some transgenic lines expressing resistance to one virus may show enhanced pathology when infected by a second virus,

although in no instance was the enhanced pathology as severe as observed by co-infection of the respective viruses (Table 1).

Table 1 Synergy of mixed infections in tobacco

Viruses	TMV	CMV	PVY
TMV	sMos, St[a]	vsMos, sSt, vsLD (C16)[b]	vsMos, St, LD
CMV	vsMos, St, vsLD (C16)	vsMos, sSt,	sMos, St sLD (C16)
PVX	vsMos, St, LD (X8)[c)]	vsVC, sN (X16; C16)	sN (X8)
PVY	vsMos, St, LD	vsMos, St, sLD (C16)	vmVC

[a] Pathogenicity: Mos = mosaic; St = stunting; LD = leaf distortion; VC = vein-clearing; N = necrosis; vm = very mild; s = severe; vs = very severe; [b] C#: Fold increase in CMV levels at 9 d.p.i. vs. CMV alone; [c] X#: Fold increase in PVX levels at 9 d.p.i. vs. PVX alone.

Table 2 Synergy in transgenic tobacco expressing viral transgenes

Transgene[a]	Virus	Synergy[b]	Resistance[c]
CMVRNA-1	PVY	Pathology & Virus Level	No
CMVRNA-1	TMV	Pathology & Virus Level	No
ΔCMVRNA-2	PVY	Pathology (Minor Effect)	Yes
TMV-30K	CMV	Pathology & Virus Level	No
TMV-30K	PVY	Pathology & Virus Level	No
PVY-NIb	TMV	Pathology	Yes

[a] Transgene: Except for ΔCMVRNA-2, which expresses RNA-2 containing a 94nt inframe deletion in the 2a gene, the other transgenic lines express intact genes. All lines except for PVY-NIb have been shown to express functional proteins;
[b] Synergy: Increase in pathology alone, or together with an increase in the level of accumulated virus;
[c] Resistance: Resistance of the transgenic line to the virus from which the transgene was derived.

The transgenic lines expressing full-length RNA-1 of CMV were susceptible to infection by CMV, and also showed synergy with PVY and TMV. In both cases, there was an increase both in pathology and the titre of the inoculated virus (Table 2). The same was true for the transgenic lines expressing the TMV-30K movement protein, which showed increased pathology and virus titres for CMV and PVY, as well as for the homologous virus TMV (Table 2). Transgenic tobacco expressing a defective variant of the TMV-30K protein did not show any synergism with unrelated viruses. In fact, those plants showed resistance to TMV and some interference with infection by other viruses (Cooper et al. 1995). Thus, transgenic plants expressing functional viral genes, and showing synergy, probably will not show the same behavior as transgenic plants expressing dysfunctional viral genes.

Even though CMV, PVY and TMV showed synergy with PVX (Table 1), none of the transgenic lines expressing CMV, PVY or TMV sequences tested here showed synergy with PVX. Thus, either viral sequences (genes) not represented in the tested transgenic plants, or combinations of viral sequences of the above viruses are required to promote synergy with PVX. In the case of PVY, a transgenic HC-Pro gene would be expected to promote synergy with PVX (Vance et al. 1995), while in the case of CMV the

2b gene (not tested) may be a possible candidate for promotion of synergy with PVX (Baulcombe, 1998). In the case of TMV, the coat protein gene was tested previously and shown not to promote synergy with PVX (Anderson et al. 1989*)*. Thus, it seems more likely that some combination of TMV sequences is required to promote synergy with PVX.

Detection of interference in transgenic tobacco

Among the 48 combinations of 12 transgenic lines infected by any of the four viruses, CMV, PVX, PVY or TMV, seven combinations showed interference of the virus by the transgene (Table 3). Five of the seven combinations again reflected transgenic lines that were not resistant to the homologous virus, and four of the lines showed reductions in virus accumulation only, with no change in the pathology induced by those viruses (Table 3). While only the TMV-54K transgenic line showed interference with the accumulation of TMV, in some experiments the transgenic line expressing the CMV-3a protein showed a delay in the rate of systemic infection by TMV and in the rate of cell-to-cell movement. However, the magnitude of this affect was not consistent between experiments (data not presented).

Table 3 Interference of virus infection in transgenic tobacco expressing viral transgenes

Transgene[a]	Virus	Interference[b]	Resistance[c]
CMV-3a	PVX	Pathology & Virus Level	No
TMV-30K	PVX	Pathology & Virus Level	No
TMV-54K	PVY	Virus Level	Yes
TMV-126K	PVX	Virus Level	No
TMV-126K	PVY	Virus Level	No
TMV-126K	CMV	Virus Level	No
PVY-NIb	PVX	Pathology & Virus Level	Yes

[a] Transgenic line expressing the corresponding viral gene; [b] Decrease in level of virus accumulation, with or without a decrease in the pathology; [c] Resistance of the transgenic line to the virus from which the transgene was derived.

Two transgenic lines gave resistance to one virus and either synergy (ΔCMVRNA-2 + PVY) or interference (TMV-54K + PVY) with another virus, while only one transgenic line (PVY-NIb) gave resistance to the homologous virus, interference with one unrelated virus (PVX), and synergy with another unrelated virus (TMV). Thus, a transgenic line expressing all three transgenes (Δ-CMVRNA-2, TMV-54K, and PVY-NIb) would be expected to show resistance to four viruses (CMV, PVX, PVY and TMV) and the effects of synergy expressed by individual virus-viral transgene combinations would be negated.

Discussion

Resistance versus interference versus synergy

To what extent is synergy a cause of concern for the use of transgenic plants expressing viral sequences to engender PDR? From the data presented here and from recent observations on the mechanism of synergy (Shi et al. 1997; Pruss et al. 1997), the risks appear relatively minor. Only a few genes were able to promote limited synergy in pathology, and there were no changes in the levels of virus accumulation. To prevent such effects, the use of particular transgenes could be avoided, dysfunctional variants of the genes could be used, or such genes could be combined with other transgenes of other viruses to inhibit the second virus involved in the synergy. Indeed, where multiple viruses infest a crop, resistance to one virus will not be sufficient to protect that crop, regardless of whether the introduced resistance is due to a gene derived from a related plant or a virus. Such plants are susceptible to non-target viruses, which will also cause loss in yields. Thus, pyramiding of resistance genes is essential in such plants to protect the crops from each of the viral pests. In fact, the unexpected interference observed between some transgenes and the accumulation (and pathology, in some instances) of non-target viruses is an additional benefit of the use of viral transgenes, which could reduce the number of transgenes required to obtain resistance to the range of viruses affecting a particular crop. Given the large number of viral-derived sequences that

will engender resistance to a given virus, different combinations of sequences from different viruses can be used to yield resistance to all of the viral pests of that crop. While such combinations may be selected to avoid synergy, if resistance is also engendered against heterologous (non-target) viruses showing synergy, then the latter could not establish an infection and would not show synergy.

Conventional versus pathogen-derived resistance

Conventional genes for virus resistance can operate by several different mechanisms (Fraser, 1990). However, few resistance genes operate by preventing the replication of the target virus. Rather, they tend to inhibit the cell-to-cell or long-distance movement of the virus, reduce the level of virus accumulation resulting in a reduced pathology (tolerance), or activate a defense mechanism restricting the virus to cells around the site of inoculation (hypersensitive response). However, it has been noted that in some cases co-infection by virus pairs involved in synergy can break conventional resistance targetted to one of the viruses (Anderson et al. 1996; Palukaitis and Kaplan, 1997). For example, tolerance to blackeye cowpea mosaic potyvirus did not prevent a synergistic reaction with CMV to induce cowpea stunt disease (Anderson et al. 1996), and resistance to CMV in cucumber was overcome by co-infection with zucchini yellow mosaic potyvirus, which interacts synergistically with CMV (Palukaitis and Kaplan, 1997). Thus, the use of single, conventional resistance genes does not necessarily provide a barrier to the effects of synergy. Moreover, in cases where conventional resistance genes are combined with cross-protection to control multiple virus resistance, if the cross-protecting virus is a potyvirus and is involved in synergy with the viral target of the conventional resistance gene, then a synergistic infection could occur. This would happen whenever infection occurs by the virus for which the plant is naturally resistant! Thus, care must be taken in combining certain resistance strategies.

Conclusions

The risk associated with synergy of infection in transgenic plants expressing viral sequences has been evaluated for 12 transgenic lines infected by four viruses. The biosafety results indicate that a low level of synergy can occur in some specific instances where resistance to only one viral pathogen is engineered into a crop that is a host for multiple virus. However, because some viral transgenes also showed interference with the accumulation (and often the pathology) of a non-target virus, the potential benefits outweigh the possible costs. In fact, if multiple transgenes from different viruses are co-expressed, the risks associated with synergy can be eliminated, which is not necessarily the case for using combinations of conventional resistance approaches. In fact, the use of selected, multiple, viral transgenes may have less risk associated with the problems of resistance-breakage and synergy than the use of combined conventional resistance strategies.

Acknowledgements

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Issues surrounding transgenic resistance to the *Luteoviridae*

Miller, W.A., Koev, G., Beckett, R.

Plant Pathology Department, 351 Bessey Hall, Iowa State University, Ames, IA 50011, USA

Abstract

The *Luteoviridae* family is among the most widespread and economically important groups of plant viruses. Field trials are only just beginning, so few biosafety data from field trials are available. Instead this talk discusses the current status of transgenic resistance to luteoviruses, potential risks, and predicts problems in durability of virus-derived transgenes. Constructs using the coat protein gene, or a pair of non-structural genes that includes the polymerase, have conferred resistance. Potential synergistic interactions between resistance transgenes derived from the *Luteoviridae* and certain invading, non-target viruses are a concern. Recombination clearly has occurred during recent luteovirus evolution, so the possibility of recombination between a transgene and an invading virus must be entertained. The large sequence variation within and between viruses in the *Luteoviridae* suggests that a given virus-derived transgene may not have sufficient homology to confer resistance to all isolates of a virus. Thus it is likely that resistance-breaking strains of *Luteoviridae* will arise. Finally, an example of pollination of a weed species by a transgenic luteovirus host indicates that pollen escape is possible. However, this risk is not peculiar to the *Luteoviridae*, rather it is a function of the host plant-weed combination. We conclude that the risks posed by transgenic resistance to *Luteoviridae* are small compared to the current situation in which these viruses run rampant. Any risks are more than offset by the benefits, which include reduced pesticide use and increased crop yield.

Introduction

Several members of the *Luteoviridae* family (formerly the luteovirus group) are of great economic importance. Examples include barley yellow dwarf (BYDV), cereal yellow dwarf virus (CYDV, formerly a strain of BYDV), potato leaf roll (PLRV), beet mild yellowing (BMYV), beet western yellows viruses (BWYV) and ground nut rosette assistor (GRAV) viruses (Miller, 1998). Annual yield losses to BYDV and CYDV amount to hundreds of millions of dollars in the wheat, barley and oat crops of North America. Annual losses valued as high as £13,000,000 occur in BMYV epidemics in the United Kingdom. The net necrosis in tubers caused by PLRV can render a potato crop virtually unmarketable. Disease caused by the complex of groundnut rosette virus and GRAV regularly devastates groundnut (peanut) crops in Africa. For these reasons, measures must be taken to control diseases caused by luteoviruses.

The *Luteoviridae* family fall into three genera: Luteovirus (formerly subgroup I), Polerovirus (formerly subgroup II) and Enamovirus (formerly Enamovirus group). With the exception of the Enamovirus genus, all of the *Luteoviridae* have a 5.7 kb positive sense genomic RNA, are confined to the phloem in the plant, and are obligately transmitted by aphids in a persistent, circulative manner. These latter properties are conferred by the genes that are homologous among all *Luteoviridae* (Figure 1):

- the coat protein (CP),
- an extended version of the CP that contains a translational read-through protein required for aphid transmission, and
- a gene contained within the CP coding sequence but in a different reading frame, which seems to be required for virus movement in the plant. The remaining portions of the Luteoviridae genomes are completely unrelated between genera..

Transgenic resistance to the *Luteoviridae*

- Current means of control are unsatisfactory. Substantial quantities of insecticides are used to control the vectors of *Luteoviridae*. Aphid migration forecasting can allow growers to time plantings so as to avoid severe infestation of young plants which are most vulnerable to disease (Harrington et al. 1989), but this is a major effort, often fraught with error. Natural disease resistance genes exist in

potato (Barker and Harrison, 1985) and barley (Ford et al. 1996), but many useful cultivars (e.g. Russet Burbank potatoes, and many malting barleys) lack resistance genes.

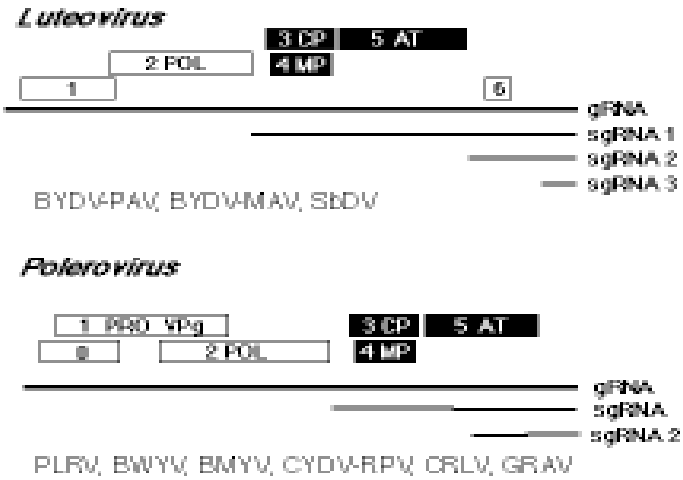

Figure 1 Genome organizations of *Luteoviridae*. Unshaded boxes: open reading frames (ORFs) with no homology to the other genus; shaded boxes: ORFs that have homology across all *Luteoviridae*. PRO, putative protease; VPg, genome-linked protein; POL, RNA-dependent RNA polymerase; CP, coat protein; AT, aphid transmission; MP, probable movement protein. Positions of subgenomic RNAs (sgRNA) are indicated below genomic RNA (bold line). Examples of members of each genus are listed.

Thus, many labs have strived toward transgenic resistance to BYDV, BWYV, BMYV, PLRV, GRAV and possibly other Luteoviruses. Several labs have constructed transgenic potatoes transformed with the coat protein genes (Barker et al. 1992; Kawchuk et al. 1990; Kawchuk et al. 1991; Van der Wilk et al. 1991). Upon inoculation with PLRV, these plants show reduced virus accumulation, reduced efficiency of aphid transmission, and slower spread of virus in the field (Thomas et al. 1997), compared to non-transgenic plants. A much higher level of resistance may have been achieved using viral replication genes (Kaniewski et al. 1994; Thomas et al. 1995). We have used this strategy to engineer resistance to barley yellow dwarf virus in oat (Koev et al. 1998). Infected transgenic plants harbor some virus, but they flower and produce seed, while the non-transgenic siblings die from virus infection before reaching 20 cm in height.

Synergistic interactions

Many of the *Luteoviridae* interact with other viruses in mixed infections to cause more severe symptoms than in single infections (reviewed by Miller et al. 1997). Examples include carrot red leaf polerovirus and carrot mottle Umbra-Virus, BYDV and CYDV (Figure 2A), beet western yellows virus and ST9-associated RNA. The BWYV-ST9-associated RNA interaction results in large increases in accumulation of both RNAs (Passmore et al. 1993). However, we found that, despite an increase in disease severity in wheat, barley and oats (Figure 2A), the levels of BYDV-PAV and CYDV-RPV RNAs in mixed infections were no higher than in infections by either virus alone (Figure 2B).

If a transgene from one virus (e.g. BYDV) contributed the synergistic enhancement of replication of the other virus (e.g. CYDV), the result would be a plant resistant to one virus (BYDV), but more susceptible to the other (CYDV). A non-luteoviral precedent exists. Transgenic plants expressing a potyviral gene synergistically enhanced replication and disease severity of potato virus X (Vance et al. 1995). In contrast, the transgene in oat (BYDV ORFs 1 and 2) had no positive (synergy) or negative (resistance)

effect on accumulation of CYDV RNA (Figure 2B). Thus, as yet, there is no evidence of synergy between transgenes and infecting viruses in the *Luteoviridae*.

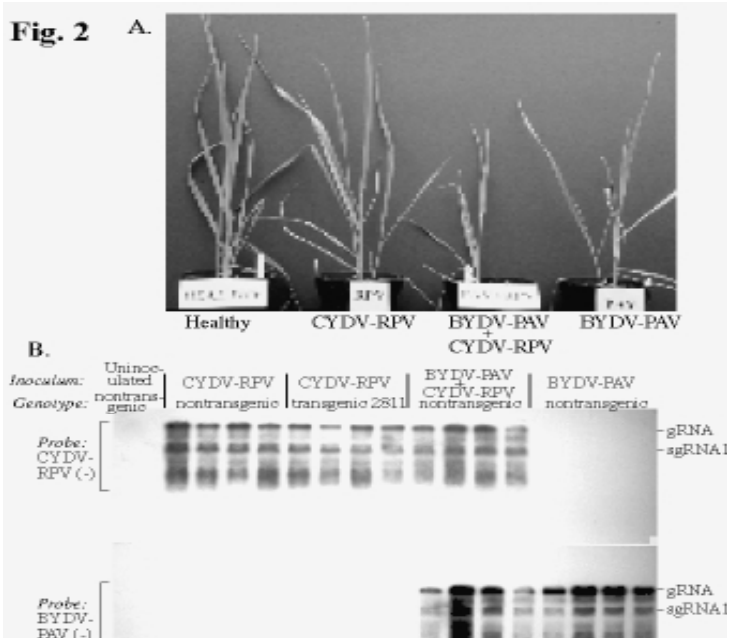

Figure 2 Interaction of BYDV (PAV strain) and CYDV (RPV strain) in oats.
A. Non-transgenic oats were aphid-inoculated with the indicated viruses. The mixed infection caused severe stunting and eventual plant death.
B. Northern blot of RNA from plants inoculated as indicated. Each lane represents a pool taken from several leaves from a single plant. Blots were probed with RNA complementary to the 3' end of virus indicated at left. While there was significant plant-to-plant variation in viral RNA levels, no clear pattern of increased RNA levels were seen in the mixed infection.

Risk of recombination?

It is clear that recombination has occurred relatively recently in the evolution of the *Luteoviridae*. The polymerases of the *Poleroviruses* and *Luteoviruses* have completely separate origins, yet open reading frames (ORFs) 3,4, and 5 are related among all of the *Luteoviridae*. ORFs 3, 4 and 5 are expressed from a sub-genomic RNA (sgRNA1) that is transcribed from the full-length genome (Figure 1). Because the initiation site of sgRNA1 is approximately at the site at which the *Luteoviridae* genera diverge, we proposed that recombination occurs occasionally during initiation of sub-genomic RNA synthesis (Miller et al. 1995; 1997). Also, there is clear evidence of recombination between the 5' end of the PLRV genome and a host chloroplast gene (Mayo and Jolly, 1991). Thus, probable sites of initiation of Luteovirus RNA synthesis should be avoided in construction of transgenic plants. These would include promoters for genomic and sub-genomic RNA synthesis. One example is the sgRNA1 promoter of BYDV-PAV which we have identified as the sequence complementary to bases 2595-2569 (G. Koev, unpublished).

Another factor which may increase risk of recombination, is expression of luteoviral transgenes in all cells of a plant with a constitutive promoter. This would allow luteoviral RNAs to accumulate outside the phloem. Because *Luteoviridae* are normally phloem-limited, this would give the RNAs opportunities to recombine with other viruses that infect mesophyll and other non-phloem cells, and which normally would have had few opportunities in evolution to encounter a luteoviral RNA. However, PLRV at least,

has been detected outside of the phloem in natural infections (Van den Heuvel et al. 1995), and other viruses may enter phloem. Thus, opportunities for recombination may occur naturally. Nevertheless, to reduce such a risk, use of phloem-specific promoters is desirable for *Luteoviridae* transgene expression.

Sequence variation

Virus-derived resistance transgenes may provide protection to only a limited number of field isolates, owing to the wide variation of sequences between genes of different strains. A certain amount of sequence similarity is necessary between the transgenes sequence and that of the virus against which it protects (Stark and Beachy, 1989). We have identified two new isolates of CYDV from Mexico and California that have serological and aphid transmission properties of CYDV-RPV (formerly known as BYDV-RPV). Indeed, the coat proteins share about 90% amino acid sequence similarity with the New York isolate of CYDV-RPV. However, the 5' regions are so divergent that probes from the 5' end of CYDV-RPV-NY RNA do not hybridize to the genomes of these new CYDV-RPV isolates (Miller and Rasochova, 1997).

In another example the coat proteins of BMYV and BWYV are 92% similar, but the polymerase, ORF1 and ORF0 proteins are only 63%, 34%, and 24%, respectively, similar in amino acid sequence (Guilley et al. 1995). Thus, the coat protein gene may confer resistance to both viruses, but the replicate or further upstream genes would not. Interestingly, another Polerovirus, cucurbit aphid-borne yellows virus (CABYV) is 68% and 64% homologous to the BMYV replicate and coat protein genes, respectively (Guilley et al. 1995). Furthermore, a new milder isolate of BMYV has been identified with a different host range, but identical RT-PCR RFLP patterns in the CP gene as BWYV (Stevens and Smith, 1996). This suggests variation within BMYV/BWYV strains that could pose problems for transgenic resistance.

In the *Luteovirus* genus, the CP, aphid transmission and movement proteins (ORFs 3, 4 and 5, respectively) of BYDV-MAV range from 65 to 73% identical to those of BYDV-PAV isolates (Ueng et al. 1992). ORFs 3, 4 and 5 are more than 85% identical among the BYDV-PAV isolates. In contrast, ORFs 1 and 2 of BYDV-MAV are more than 97% identical to those of all BYDV-PAV isolates except BYDV-PAV-129, which is only 88% identical to the others in ORF2 and 79% identical in ORF1. Thus, BYDV-PAV-129 diverges more from the other BYDV-PAV isolates in the 5' half of the genome than does BYDV-MAV. Here, polymerase-derived resistance from a common BYDV-PAV would confer resistance to BYDV-MAV but possibly not to BYDV-PAV-129, whereas CP-mediated resistance might be the other way around.

The main lesson from these observations is that sequence variation between homologous genes of strains of a virus or between viruses can be large and unpredictable. The amount of homology between genomes also varies hugely depending on which gene is compared. Thus, use of virus-derived transgenes may provide resistance to only a limited number of isolates of a given virus. Numerous isolates that differ substantially in sequence from the transgene may already exist in the field and readily break the resistance. This is especially likely for BYDV/CYDV and BMYV/BWYV viruses. This may actually decrease concern about new recombinants arising with transgenes. The harmful, fit recombinants that we worry about being generated with transgenes, already abound and are continually being generated by natural means. In contrast, because all known PLRV isolates from four continents are highly conserved (Keese et al. 1990), virus-derived resistance transgenes may provide a more durable resistance to PLRV. Although more divergent PLRV isolates may be out there, awaiting discovery.

Pollen escape

A concern for all transgenic plants is whether pollen may escape from the transgenic plant to fertilize a weed of a related species. This has been discussed for several *Luteoviridae* hosts by Miller et al. (1997). We tested the ability of cultivated transgenic oat (*Avena sativa)* to pollinate the closely related noxious weed, wild oat, (*A. fatua)* by manually pollinating emasculated *A. fatua* plants with pollen from transgenic oat. Seed set was very inefficient. Only two seeds were obtained from 15 plants. However, they both yielded fertile hybrid, transgenic plants (Figure 3). The Southern blots reveal complex banding patterns that are identical in parental and hybrid progeny genomes. All of the bands in all oat transformants segregated as a single transgene (Koev et al. 1998). Whether such an outcrossing event is likely to occur in the field in this self-pollinating species is unknown, but the data shows there are no physiological barriers.

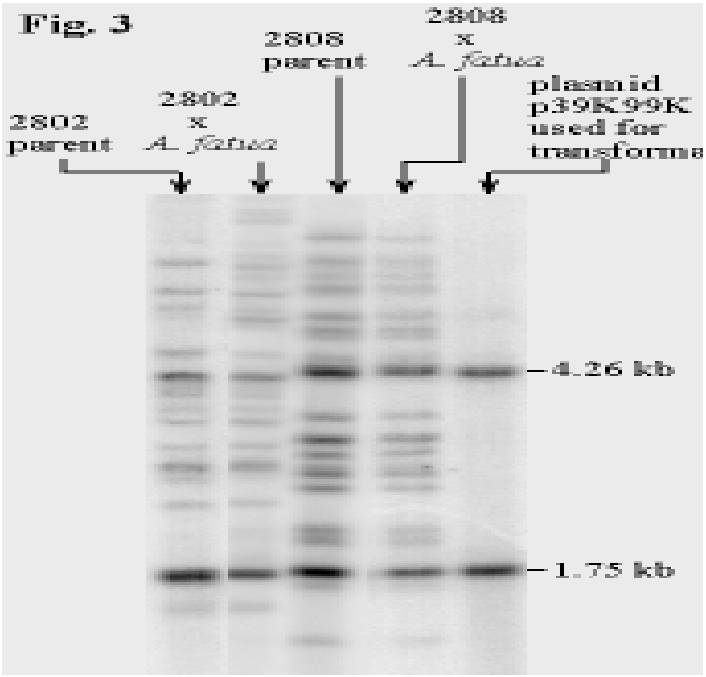

Figure 3 Southern blot of DNA from transgenic *A. sativa* plants (lines 2802, 2808) and from hybrid progeny of *A. sativa* x *A. fatua* crosses. Plants were transformed with the BYDV-PAV sequence spanning the 5' half of the genome comprising ORFs 1 and 2 (Koev et al. 1998). The virus insert from this plasmid, p35S39K60K, was used as probe. Expected sizes of inserts are at right. Other bands are stably inherited rearrangements that occur during particle bombardment. *A. fatua* were emasculated and pollinated manually with pollen from the transgenic *A. sativa*. Two seeds were obtained from 15 plants.

Summary

It is too early to draw conclusions about the risks of transgenic resistance to luteoviruses. We are aware of published reports of transgenic resistance only against PLRV and BYDV. Years of close monitoring of field trials and commercially released cultivars will be necessary to determine whether any new recombinants, synergistic interactions, or resistance-breaking virus strains arise. The costs and benefits to the agro-ecosystem must be monitored over the years. We strongly suspect that the benefits will more than justify the perceived risks.

Certainly we must move ahead with transgenic approaches. Pesticides currently used to control *Luteoviridae* are expensive and harmful to desirable insects, such as ladybird beetles, and to fish and wildlife (Flickinger et al. 1991). Thus resistance approaches are preferred. Conventional breeding seems to lack adequate sources of resistance. The first wave of transgenic resistance will use virus-derived transgenes, and initial results look promising. However, owing to perceived risk of recombination, possible synergistic interactions, and the much more likely risk of resistance-breaking strains of viruses arising, it is worth pursuing other types of transgenic resistance. These could include transferring natural resistance genes between species by transgenic methods, use of broad-spectrum antiviral genes (Lodge et al. 1993; Masuta et al. 1995; Watanabe et al. 1995), or engineering aphid resistance genes (Hilder et al. 1995).

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Pathogen induced transgene instability in *Brassica napus*

Dale, P., Covey, S., Kreike, M., Page, A., Pinder, R., Al-Kaff, N.

John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Abstract

Transgene and resident gene expression can be influenced by interactions between homologous DNA sequences in plants. There is interaction between different transgenes and between resident genes and transgenes. DNA sequences from the pathogenic cauliflower mosaic virus (CaMV) have been found to interact with the CaMV *35S* promoter and terminator sequences used to control transgene expression. *Brassica napus* plants infected with CaMV and containing the *35S* sequences to regulate transgene expression, display transgene silencing. The nature of the pathogen induced instability is influenced by the plant species, viral isolate and environmental factors. Various experiments are underway to determine the significance of this phenomenon for biosafety and the utility of the *35S* regulatory sequences in transgenic plants.

The importance of transgene expression and stability

A better understanding of what affects the way transgenes work is important for the following reasons:

- to determine their utility and usefulness in agricultural crops
- to enable the biosafety assessment process to be better informed scientifically and
- to improve our basic understanding of the underlying principles governing the action and interaction of all genes.

DNA homology interactions

With our developing experience of transgene genetics, it has become apparent that there are various interactions between homologous DNA sequences within plants. Homology between transgenes and endogenous genes can lead to cosuppression of both types of genes, and is now a widely used in sense and anti-sense suppression of gene action (e.g. modified ripening of tomato). The variation in expression levels between plants carrying different numbers of copies of a transgene construct is probably also influenced by the presence of multiple areas of DNA homology, although there are likely to be other factors involved (e.g. position effects). The multiple use of particular promoters can also be associated with silencing of promoter function (e.g. promoter methylation). For relevant references, see Meyer (1995).

DNA homology between a transgene construct and plant pathogen

To further develop our understanding of the principles governing the expression of transgenes, and the role of DNA homology in that process, we explored the interaction between transgene constructs and homologous DNA sequences from the DNA virus, cauliflower mosaic virus (CaMV). The *35S* promoter and terminator sequences from CaMV are widely used in transgene constructs, to genetically modify crop plants. Unlike plants containing multiple transgenes, where there might be 2-10 transgene copies in a plant, a *Brassica* plant infected with CaMV can contain up to 100,000 viral particles in each infected cell (Maule et al. 1983). Because the infecting virus generates many copies of the *35S* regulatory sequences, and carries homology to the *35S* genetic control sequences in the transgene construct, we expected that it would display homology related DNA interactions between virus and transgene.

The complete data have been presented in Al-Kaff et al. (1998a) and Al-Kaff et al. (1998b). The observations are summarised as follows:

1. Infection of transgenic oilseed rape with CaMV causes transgene instability,
   a) with the *35S* promoter, transcriptional silencing
   b) with the *35S* terminator, posttranscriptional silencing
   c) with the *nos* promoter (no homology to CaMV), no transgene silencing, but evidence of enhanced transgene expression.

2. The precise nature of the interaction is influenced by the plant species, viral isolate and the environment.

Significance of this observation?

The consequences of these observations for the development of transgenic plants containing *35S* regulatory sequences, and indeed the significance of other transgene - pathogen interactions are currently the subject of intensive investigation. The phenomenon raises a number of important questions about the *35S* promoter in particular, which is present in a high proportion of all transgenic plants (80%?). Some obvious questions, and brief comments are as follows:

1. How common is CaMV infection in oilseed rape crops in agricultural production? Hardwick et al. (1994) in a survey of UK winter oilseed rape crops in 1992 found 14% of crops contained CaMV with 5% of plants infected, and in 1993 they found 25% of crops contained CaMV with 7% of plants infected. The ELISA method of virus detection was used.
2. How will the transgene silencing affect agriculturally important transgene characters, including herbicide tolerance? Most of the studies so far have been on transgene expression at the molecular level. Studies are currently aimed at understanding its effect on the whole plant phenotype.
3. Is it a phenomenon unusual to CaMV infection of *Brassica* species? Again this is the subject of further study. It is known that *Brassica* species do have a gene silencing mechanism that inactivates CaMV infection (Covey et al. 1997) and functions as a viral defence mechanism.

Conclusions

More studies need to be carried out into the consequences of pathogen induced transgene instability, for biosafety assessment. The impact of transgene instability, especially silencing of transgene expression, will depend principally on the nature and function of the transgene(s) involved. If herbicide tolerance transgenes in oilseed rape become ineffective because of transgene silencing, then the crop concerned, or more specifically individual plants within a crop, will become herbicide susceptible. This is unlikely to have a significant impact on biosafety, but may influence agronomic management. However, if the function of a transgene is to down-regulate an undesirable plant compound, such as a toxin or allergen, then pathogen induced instability of that process would need to be an important feature in any biosafety assessment before marketing approval.

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Resistance management strategies for *Bt* toxin transgenic plants

Siegfried, B. (Moderator)

Dept. of Entomology, University of Nebraska, USA

The workshop to address resistance management strategies for *Bt* toxin transgenic plants was comprised of speakers representing industry, academia and governmental research organizations. The presentations encompassed a discussion of research efforts to develop resistance management strategies for the three major crop plants (maize, cotton, and potatoes) that have been transformed to express the *Bt* toxin.

Common themes

A number of common themes were discussed by a number of presenters in the workshop. Perhaps the most prevalent was a recognition that resistance management of transgenic *Bt* plants is a necessary component of product stewardship. The advantages of *Bt* transgenic plants over more traditional pest control options were documented by a number of speakers and included both economic benefits to growers as well as environmental benefits that would result from minimizing the use of more environmentally disruptive synthetic chemicals. Without a proactive stance on resistance management, however, it is likely that large-scale introduction of *Bt* transgenic plants will rapidly lead to the development of resistance to *Bt* within pest populations. The consequences of not employing sound resistance management strategies were illustrated in David Ferro's (University of Massachusetts) discussion of Colorado potato beetle resistance to all synthetic, broad spectrum insecticides registered by the US Environmental Protection Agency for its control.

A second common theme relates to the high-dose refuge concept for resistance management. This strategy was illustrated by Richard Hellmich, USDA Corn Insects and Genetics Research Unit. The concept is dependent on high levels of *Bt* expression in the transgenic plants such that all susceptible and most, if not all, insects that carry one copy of a resistance gene (i.e., heterozygotes) are killed. A high-dose strategy alone would exert an extremely high selection pressure for resistance, and therefore, the high dose must be deployed in conjunction with a refuge. The refuge provides unselected susceptible insects that mate with rare resistant individuals that survive exposure to the *Bt* plant. This dilutes the resistant genes by insuring that resistant homozygotes only mate with susceptible homozygotes. The progeny from this mating would be heterozygous and because of the high dose of toxin, the resistance allele would be functionally recessive.

Although the high-dose refuge strategy was widely recognized as the primary resistance management strategy by most workshop participants, Shi-Rong Jia (Biotechnology Research Center, Chinese Academy of Agricultural Sciences) indicated that for transgenic *Bt* cotton in China, there were sufficient alternative host plants for the primary pest species, *Helicoverpa armigera*, that a managed refuge would not be necessary.

Another common component of resistance management for transgenic *Bt* plants involved the development of plant cultivars with *Bt* toxins that affect independent target sites. Sue MacIntosh of Plant Genetic Systems disused the development of a *Bt* corn product that expresses the Cry 9c gene which has a distinct site of action relative to other *Bt* toxins currently available in other transgenic maize varieties. Although the mode of action of the Cry 9c protein is very similar to the CryIA proteins, Cry 9c binds a different site in the midgut of the target insect. The availability of multiple *Bt* toxins with independent targets offers the opportunity to pyramid two insecticidal proteins in the same plant which has been proposed as a second approach to managing resistance in conjunction with the high-dose refuge. Shi-Rong Jia tested a similar approach in transgenic tobacco which combined a *Bt* gene with CpTI gene and concluded that the two genes will delay resistance development relative to the single *Bt* gene.

Other necessary components of resistance management strategies discussed by the workshop participants included a recognition that cooperation among various stakeholders in the technology, including growers, industry and regulatory agencies was critical to the successful implementation and adoption of any resistance management program. The effort on the part of a USDA sponsored regional project (NC-205) was cited by both William Hutchison (University of Minnesota, Department of Entomology) and Richard Hellmich (USDA-ARS, Corn Insects and Crop Genetics Research Unit) as an important example of such

cooperation. Additionally, a strong educational component that makes growers aware of the consequences of non compliance with resistance management programs was also considered important. Finally, it was recognized that because of information gaps in current understanding of RM theory, programs must be flexible in order to incorporate new information as it becomes available.

Uncertainties

In addition to a general recognition of the essential components necessary to establishing sound resistance management strategies, there were also a number of uncertainties identified that could have important consequences to the success of such programs. Refining these areas will require further research, the results of which could potentially impact present and future resistance management programs.

Perhaps the most critical area of uncertainty revolves around the size and placement of refuges that are necessary to provide enough homozygous susceptible individuals to dilute resistance genes and maintain susceptibility of exposed populations. A number of speakers including William Hutchison, Richard Hellmich and Graham Head (Monsanto Company) discussed the importance of mathematical models to make predictions about refuge size and also the parameters that are used to derive estimates for refuge size based on these models. Many of these parameters such as the degree of random mating, initial frequency of resistance alleles, and fitness of heterozygotes can have profound effects on the model outcome. Providing accurate estimates for these parameters is essential to establishing effective resistance management strategies. Graham Head also presented data indicating that the willingness of growers to accept the refuge concept could be affected by recommendations about size, and emphasized the importance of obtaining grower acceptance in order for the refuge to have a positive impact on resistance management.

A second area of uncertainty identified by a number of speakers concerned the definition of high-dose and methods used to evaluate new *Bt* products to insure that the plants were expressing a high dose. Products that do not satisfy the high-dose definition could potentially increase the fitness of heterozygotes resulting in increased rates of resistance development. However, without strains of insects resistant to *Bt* it is not possible to determine whether a given *Bt* product is capable of causing mortality to heterozygotes.

The topic of resistance monitoring was recognized by a number of speakers as an important component of resistance management programs although the exact nature of the monitoring programs that would provide enough sensitivity to detect small changes in resistance frequencies remains uncertain.

In addition to uncertainties that directly affect the success of resistance management programs, Angelika Hilbeck (Swiss Federal Research, Station for Agroecology and Agriculture) presented results indicating that insects which consume *Bt* toxin may adversely affect predatory insects which utilize them as prey. Her discussion also raised issues concerning the large-scale utilization of insecticidal *Bt* plants on secondary pest species either due to release from competition by the target pest or by eliminating exposure to previously used synthetic pesticides.

Conclusions

Despite the uncertainties associated with establishing resistance management strategies for *Bt* transgenic crop plants, there was general agreement on the methods necessary to initiate programs that will delay the onset of resistance in target pests. These programs must remain flexible in order to incorporate new information as it becomes available and require input from all stakeholders to insure their success.

Establishing research priorities for managing insect resistance to transgenic corn

Hellmich, R.L.[1], Siegfried, B.[2]

[1] USDA-ARS, Corn Insects & Plant Genetics Research Unit, and Department of Entomology, Iowa State University, Ames, IA 50011, USA;
[2] Department of Entomology, University of Nebraska, Lincoln, NE 68583, USA

Summary

Managing resistance of European corn borer, *Ostrinia nubilalis* (Hübner), to *Bt* corn is necessary because *Bt* corn is valuable and all stakeholders want to preserve its efficacy. Currently, the resistance management strategy of choice has two components high-dose and refuge. There are, however, potential problems with this strategy. Research is underway to try to better define these problems and to offer solutions. A resistance management forum, like the NC-205 Resistance Management meeting, greatly facilitates cooperation among all stakeholders and expedites solutions to possible problems. Genetic factors, such as frequency of resistance alleles, number of resistance genes, functional dominance of resistance genes, and fitness of resistant insects, are not known. These are high priority research areas. Developing efficient techniques to monitor for resistance also is a high priority. Biological factors identified as high research priorities include larval movement, adult mating, migration, and refuge.

Introduction

European corn borer is a serious pest in the Corn Belt that is second only to the rootworm complex, *Diabrotica* spp., for numbers of corn hectares treated with insecticides. In Iowa untreated *O. nubilalis* larvae cause average losses of 13 bushels per acre in both first and second generations (Ostlie et al. 1997). Scouting and properly timed applications of chemical or biological insecticides can be effectively used to manage first generation *O. nubilalis* larvae. There are no economically effective means, however, for managing the second generation of this pest; insecticide applications are difficult to time because of a prolonged oviposition period (Mason et al. 1996).

Corn hybrids expressing an endotoxin from a soil bacterium, *Bacillus thuringiensis* (Berliner) (*Bt*), were sold commercially for the first time in 1996. *Bt* corn represents a significant departure from traditional control procedures because these plants produce insecticidal proteins throughout the growing season. *Bacillus thuringiensis* has been used for decades as a biological insecticide for the control of lepidopteran, coleopteran, and dipteran pests. *Bt* is environmentally friendly because it breaks down rapidly and has no effects on mammals, birds, aquatic life, or beneficial insects.

Before retiring, USDA research entomologist Dr. William B. Showers suggested that most producers do not realize how much yield corn borers steal from them. He called them “silent thieves”. He predicted that once producers realize that corn borers reduce yields 10% or more, they would switch to *Bt* corn in high numbers. His predictions apparently are becoming true.

Yield protection advantages from *Bt* corn indeed are very impressive. In Iowa yield protection from European corn borer injury commonly ranges from 5 to 15%. In Kansas in areas where European corn borer and southwestern corn borer occur together the yield protection can exceed 25% or more (Buschman et al. 1997). In 1998 approximately 10% of the corn planted in the USA was *Bt* corn. These numbers could double in 1999. Widespread use and the dramatic control of corn borer by *Bt* corn has many scientists concerned about high selection pressure from *Bt* toxins and the subsequent adaptation by pest insects to these toxins.

Cry proteins and *Bt* corn

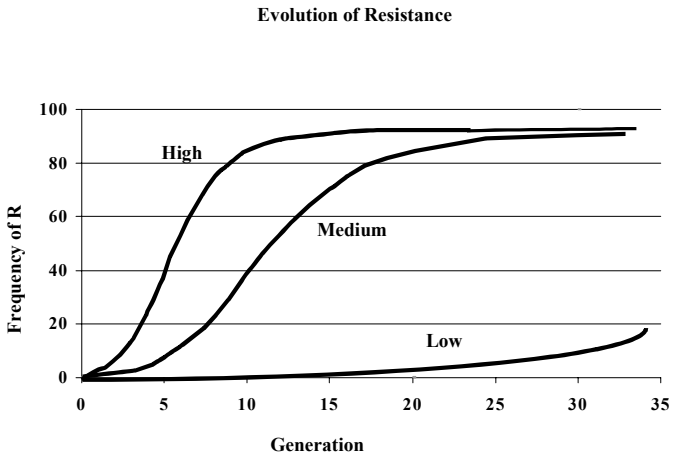
Bt corn hybrids produce insecticidal crystalline proteins, called Cry proteins, which are specific for lepidoptera. Once ingested, the Cry protein dissolves in the alkaline midgut of a larva and is proteolytically activated into a toxin. The toxins cross the peritrophic membrane and bind to receptors on the midgut epithelium. This leads to pore formation, cell lysis, and death within 48 hours from septicemia.

Bt corn is made by inserting *cry* genes into the corn genome through various transformation techniques. Successful transformations, called events, are carefully screened for *Bt* production and agronomic traits. The five commercially available events are shown in the table below. Endotoxins expressed in the transgenic plants include Cry1Ab, Cry1Ac (both derived from *B. thuringiensis* subsp. *kurstaki*), and Cry9C (derived from *B. thuringiensis* subsp. *tolworthi*). The *Bt* hybrids have two expression profiles based on gene promoters. Plants with the cauliflower mosaic virus (CaMV) 35S promoter express endotoxins throughout the entire plant, whereas those with the combination of the maize phosphoenolpyruvate carboxylase (PEPC) and a maize pollen specific promoter express in green tissue and pollen.

					***O. nubilalis* control**	
Event	**Registration**	**Protein**	**Promoter**	**Company**	**1st Gen.**	**2nd Gen.**
176	Aug-95	Cry1Ab	PEPC	Mycogen/Novartis	Excellent	Good
*BT*11	Aug-96	Cry1Ab	CaMV/35S	Monsanto/Novartis	Excellent	Excellent
MON810	Dec-96	Cry1Ab	CaMV/35S	Monsanto	Excellent	Excellent
D*BT*418	Mar-97	Cry1Ac	CaMV/35S	DeKalb Genetics	Excellent	Good
CBH351	Apr-98	Cry9C	CaMV/35S	AgrEvo	Excellent	Excellent

Resistance risks

Most corn borers that feed on *Bt* corn die, but potentially there are individuals with resistant genotypes (one or a number of genes) that will survive. There are a wide variety of mechanisms responsible for modifying the reproductive success of a genotype which collectively are called selection.



Insect geneticists have used simple two allele models, based on Hardy-Weinberg principles, as tools to gain information about selection for resistance (Gould, 1986). These models suggest that widespread use of *Bt* corn could result in a very high selection pressure for a resistant population of insects. Under some scenarios resistance could occur within 5-10 generations. The figure shows high, medium and low levels of selection. All *Bt* corn stakeholders want to avoid the high selection pressure conditions and delay resistance as long as possible. The resistance management strategy that is currently recommended for delaying insect resistance to transgenic plants has two components high dose and refuge (Roush, 1998). Companies that provide the various *Bt* corn events, through technology and careful screening, have

developed *Bt* corn plants that produce extremely high levels of *Bt* toxin. A proposed definition of high dose is a toxin titre that is 25 times the concentration needed to kill susceptible larvae.

The most important objective of high-dose strategy is to kill all susceptible insects and most, if not all, insects that carry one copy of a resistance gene, that is, any heterozygous individuals. Hardy-Weinberg theory indicates that heterozygous individuals carry the majority of resistance genes. Thus, survival of even a low percentage of heterozygous insects compromises the high-dose strategy and greatly accelerates selection for resistant insects.

A high-dose strategy alone would be disastrous because it would exert an extremely high selection pressure for resistance. This is the reason the high-dose strategy is complemented with a refuge strategy. The refuge provides unselected susceptible insects that mate with rare resistant insects. This in effect dilutes the resistance genes. There should be an overwhelming number of susceptible insects from refuge corn that will mate with potential resistant moths. Currently, at least a 500 to 1 ratio of susceptible to resistant moths is recommended. Sometimes it is hard to convince growers that a refuge strategy is needed. They have been told for years by entomologists that they should kill insects. Now they are told to keep some of the insects alive. The talk thus far has set the stage. *Bt* corn has many benefits - environmental benefits, yield protection and more. All stakeholders want to keep it as a viable product for as long as possible. Selection for resistant individuals could be very high. A high-dose/refuge strategy is recommended for managing insect resistance development. Now the NC-205 research committee and its role in resistance management is discussed.

Resistance management forum

Scientists involved with insect resistance management know that there are more research possibilities than there are funds. Sometimes it is difficult to choose which types of research should be conducted. Producers emphasize research that increases income; industry emphasizes research that increases product value; academics emphasize research that results in publications. Often such research priorities do not overlap. A Regional Research Committee has tried to build bridges between industry, producers, academics and regulators. The NC-205 committee formally addresses research on the "Ecology and Management of European Corn Borer and Other Stalk-Boring Lepidoptera." For the past three years this committee has sponsored five meetings with industry and the Environmental Protection Agency (EPA) to discuss resistance management issues. The committee also has sponsored a number of symposia and conferences.

The NC-205 Resistance Management meeting provides a forum for all parties to discuss general and specific issues concerning managing the resistance of European corn borer. The meetings provide opportunities for sharing information, fine-tuning programs, establishing research priorities, reducing redundancies, and building trust among all participants. Topics discussed include monitoring for insect resistance, education, extension, grower surveys, managing resistance of other insects (especially rootworm), ongoing research, and future needs for research. In 1997 NC-205 met with the Rootworm Technical Committee (NCR-46) in order to lay the foundation for programs that consider resistance management for both European corn borer and the rootworm complex. The NC-205 Resistance Management meetings were attended by university researchers and extension specialists from 20 states, including each state in the corn belt, representatives from government agencies (Agricultural Research Service, Extension and Education Service (CSREES), and EPA), seed corn industry, and gene suppliers (DeKalb, Dow Elanco, ICI-Garst, Monsanto, Mycogen, Novartis, Pioneer, and AgrEvo), and crop consultants. The first product from these meetings is a NC-205 resistance management extension publication (NCR 602) entitled "*Bt* Corn & European Corn Borer: Long-term success through resistance management". Novartis Seeds has adopted the resistance management plans recommended in this publication.

Research priorities

The most controversial recommendation made by the NC-205 committee involves refuge amount. Presently the committee recommends 20-30% unsprayed refuge or 40% sprayed refuge. These numbers are considered too conservative by some industry representatives and not conservative enough by several academics. There are many different ways to assess refuge values, and all of them rely on imperfect data. Sensitivity analyses of models, however, suggest that genetic variables that especially influence refuge

size include gene frequency, number of resistance genes, functional dominance, and fitness of resistant insects (Hurley et al. 1998). Refuge recommendations can change drastically when any of these factors or combination of these factors are modified. In most cases there is not sufficient data to reliably estimate values for these variables. As of September 1998 there are no known cases of European corn borer resistance to *Bt* corn. Thus, information on gene number, functional dominance, and fitness is not available. Obviously there are research needs in this area. Researchers from Kansas, Minnesota, Iowa, and Delaware are conducting genetic studies to try to fill these gaps. Researchers in Nebraska and Minnesota are developing more efficient methods for monitoring for *O. nubilalis* resistance.

Members and collaborators of NC-205 also have focused on biological factors that influence resistance management of the European corn borer. Researchers from Iowa, Delaware, New York, and Kansas are investigating non-corn sources of refuge. The value of non-corn refuge sources could vary among different regions of the USA. This could have important implications for refuge amounts required in different corn growing regions.

Researchers from Nebraska and Iowa are investigating movement of late instars from non-transgenic plants to *Bt* corn. This research is important because late-instar tolerance for high levels could compromise the high-dose strategy. Research is ongoing in Nebraska and Pennsylvania to determine how far moths move before they mate. This research is necessary to determine how close refuge must be planted to *Bt* corn to promote random mating of susceptible and resistant moths. Related to this, researchers in Kansas, Iowa, Illinois, and Minnesota are investigating planting schemes that will allow farmers to promote random mating and at the same time be practical for the farmers. Population genetic studies are underway in Minnesota to measure European corn borer migration rates. This research would help predict how fast resistant populations of corn borers would spread, if resistance should develop.

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A science-based development of a resistance management strategy

MacIntosh, S.

AgrEvo USA Company, Des Moines, Iowa

Abstract

Using the new product, StarLink™ corn hybrids, it will be demonstrated how one company, Plant Genetic Systems (now a company of AgrEvo GmbH), made a strategic decision to provide a new insect resistance management (IRM) option for growers. StarLink™ corn expresses a new protein, a unique *Bt* Cry9C protein, which has a different binding site than the popular Cry1A proteins. The new trait is biologically based with environmentally friendly qualities. These new corn hybrids are being introduced with a detailed IRM plan that incorporates the latest scientific data. Pyramiding of two insecticidal proteins is now a viable option with a new protein, *Bt* Cry9C, that can be combined with a Cry1A protein. StarLink™ corn hybrids provide excellent efficacy and reliability which protects the yield potential of the corn crop and ultimately a great value to the grower.

Introduction

Several different topics will be presented, first a discussion on both microbial *Bt* and pesticidal plants and the advantages and/or disadvantages of moving from one technology to the other. A general overview of *Bt* crops resistance issues will be provided followed by a focused discussion on a new *Bt* corn product known as StarLink™ corn hybrids, a product that incorporates a new *Bt* Cry9C protein (Lambert et al. 1996). The important differences between the well-known Cry1A proteins and this new Cry9C protein will be highlighted especially as it relates to IRM. Emphasis will be made on the long-term view to maintain this technology, while balancing a grower's perspective of short-term needs. Overall the goal must be to preserve insect susceptibility forever.

Bacillus thuringiensis-based product

Bacillus thuringiensis has proved to be the most successful organism for biological control (Marrone and MacIntosh, 1993; Adams et al. 1994). *Bt*, as it is often known, has multiple insecticidal crystals which are packaged within a bacterial crystal. These proteins display a rather narrow insect activity range, yet this narrow range is quite important from an environmental safety standpoint (Adams et al. 1994; Höfte and Whiteley, 1989). In addition, the short residual activity of Cry proteins, which quickly degrade in nature, is a quality regardless of whether the proteins are used in a formulated product or whether they are present in a *Bt* crop that has been incorporated into the soil at the end of a growing season. *Bt* strains have a long history of safe use. For more than thirty years, the *Bt kurstaki* strain has been commercialized and used throughout the world. And yet, despite that long history, there have been only a few examples of insect resistance (Marrone and MacIntosh, 1993; Adams et al. 1994). In fact, many scientists once believed that *Bt* sprays would never display insect resistance, but, obviously, that proved not to be the case. This simple fact that biological substances can cause the evolution of insect resistance holds some very important lessons for how insecticidal products should be used, regardless of the biological or chemical nature of the substance.

Sprayable *Bt*-based products also have some disadvantages (Adams et al. 1994). Due to the short residual activity, there is often a lack of consistent efficacy. If the product is sprayed at the right time, in the right weather, under the right conditions, product performance is excellent. But often the result is poor efficacy. Furthermore, these products are costly when compared to many synthetic insecticides.

Resistance to *Bt*-based formulated products

The resistance history associated with *Bt* products has been rather short. It was in the early 1990's that the diamondback moth, *Plutella xylostella*, was found to have resistance towards *Bt kurstaki* in Hawaii (Tabashnik et al. 1990). This is the only insect to date that has displayed any resistance to sprayable *Bt* products (Adams et al. 1994; Tabashnik et al. 1997). The resistance resulted from extreme overuse of the product. In Hawaii, for example, watercress fields were sprayed twice weekly for four years before resistance was discovered.

Transgenic *Bt* crops

Bt-based crops have a number of improvements over *Bt* sprays (Peferoen, 1997). The insecticide is found within plant cells; therefore, insect behavior is not a factor. For example, *Ostrinia nubilalis*, the European corn borer, has a tendency to tunnel into the crop quite quickly. With the *Bt* protein expressed in all plant cells, this tunneling has no impact since the insects are exposed to the insecticide no matter what tissues are initially fed upon. Insect control is found throughout the plant by use of specific promoters, or the expression of *Bt* protein can be targeted into the most valuable tissues using tissue specific promoters.

Safety features of plant pesticides are many. There has been an overall decreased use of synthetic pesticides, reducing the environmental issues surrounding these pesticides. The targeted activity of *Bt* protein causes little or no effects on beneficial insects (EPA, 1988). Transgenic plants offer new options for growers, the possibility to utilize these tools in an integrated pest management system, and, more specifically, in an IRM system.

Resistance management for *Bt*-based crops

Resistance management is not new to industry; in fact, industry has been extremely proactive with regard to *Bt*-based products. The *Bt* Management Working Group was established just 10 years ago, in 1988 (Marrone and MacIntosh, 1993). This Working Group consisted of a group of companies that were involved in both formulated sprayable products and transgenic *Bt* products. Well over $400,000 was spent on basic and applied research focused on a better understanding of resistance development and means to delay the evolution of resistance to *Bt*-based products. The Insect Resistance Action Committee, also established in the late 1980s, focuses more on chemical pesticides, but is also keen to preserve all pesticidal technologies by delaying resistance development through funding basic and applied research. One of the more recent groups working on IRM is the Health and Environmental Safety Institute, which has convened an expert panel on *Bt* IRM. This group first met last fall and will have their work product available by the end of 1998.

As previously stated, one of the major challenges for pesticidal plant products could be pest adaptation, and for that reason practical IRM plans must be developed. An IRM plan must be scientifically valid, but also contain practical aspects in order to gain acceptance by the grower. Another challenge is the recognition that pesticidal plants do not eliminate all pesticides but instead focus the use towards fewer applications of safer pesticides. Probably the greatest challenge is grower education, which is critical for the success of this technology. Since growers are the ones implementing this new technology they must be highly informed to understand not only the threat of insect resistance but how best to delay its onset. IRM issues are diverse, below the most crucial aspects are highlighted.

The biology of the pest-crop interaction is critical. The different issues for each combination of a pest and a crop will demand specific precautions. The biology of the pest-crop interaction will dictate how a resistance management plan is implemented. Although there are practical aspects, the decisions must be based on the latest and best scientific data. Choosing the right resistance strategy and the right strategy deployment is the foundation of IRM (Marrone and MacIntosh, 1993). The best scientific evidence for the protection of *Bt* crop technology has focused on a high dose-refugia strategy (Peferoen, 1997; Mellon and Rissler, 1998; Roush, 1994; Gould, 1998). High insecticidal dose is a feature of nearly all *Bt* crops on the market today. The goal is to kill all heterozygous resistant insects, which would be the major carriers of resistance genes (Roush, 1994; Gould, 1998). The unsprayed non-*Bt* crop refuge should provide sufficient numbers of susceptible insects so that they will mate with those rare resistant individuals that potentially survive the *Bt* crop. Obviously, this crop refuge must be placed close to the *Bt* crop for random mating to occur at a high frequency. And, in addition to both of these aspects, other management practices should be encouraged that would reduce selection pressure on the target pest population. How these strategies are deployed and how educational information is shared with growers and other stakeholders will be critical for success. Strict grower compliance of resistance management strategies is required by the EPA, if good faith efforts are not made, the agency may revoke a product's registration.

Monitoring plans are another aspect of IRM. Usually monitoring is approached from two directions: Assessing unexpected plant damage and targeted insect population sampling.

For the future, research is broadly applied and covers all aspects discussed in this section. Probably the most pressing area of research is in new toxin discovery to allow insecticidal protein pyramiding, which according to experts, appears to be the best long-term option for resistance management (Roush, 1994; Gould, 1998).

Pyramiding insecticidal proteins

All experts acknowledge that putting two insecticidal proteins in the same plant is a far superior method of resistance management than single protein crop plants (Roush, 1994; Gould, 1998). These two insecticides must have distinct modes-of-action or sites-of-action to utilize the theory that is referred to as redundant killing. The theory of "killing" an insect twice, from two different toxic mechanisms contained within the same plant, greatly reduces the chance of the insect overcoming the activity of both toxins and becoming resistant. For *Bt* crops the discussion focuses on distinct insect midgut binding sites - if one binding site is altered, then the other binding site remains functional. A high dose for both proteins is still required and that causes somewhat of a challenge for measuring these proteins. Immunoassay data, as from an ELISA, should be sufficient when combined with bioassay data to establish a high dose for both proteins. One of the advantages to the pyramiding toxin strategy is that the refuge size can be reduced, possibly by 4-5 fold, and still maintain a long-term product life according to computer simulated models (Roush, 1994).

To illustrate what is meant by binding sites and Cry protein interaction with these binding sites, a review of the binding site complexity of the *Heliothis virescens*, a major pest of cotton, is provided in Figure 1. This work was done almost ten years ago by researchers at Plant Genetic Systems (Van Rie et al. 1989). In *Heliothis virescens*, there are three different populations of binding sites for the Cry1A proteins. The Cry1Ac protein binds to all three of these sites, Cry1Ab binds two of the sites, and the Cry1Aa protein binds to only one of the binding site populations. To look at this from a different direction, each site binds different combinations. One of those three sites will bind all three of the Cry1A proteins, one site will bind two of them, that is, the Cry1Ab and Cry1Ac, while one site only binds the Cry1Ac protein. The cartoon in Figure 1 illustrates this concept. In addition to the Cry1A proteins, the Cry2A proteins are also included in this diagram. Although there is some conflicting evidence, it is believed that Cry2A binds to a separate site than either of the Cry1A proteins. Imagine the tube is the midgut of a *Heliothis virescens* insect, containing various binding sites on the midgut membrane. The Cry1A binding sites are indicated in solid black and Cry2A binding sites are striped with the Cry proteins marked with various cross hatching - Cry1Ac is indicated by small squares, Cry1Ab by wavy lines, and Cry1Aa by vertical hatches. One of the sites to the right binds only Cry1Ac. Another site in the middle binds both Cry1Ab and Cry1Ac proteins. Finally there is another population of sites that binds all three proteins. A completely unique site is the Cry2A binding site, and the Cry2A protein (polka-dots) is bound to that site.

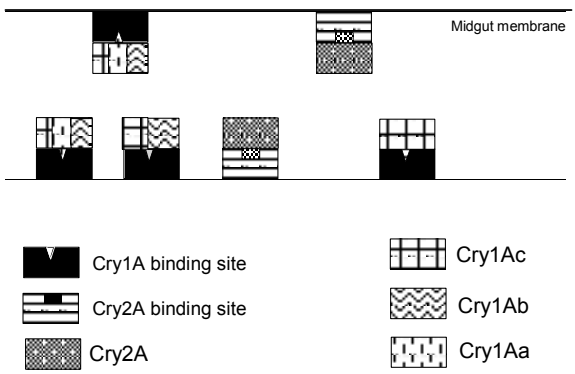

Figure 1 Binding sites for *Heliothis virescens*

What is the impact for the insect control? Should resistance develop to Cry1Aa, inactivating the Cry1Aa binding site, then one would expect at least partial cross resistance to the Cry1Ac and Cry1Ab proteins since Cry1Aa shares the same binding site with those two proteins. The effect on the binding would likely be either an altered affinity or reduction in binding site number leading to a reduction or loss in susceptibility. For another example, imagine that partial resistance develops to the Cry1Ac protein affecting only the site that specifically binds Cry1Ac. Although a reduction in Cry1Ac activity would be expected, theoretically there should be no effect on the other two proteins, Cry1Aa or Cry1Ab since they do not bind to the altered site. It is important to remember that other resistance mechanisms are possible, such as proteolytic cleavage of the Cry protein. An increase of a specific insect midgut enzyme might inactivate all Cry proteins, but to date, the majority of resistance mechanisms have been binding site related.

StarLink™ corn, *Bt* Cry9C protein technology

The *Bt* Cry9C technology, known as StarLink™ corn, was developed by PGS/AgrEvo. The Cry9C gene was isolated from a *Bacillus thuringiensis* subsp. *tolworthi* strain. This *Bt tolworthi* strain was isolated from the Philippines in 1991 and has never before been commercialized in a formulated product (Lambert et al. 1996). Instead, the *cry9C* gene was cloned and inserted into bacteria and into plants, with transgenic Cry9C corn as the first product. The first corn field trial was performed in Europe in 1994, and in the USA in 1995, with a US product launch in 1998. As is illustrated in Figure 2, the Cry9C technology in StarLink™ corn is exclusively found from PGS/AgrEvo. PGS/AgrEvo choose a different strategy in launching StarLink™ corn, strictly based on the potential threat of IRM. The marketplace was filled with Cry1A products and the company felt strongly that a product with a novel insecticidal gene must be introduced into the market to provide the option of insecticidal pyramiding.

Cry1A *Bt* Corn	Cry9C *Bt* Corn
• Mycogen NatureGard ™ • Ciba Maximizer ™ • N-K Bite Protection • Monsanto YieldGard ™ – Cargill, Pioneer, Asgrow... • DeKalb DeKal *Bt* ™	• Plant Genetic Systems / AgrEvo StarLink™

Figure 2 *Bt* Corn Hybrids

Cry9C protein has a number of attributes. It is not surprising that the sequence homology is only about 50% with the Cry1A proteins (Lambert et al. 1996). As might be expected from this lack of homology, the insect spectrum is also quite unique (Lambert et al. 1996). Two common corn pests, *Ostrinia nubilalis* and *Diatraea grandiosella,* are sensitive to both the Cry9C and the Cry1A proteins. In addition, several other insects that are sensitive to the Cry9C protein include *Agrotis ipsilon, Spodoptera exigua, Plutella xylostella, Heliothis virescens*. Another interesting finding is that there is no activity toward *Heliocoverpa zea* from Cry9C. This has a very important aspect in terms of the southern US where sales of Cry1A-containing corn has been restricted due to the presence of the Cry1A-containing cotton. Therefore, StarLink™ corn has no southern US restrictions.

Despite these differences the mode of action is equivalent to the other *Bt* proteins with one important difference, a unique insect midgut binding site (Lambert et al. 1996; Denolf et al. 1993). Separate binding sites were identified for the Cry9C protein as compared to Cry1A or Cry1C proteins. This has been found in a number of different insects, in *Ostrinia*, *Plutella*, and *Spodoptera*. The PGS researchers have done a series of competitive binding studies to show that there is no overlap between the Cry9C and Cry1A binding sites, except for one example. There appears to be some overlap with the Cry1C binding site in *Spodoptera*, where a lower affinity site binds both Cry1C and Cry9C. The studies indicate that there are more than one site for Cry1C and that Cry9C binds to one of these binding site populations.

Returning to another cartoon to illustrate the binding site specificity, Figure 3 shows two different binding sites for *Ostrinia*. The Cry1A binding site is in black, while the striped box is the Cry9C binding site, to indicate these differences. As might be expected from the *Heliothis* example, Cry1Ac and Cry1Ab proteins bind the same site in *Ostrinia*. Different combinations for the Cry1Ab/Cry1Ac binding sites are shown in Figure 3. The Cry9C binding site population is quite unique, binding only the Cry9C protein and not the Cry1A proteins. Likewise, the Cry1A binding site will not bind the Cry9C protein.

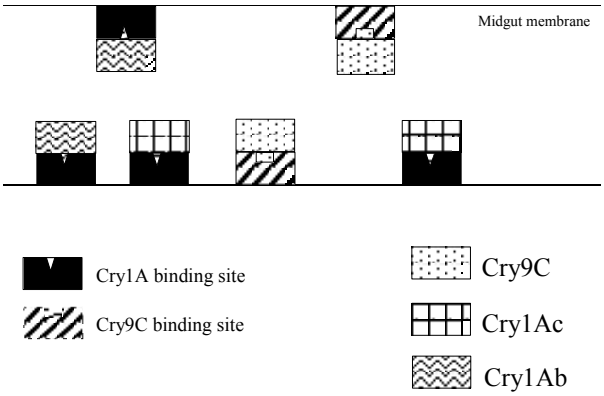

Figure 3 Binding sites for *Ostrinia nubilalus*

Insect bioassay data further supports the lack of binding site overlap. At the University of Minnesota, Bolin et al. have developed a Cry1Ac-resistant colony of *Ostrinia* (Andow et al. 1998). They used laboratory selection to develop this colony, and after 37 generations of selection, they measured a resistance ratio of about 20 for Cry1Ac protein. They found that the Cry1Ac-resistant colony was equal susceptible to Cry9C protein as the susceptible *Ostrinia* colony (P. Bolin, personal communication).

Plutella provides yet another example to support the separate binding site theory. Competition binding experiments were performed by researchers at PGS in Gent, Belgium (Lambert et al. 1996). They found that Cry1Ab and Cry1Ac proteins could compete for the same binding site, and there was a separate binding site for the Cry9C protein. Insect bioassays (Lambert et al. 1996) fully support this data using a Cry1Ab-resistant population of *Plutella* (Ferré et al. 1991). The Cry9C protein was equally active towards the resistant colony as to the susceptible *Plutella* population. Research is underway to assess the effect of the Cry9C protein towards other resistant insect populations from around the world.

Resistance management plans for StarLink™ corn

Despite the fact that Cry9C has not been commercialized in any other formulated product, AgrEvo USA/PGS takes resistance management very seriously, and, in fact, embraces the view that IRM is a product stewardship issue. Only a select number of insecticidal proteins are available for plant transformation, despite the fact that there has been a large discovery process over the last 10 years. These proteins are valuable and as a company, we feel it is very critical to monitor and maintain these products for many years of use.

StarLink™ corn hybrids were launched this year and the IRM plan is detailed below. StarLink™ corn contains a very high dose of the Cry9C protein within, greater than 25 times the LC_{99} for *Ostrinia*, the level recommended in a recent EPA Scientific Advisory Panel meeting. Actual bioassay data from resistant colonies of insects to support this level is limited, but it is the best choice from a conservative perspective. StarLink™ corn has season-long protection in all tissues due to the presence of a 35S promoter that drives the *cry9C* and *bar* genes (Jansens et al. 1997). The *bar* gene provides resistance to glufosinate herbicides, known as Liberty® or Basta®. A non-*Bt* corn refuge is required, but a couple of options are given. One option is the use of a 25% unsprayed refuge where no insecticide would be

sprayed, whereas a 40% sprayed refuge is a second option where any non-*Bt* insecticide could be applied. In either case, it is important that the refuge location be within one kilometer of a *Bt* corn field in order to ensure that random mating occurs between susceptible insects from the refuge and any rare resistant insects that might come out of the *Bt* corn field. Other IRM practices are also encouraged. Some examples include crop rotation, insecticide rotation, and the removal of crop residues.

Information and education, which have been highlighted several times already, is critical for compliance. We have a responsibility to educate and inform a wide range of different people so that they can balance short-term gains that this technology offers with the long-term success that we all desire. This information is being provided in a number of different forms by AgrEvo/PGS, in written brochures, pamphlets, verbally through meetings with sales, agronomists, growers, distributors, and electronically through the World Wide Web. Efforts are underway to reach every level. The grower, of course, is the one who implements the strategy, but they are influenced by a wide range of different people, including distributors, economists, sales, and maybe most important, corporate management. If management does not support IRM in seed companies and in technology providers like PGS/AgrEvo, then financial gains will be short lived rather than the potential for long-term success.

Monitoring is important to evaluate what is occurring at the field level. The primary focus must be to assess unexpected and serious plant corn damage. It is at this level where the very first resistant insects might be detected. Plant damage has to be carefully assessed since it can occur from many different sources. A grower should expect up to 3% off-types in the field, as found with traditional breeding efforts. The grower may forget how he originally planted his plots and without accurate records the plant damage may be occurring in a regular corn hybrid plot that does not include a *Bt* corn hybrid. To evaluate StarLink™ plants in the field, a qualitative field test kit is being developed and validated in the field this summer. Alternately, the damage may be due to a different insect, one that is not sensitive to the *Bt* protein. Careful review of the plant damage by an entomologist or extension personnel would be necessary. If all other possibilities are ruled out, surviving insects will be tested for their sensitivity to the Cry9C protein to determine if a Cry9C-resistant insect caused the damage.

The secondary efforts of monitoring are to test insects in areas of high use. This would be based on annual sales data to focus on regions where the product is launched at a very high percentage. One option is to only monitor those areas where StarLink™ corn exceeds a 25% market share. Any lower market share would have little effect on selection pressure. A baseline susceptibility study provides a basis for the range of the distribution of insect susceptibility. From this data, a discriminating dose assay is being developed, by choosing a Cry9C dose that will kill all susceptible target insects but allow survival of resistant individuals. This type of assay would allow efficient testing of large numbers of insects.

AgrEvo/PGS funds wide ranging research programs throughout the world. A variety of different aspects of IRM are being studied to try to understand more about resistance management, how it occurs, and how it be can delayed or possibly avoided. Some studies focus on the biology of the insect, with efforts to capitalize on insect movement. How an insect moves between the refuge and the *Bt* crop can be utilized to optimize resistance plans. Determining the best way to deploy the refuge is another important research topic. Where should it be deployed? How should it be deployed? Would in-field blocks have more advantages than the use of in-field strips? Are there alternative hosts crops that could be utilized so that the actual size of the refuge could be minimized, while maintaining the number of insects sufficiently high to dilute any resistant insects? Are there incentives that can be applied to encourage compliance by the grower? Are there other resistance management alternatives than the popular high dose-refugia strategy? Obviously, there is quite a tremendous amount of effort going into these areas of research, to enhance our understanding of IRM so that as many effective options as possible can be provided to the grower.

The future - dual gene, pyramided plants

Another area of active research is the discovery and basic research about protein structure and function of other insecticidal genes that could be applied to dual gene, pyramided plant products. Cry1A proteins are present in a number of different products, and the addition of the Cry9C gene this year in StarLink™ products offers the only current option to providing a pyramided product. Corn hybrids, of course, allow simple integration of additional traits. The Cry1A protein could be used on one side of the pedigree with Cry9C on the other side. In fact, these hybrids are being tested in research plots in summer 1998.

Registration issues may possibly interfere with the introduction of pyramiding technology. In the USA, we have quite a different situation than that of the EU. If two traits are registered in the USA, pyramiding of insecticidal proteins into new products is allowed with minor government notification. In other words, if a Cry1A-containing corn product was combined with the StarLink™ corn, as long as the requirements of both registrations is adhered to, no further registration is necessary except for a notification process. But in the EU, a separate registration is currently required, which means a separate evaluation, and, most importantly, a long delay in the introduction of these improved products.

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Reconciling science, economics and practicality in resistance management for transgenic insect-protected crops

Head, G.

Monsanto Company, St. Louis, MO

Abstract

Insect resistance management for transgenic insect-protected crops is generally recognized as a critical part of the stewardship of these products. Given the benefits at stake and the increasingly global nature of transgenic crops, it is critical that a suitable framework for insect resistance management plans be devised. Considerable work has gone into the underlying science but critical economic aspects have received less attention. In this paper, I discuss the important scientific and economic elements of resistance management and give an example in which these elements have been successfully reconciled, producing resistance management plans that contain adequate safety margins while still being acceptable to growers. Once the importance of these elements is recognized, then it also follows that resistance management strategies can be, and must be, adapted to the specific needs of different crops and geographic regions.

Introduction

The world is in the midst of an agricultural revolution driven by biotechnology. By genetically engineering genes from various sources into crop plants, plants that have insect protection, herbicide resistance, or one of a number of other traits have been produced. The most successful of the insect-protected products contain genes from the soil bacterium *Bacillus thuringiensis* (*Bt*). The resulting plants express proteins that are toxic to (depending on the gene) one or more of a variety of lepidopteran and coleopteran pests. In less than five years, the number of acres devoted to these crops has risen to about 20 million in the USA alone. These transgenic products can be effective enough in preventing insect damage to eliminate the need for insecticidal sprays in some cases and, in general, they greatly reduce the need for traditional insecticides. Other benefits of the technology include reduced environmental contamination, reduced worker exposure to hazardous chemicals, economic benefits to the growers, and a shift toward more sustainable agricultural practices.

Given the benefits of these products, product stewardship is vital to ensure their durability. Insect resistance management (IRM) is a critical component of that product stewardship. Companies like Monsanto have been researching and refining IRM plans for over a decade in collaboration with academics, regulators and other stakeholders in the technology. Based on this research, and experience garnered in the commercialization of these products, it is becoming clear that science, economic considerations, and the practical needs of growers, and the complex interactions among these components, all play important roles in IRM for transgenic *Bt* crops. In this paper, I outline the essential elements involved in the science, economics and practicalities of IRM, and illustrate how they can be reconciled. I also discuss how these elements can and do vary with the crop and geographic region involved, and the consequences that this variation has for IRM recommendations.

Scientific elements of IRM for transgenic *Bt* crops

The rate at which insect resistance evolves to *Bt* crops will be determined by the same factors that affect resistance evolution to traditional insecticidal products. Critical factors can be divided into aspects of the genetics of resistance (initial frequency of the resistant allele, degree of dominance of that allele, and fitness costs of resistance), the nature of the product and how it selects for resistance (pattern of *Bt* expression in the crop plant and penetration of the product), and elements of insect behavior that mediate how the product affects the target insects (insect movement and mating). These factors have been reviewed in detail by other authors (FIFRA SAP, 1998; Gould, 1998) so I will only touch on them briefly here.

Frequency of resistance alleles - Researchers have estimated initial allele frequencies in laboratory selection experiments but, because of sampling restrictions, the allele frequency must be greater than about 10^{-4} to be measured accurately in these experiments (Gould, 1998). Thus direct assessments of

allele frequencies in field populations are preferred. Work on *Heliothis virescens* collected from cotton indicated that resistance allele frequencies may be as high as 10^{-3} (Gould et al. 1997). Recent surveys of *Bt* field corn and sweet corn suggest lower resistance allele frequencies. An Illinois survey of 327 acres of whorl stage *Bt* corn found only 2 live European corn borer (ECB) on *Bt* plants out of an estimated population of 4.5 million ECB (Weinzierl et al. 1997). Based on these efforts, resistance allele frequency was estimated to be in the range of 7×10^{-4} to 2×10^{-7}. Hutchison and Andow (pers. com.) surveyed ears of *Bt* sweet corn, and estimated the resistance allele frequency to be between 8.3×10^{-3} to 6.9×10^{-5}.

Dominance of resistance allele - Dominance reflects the nature of the allele itself and the efficacy of the product. Higher dominance will tend to lead to more rapid resistance evolution. In many but not all cases of *Bt* resistance studied so far, resistance is partially to completely recessive (Tabashnik, 1994; Tabashnik et al. 1997). The only published case of dominant resistance led to only 25-fold resistance (Gould, 1998; Gould et al. 1995), meaning that such alleles would not be sufficient to allow survival on a high expressing *Bt* product. These results suggest that resistance to transgenic plants likely will be inherited as a partially or wholly recessive trait with dominance in the range from 0.01 to 0.05. Furthermore, the high dose nature of most transgenic *Bt* products increases the likelihood that resistance alleles are functionally recessive (see below).

Fitness of resistant insects - Work on the fitness of *Bt*-resistant lines derived from cases of field resistance (Groeters et al. 1994), and from laboratory selection experiments designed to create lines that might survive on transgenic plants (F. Gould, pers. com.), indicates high fitness costs of resistance to *Bts*. With the latter, few if any *Bt*-resistant *Heliothis virescens* survive beyond the fourth instar even when raised on non-*Bt* plants.

The nature of the product - The pattern of *Bt* protein expression in a transgenic corn product, and particularly the magnitude and consistency of that expression, will affect resistance development. The preferred expression pattern is a season-long, high dose that is sufficient to control target insects that are heterozygous for any resistance genes (FIFRA SAP, 1998). By delivering a dose sufficiently high to kill all or nearly all of the susceptible heterozygotes, resistance becomes functionally recessive. Metrics for determining whether a transgenic product expresses a high dose have been devised (FIFRA SAP, 1998). For example, information available today on Monsanto's *Bt* corn (MON 810) indicates that it is high dose for the important target pests (Andow and Hutchison, 1998). This information includes field surveys demonstrating very high performance, studies showing control of later instar target insects, and work on laboratory-selected European corn borer (ECB) colonies that tolerate high levels of *Bt* in diet assays showing that these insects do not survive on MON 810 plants (Principle investigators: W. Hutchison at University of Minnesota, C. Mason at University of Deleware, and R. Higgins at Kansas State University).

Product penetration and product cycle - The amount of a particular *Bt* crop planted in any given area also is a key determinant of resistance evolution. High adoption rates by individual growers, and across all growers, would lead to more rapid resistance. Generally, this is not yet the case. For example, overall penetration of *Bt* corn into the corn market in the USA is in the range of 15-20%, and surveys indicate that individual growers that purchase *Bt* corn plant about 30% of their farm to *Bt* (Harvest Research Company, 1998). Penetration is projected to eventually reach 50-70% but this will take several years. In the meantime, companies are developing second generation *Bt* products and these are in field tests. Indications are that these new products will become commercially available within the next four years.

Insect behavior - The way in which insects move between *Bt* and non-*Bt* plants determines insect exposure to the *Bt* toxin (Hoy et al. 1998). Both larval interplant movement, and longer range adult movement, have important effects on resistance evolution, by affecting both the selection pressure for resistance and the likelihood that resistant individuals will mate with susceptible insects. For example, studies of the biology of the major corn and cotton pests indicate that they tend to disperse relatively long distances (see, for example, the SAP document). This makes it likely that pockets of resistant insects will be diluted out by susceptible immigrants.

What the science says about IRM

Although the scientific elements that affect the development of resistance are many and they interact in complex ways, several points are clear. All of the elements will vary considerably among crops and

regions, so IRM strategies will have to vary accordingly. This is a particularly important point given the global nature of *Bt* crop deployment. A continuum can be identified in terms of resistance risk that ranges from low (high dose product, functionally recessive resistance, low product penetration) to high (low dose product, partially dominant resistance, no cost of resistance, low pest mobility, high penetration). The need for effective IRM will follow that same continuum. But, at the same time, these scientific elements do not provide the full picture of how IRM should look. Grower practices and decisions ultimately decide what is possible and what will be effective in terms of IRM.

Agronomic practices

The agronomic practices of growers in a particular crop will shape the IRM needs and constrain the IRM strategies that can be used. This is true not only when looking at gross differences, but also at subtler levels. Extreme differences exist, for example, between the way in which corn and cotton are grown in the USA versus India and China, with far smaller average farm sizes and more diverse cropping systems in the latter countries. In the USA, with large, continuous acres of *Bt* corn and cotton, the risk of resistance is relatively high compared to the Asian cases and the use of structured refuges becomes more important. At the same time, coordinating structured refuges may not even be possible in the case of many small farms and millions of individual growers. At a finer level, again consider the case of corn in the USA. Depending upon the region, corn may be grown continuously or in rotations, in blocks or in center pivots, with varying amounts of tillage. Any IRM requirements must accommodate these differences, which points toward the need for flexibility. Failure to make IRM fit with agronomic practices will result in ineffective implementation.

Grower needs and economics

Just as a grower's decision of whether to grow a particular crop or variety is based on an economic analysis, IRM too is inherently an economic decision in which an investment in IRM and is weighed against a long-term gain in product durability (May and Dobson, 1986). This fact, however, is not always taken into account in discussions of IRM. Despite attempts by some to explicitly include the costs of IRM in models of IRM strategies (Hurley et al. 1998), others have ignored or downplayed the economic realities and instead focused upon the need to guard against scientific uncertainties (Andow and Hutchison, 1998; Gould, 1998).

As a simple example of the factors involved, consider cotton and corn in the USA. In cotton, chemical costs average as much as $90 per acre in areas like the Delta region of the southern USA. Use of *Bt* cotton can replace as much two thirds of this cost, while providing better protection against the major lepidopteran pests than chemical alternatives. Planting a refuge requires a grower sacrifice most or all of the yield from the relevant acreage, while possibly also increasing chemical use. Similarly, with corn, Monsanto studies from the 1997 growing season show that there was an average yield advantage for MON 810 versus non-*Bt* hybrids of 10.8 Bu/A, providing the grower with a per acre dividend of $17. Growing a refuge then sacrifices that dividend on the acres that are planted to non-*Bt* crops.

These examples also highlight the fact that the cost to growers will be greatest in areas where insecticide use would otherwise be heaviest (for example, the Delta area for cotton or the south-central states in the USA for corn), and this is where the need for transgenic *Bt* products also is the greatest. Note that these costs will not only affect the growers. Because traditional insecticides will be used on many of the non-*Bt* acres, the other benefits of these products also will be lost on the affected acres (reduced environmental contamination, etc.).

More generally, just as the need for IRM will vary among crops and geographic areas, so will the costs of IRM for growers. Crops and regions with a strong need for *Bt* crops as a control measure, whether because of high crop losses to insect damage or few alternatives, will be particularly affected. Failure to recognize these costs will have the same result as ignoring grower practices; growers will be unwilling and possibly unable to implement the IRM practices. Past experience tells us that insect resistance tends to be more a function of how a product is used and misused, than a function of the product itself or the pest species (Roush, 1994). For example, foliar *Bt* sprays have been used in some areas of the USA for decades without resistance arising, but in areas where they are overused, resistance to multiple *Bt* proteins has arisen (Tabashnik, 1994; Tabashnik et al. 1997).

How grower economics affects grower decisions and IRM

Growers make several major decisions that critically impact IRM, including whether to buy a transgenic *Bt* product, whether insecticidal sprays are needed as well, and to what degree they will comply with any IRM requirements. These decisions are all shaped by the economics factors outlined above. To illustrate these impacts, consider the following study. In July 1998, the National Corn Growers Association (NCGA) funded a cooperative study to understand current and future grower practices. Harvest Research Company conducted a phone survey of 504 growers who planted MON 810 hybrids. Growers were selected from three corn regions in the USA. Area 1 was defined as eastern Nebraska, South Dakota, Iowa, Minnesota, Wisconsin, Illinois, and Indiana. Area 2 represented growers from northwest Kansas, western Nebraska, and northeast Colorado. Area 3 included growers from New Mexico, Texas, southwestern Kansas, and southeastern Colorado. A series of questions were asked to understand how much structured refuge would be acceptable to growers. More than 96% of the growers surveyed had planted refuges of the currently required size or larger in 1998 (5% unsprayed/20% sprayed). Respondents were asked to what degree they would comply with each of two higher options: a 10% unsprayed / 20% sprayed option and a 25% unsprayed / 40% sprayed option (Table 1). The vast majority across all regions (83%) would establish at or above the 10/20 option. Only 5% would not establish a refuge at all. In contrast, when presented with requirements of 25% unsprayed / 40% sprayed option, anticipated compliance dropped to 56%. In fact, the proportion of growers not establishing a refuge doubled to 20%. This is particularly striking in Area 3, which is a high insecticide use area where the cost of refuge acres is potentially high to growers. In addition to these changes in compliance, about 30% more growers said that they would spray their refuge acres than currently do if IRM requirements increased in this way. Thus, rather than reducing the risk of resistance, overly restrictive requirements would lead to non-compliance and an increased risk of resistance development, as well as increasing spray use and thus sacrificing one of the major benefits of transgenic *Bt* products.

Table 1 Results of grower surveys. Question to growers: To what extent would you comply with a new larger refuge requirement of either 10% unsprayed / 20% sprayed or 25% unsprayed / 40% sprayed?

		Percent of respondents			
Option	**Level of Compliance**	**Total**	**Area 1**	**Area 2**	**Area 3**
10/20	At or above recommendations	83%	88%	78%	80%
	Less than recommendations	11%	9%	12%	9%
	Not establish refuge at all	5%	3%	7%	5%
25/40	At or above recommendations	56%	67%	46%	50%
	Less than recommendation	33%	28%	39%	29%
	Not establish refuge at all	10%	5%	14%	16%

Achieving a balance in IRM

Despite the inherent conflict between minimizing the risk of resistance and minimizing economic impacts on growers, IRM plans that balance these needs can be created. Consider the situation in the USA corn belt. Mathematical models have been developed that indicate what reasonably conservative IRM requirements should be. For a high dose product, particularly in combination with refuges, even a 5% unsprayed refuge will delay resistance significantly (Table 2; Onstad and Gould, 1998), while a 10-20% refuge should be adequate for a less than high dose product.

Table 2 Years for resistance to develop in a *Bt* corn model (Onstad and Gould, 1998)

% Refuge	High Dose	Not High Dose
0	1	3
0.1	5	3
1	22	5
5	83	11
10	>100	19
20	>100	35

And these models, and thus the estimates derived from them, contain large margins of safety in the critical variables (Table 3). Thus, from the point of view of resistance risk, a 5-10% unsprayed refuge in combination with other IRM strategies should be effective. As discussed above, the vast majority of corn growers view such a refuge size as reasonable and would comply with such a requirement, so this also would be a practical solution. Clearly then, IRM plans can be developed that balance the needs of all stakeholders. The nature of this balance will be crop and region-specific, but the same scientific and economic components always will be important.

Table 3 Approximate margins of safety built into the models used to derive IRM recommendations for the high dose Yieldgard *Bt* corn in the US corn belt

	Assumed Value	True Range	Margin of Safety
Nature of resistance			
Dominance at high dose	0.01	≈ 0-0.001	10-fold
Fitness of resistant insect	1.0	≈ 0-0.7	1.5-fold
Nature of product			
High dose definition	25 x LC99	6-10 x	2.5-fold
Penetration	100%	30-70%	1.5-fold
Product cycle	>10 years	4-7 years	1.5-fold

Conclusions

IRM must have a strong science base because the underlying questions are scientific in nature, but scientific considerations must be balanced with an understanding of grower economics and how economics affect behavior. In addition, there must be a recognition that all of these factors vary among crops and geographic regions, and that IRM plans must vary accordingly. Only this can achieve what all stakeholders in this technology want - a lasting, effective IRM strategy. And these goals are reasonable. As illustrated, plans can be developed that include large margins of safety while still being acceptable to growers. On the other hand, unnecessarily conservative IRM requirements will result in either or both of two undesirable events: replacement of transgenic crops with insecticidal sprays that are far less environmentally friendly, or non-compliance by growers and an increased risk of insect resistance.

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Resistance management for *Bt* corn: progress and challenges to consensus in US policy

Hutchison, W.D., Andow, D.A.

Department of Entomology, 1980 Folwell Ave.,University of Minnesota, St. Paul, MN 55108, USA

"...scientific knowledge is built up, not by maximizing certainty or striving awkwardly for consensus, but by minimizing uncertainty." (Robert May, Britain's Chief Scientific Advisor - Nature, 1998)

Abstract

The introduction of corn hybrids genetically altered to express various protein toxins produced by the bacterium, *Bacillus thuringiensis* (*Bt*), has provided a significant technological advancement for corn pest management, balancing high efficacy with reasonably low cost to growers. Demand for *Bt* corn remains high, with a projected USA planting of ca. 15 million ha by 2000. In the USA, the primary concern of most scientists is the possibility that targeted pests, such as the European corn borer, *Ostrinia nubilalis* (Hübner), could rapidly develop resistance to *Bt* if market penetration of *Bt* corn remains high. Following several joint meetings with university, industry, USDA, and US-EPA representatives, facilitated by the NC-205 Regional Research Project on Ecology and Management of Stalk-boring *Lepidoptera*, a proactive resistance management plan was developed in 1996 (Ostlie et al. 1997). The plan was widely distributed among all stakeholders during 1997-1998. It relies upon a 'refuge/high-dose' strategy, suggesting that a relatively high proportion of the European corn borer larval population (20-30%) not be exposed to *Bt*. In most production areas this translates to a 'refuge' area of 20-30% non-*Bt* corn. If a grower anticipates the need to use insecticide in the corn refuge, the total non-*Bt* corn refuge should be 40%. This recommendation is based on both available data, and the analysis of several resistance management models, assuming a long-term planning horizon of 20+ years for the technology. In this paper we discuss how a risk-based strategy has evolved in the USA, as well as how multiple and conflicting objectives among stakeholders continue to challenge the goal of unanimous consensus and subsequent grower adoption of *Bt* corn resistance management.

Introduction

The advent of transgenic crops, particularly those engineered to express various insecticidal endotoxins of the bacterium *Bacillus thuringiensis* (*Bt*), has been met with a diversity of views, including optimism by growers (Pilcher and Rice, 1998; Pocock, 1998) to concern by environmental advocates, particularly in Europe (Williams, 1998). With transgenic corn (*Bt* corn), most stakeholders, including growers (Rice and Ostlie, 1997; Pilcher and Rice, 1998), industry representatives (Sachs, 1998) and public-sector scientists (Ostlie et al. 1997; Rice and Pilcher, 1998), are aware of significant positive short-term benefits for individual farmers. The technology provides the most convenient, efficacious management approach – generally accepted to be superior to previous integrated pest management programs for economically important stalk-boring *Lepidopteran* pests (Ostlie et al. 1997).

Bt corn was developed primarily to provide more consistent control of the European corn borer, *Ostrinia nubilalis* (Hübner) (*Lepidoptera: Crambidae*). Nationally, European corn borer is considered the primary insect pest of field corn in the USA with losses commonly estimated to exceed $1 billion per year (Mason et al. 1996). Other pests such as the south-western corn borer, *Diatraea grandiosella* Dyar (Chippendale and Sorenson, 1997) and corn earthworm, *Helicoverpa zea* (Boddie), can also be controlled at various levels depending on the *Bt* event (Buschman et al. 1997a,b; W.D. Hutchison, unpublished data). In 1997 field tests in Iowa, Minnesota and Nebraska, where European corn borer is the primary pest, *Bt* events expressing the Cry1Ab gene provided yield increases ranging from 0 to 116 bushels/ha in Iowa (Rice and Pilcher, 1998). In Kansas, where both European and south-western corn borer occur, maximum yield increases ranged from 89 to 199 bushels/ha (Buschman et al. 1997a,b).

Costs and benefits to growers are dependent upon actual insect infestation level, and *Bt* seed premiums for a given region. Seed premiums are usually scaled to reflect pest damage potential and history in a specific region. However, the higher the seed premium, the more careful a grower must gauge the decision to plant or not plant a *Bt* crop. This decision is also complicated by the fact that most seed purchases are completed by November-December, preceding the growing season that seed will be planted. Thus, the decision is made as a preventative tactic, conducive to farmers who prefer an insurance-based approach to overall risk management for their enterprise. Although *Bt* seed premiums have ranged from $10-20/ha, added competition among companies in 1998 resulted in low premiums in some areas (WDH, unpublished data), permitting companies to increase or maintain market share. Given the high efficacy and low premiums, market demand will likely remain high in the USA.

Given the numerous advantages of *Bt* corn, including a high level of efficacy, and potential widespread market penetration, a central concern with the technology is the risk of insect pests developing resistance to *Bt* (Alstad and Andow, 1995; Onstad and Gould, 1998; Gould, 1998; Andow and Hutchison, 1998). Because the European corn borer is the most widely distributed pest of *Bt* corn, the focus of this article will be a review of the development of resistance management plans for European corn borer in the USA during the past four years. Specifically, we review

1) unique aspects of the *Bt* corn market as they affect resistance management,
2) rationale for resistance management plans developed to date,
3) benefits of multiple stakeholders working together to forge a plan, and
4) suggestions for future research and dialogue.

Unique market features of *Bt* corn

Unlike *Bt* potato and cotton, *Bt* corn encompasses a diversity of events, hybrids, *Bt* proteins and corporate interests. *Bt* corn can also exploit a much larger potential US market of ca. 30 million ha (75 million ac). In 1998, approx. 6.1 million ha (20% market share) of *Bt* corn was planted; projections for 1999 and 2000 are 10 and 14 million ha, respectively, approaching 45% market share (Novartis, 1998a). By contrast, *Bt* cotton and potato were planted to approximately 1.0 million and 16,190 ha, respectively, in 1998.

Most corn events to date, have been developed using the Cry1Ab gene (Novartis, Mycogen, Pioneer, Monsanto) (Koziel et al. 1993; Armstrong et al. 1995), with DeKalb (Monsanto) hybrids having the Cry1Ac gene and the recently registered AgrEvo hybrid expressing Cry9c. Although there are still several *Bt* corn companies, the merging and acquisition trend appears to still be strong. The trend for fewer companies has potentially positive and negative effects relative to resistance management. One advantage of fewer companies is that it could be easier for public-sector and government regulatory groups to reach consensus on risk-based approaches to resistance management. A potential disadvantage, however, is that although competition among subsidiaries is currently strong, continued mergers could eventually result in less competition, and possibly increased seed costs. Presently, because of the multiple companies involved, the trend has been to keep seed premiums low in an effort to increase market share.

Current resistance management plan for US *Bt* corn

With the North Central regional NC-205 publication on *Bt* corn resistance management (Ostlie et al. 1997), and in subsequent publications (Andow and Hutchison, 1998; US-EPA, 1998), a structured "refuge/high-dose" strategy has continued to be affirmed as the most effective approach to substantially delay the evolution of European corn borer resistance to *Bt*. The refuge is defined as a high proportion of the European corn borer larval population (20-30%) that is not exposed to *Bt*; in most production areas this translates to a "refuge" area of 20-30% non-*Bt* corn. If a grower anticipates the need to use insecticide in the refuge corn, the total non-*Bt* corn refuge should be 40% (Ostlie et al. 1997).

The high-dose component of the strategy has been provided by the corn seed industry. Although several definitions have been offered for a high dose, an operational definition (US-EPA 1998) states that the dose should be high enough to be 25x the dose necessary to kill all susceptible (ss) and most heterozygous resistant (rs) individuals. With *Bt* corn, some events, such as "176" clearly do not meet the high dose criterion once plants approach antithesis (e.g., Onstad and Gould, 1998), resulting in a mosaic

of high, moderate or low doses of *Bt* in some production areas. However, for most of the production, to date, hybrids have been planted with what most US entomologists would accept as high-dose events for European corn borer.

This resistance management plan, along with the relatively high levels of refuge requirement, is based upon the best available data on European corn borer ecology, migration, laboratory studies of resistance evolution (Huang et al. 1997; Bolin et al. in revision), and the analysis of several resistance management models (Alstad and Andow, 1995; Onstad and Gould, 1998; Caprio, 1998; T. Hurley, unpublished data). A critical point is that the plan assumes a long-term usefulness of the technology, such as 20+ years. Key assumptions of the refuge/high-dose approach include: a high dose for the targeted insect pest (as defined above), refuge is effective at providing a high number of susceptible moths to mate with any possible survivors from nearby *Bt* corn, low initial frequency of resistance alleles (e.g., $< 1x10^{-3}$), and random mating between refuge and *Bt* corn and therefore no inbreeding.

Resistance evolution - resistance management under uncertainty

As with other management decisions under uncertainty, there are two critical components of the decision-making process that should be kept separate, and then combined for final policy or management recommendations. Decision analysis methods allows one to partition the two components of risk assessment (e.g., Carlson, 1970; McNamee and Celona, 1987), specifically:

1. Uncertainty/Risk (addressed via probability theory)
2. Multiple/Conflicting Values and Objectives (addressed via utility theory).

The US process has benefited from distinguishing each component during most discussions. However, the process can be difficult as different interpretations or perceptions of either component may exist, depending on scientist and stakeholder experience or affiliation. The previous quote by Robert May (Nature, 1998) captures some of the essence of the dilemma from a scientist's perspective. That is, scientists are more comfortable with the first component of risk assessment, dealing with uncertainty and risk, and accepting a certain level of uncertainty (experimental error) in the conduct of experiments, or with the use of models of complex systems. However, scientists are generally not in a position to understand or assign value to multiple or conflicting objectives of stakeholders involved, e.g., growers with short-term or long-term goals, the value of technology to small versus large growers, or small versus large seed companies. These latter questions contribute to component 2, and often become the most difficult to evaluate during consensus building forums. Despite May's "awkward" characterization of the consensus building process with multiple stakeholder involvement, reaching a consensus on this issue is nevertheless critical if we are to achieve substantial delays in the evolution of resistance to *Bt* throughout most of the USA corn belt. In the following sections, we summarize key results and issues that have influenced the decision-making process in developing a US resistance management plan for *Bt* corn.

Component 1: Reducing uncertainty and risk

A systematic and systems approach to reducing risk, must begin with experimental data and subsequent analyses. Two types of information have been generated to provide answers to justify a scientific basis for a refuge/high-dose strategy designed to reduce the risk of rapid resistance development: experimental data (laboratory and field results), and computer simulation models of resistance development.

Recent experimental results include:

a) European corn borer populations collected in Minnesota, Iowa and Kansas show a consistent pattern of genetic variability necessary to develop moderate levels of resistance to both Cry1Ab and Cry1Ac *Bt* toxins (Huang et al. 1997; Bolin et al. 1999),

b) Dispersal studies indicate relatively low levels of European corn borer adult movement from emergence sites, with much of the adult movement occurring at < 100 m (D.A. Andow and D.N. Alstad, unpublished data), indicating that non-*Bt* corn refuge areas need to be planted in close proximity to *Bt* corn (probably within a ¼ section or 300 ha),

c) European corn borer shows limited survival on wild or alternative hosts, suggesting that most of the refuge area in most US production regions will need to be non-*Bt* corn (R. Hellmich, unpublished data), and

d) A recent N. American study of 45 European corn borer populations indicates a surprisingly high level of inbreeding within populations separated by an average distance of 300 km (D.A. Andow and D.N. Alstad, unpublished data). This last result suggests that N. American European corn borer is not panmictic, but reflects limited gene exchange among locations, indicating that we may not always be able to assume random mating between refuge areas and *Bt* corn.

Table 1 Influence of heterozygous rs survival and inbreeding on evolution of resistance to *Bt* by European corn borer; Caprio (1998) model[a,b]

Refuge Area	rs-survival	Inbreeding Coefficient	Generations to Resistance
5%	0.01	0.0	58
		0.04	20
25%	0.01	0.0	300+
		0.04	80

[a] Model available at: www.msstate.edu/Entomology/PGjava/ILSImodel.html;
[b] Assumptions: initial resistance allele frequency = 1 x 10^{-6}, ss survival = 0.0, rr survival = 1.0, rs survival a proportion of rr survival, and random mating if inbreeding coefficient = 0.0

Results from simulation models of resistance development cannot be used to directly assess the number of years to resistance, but can be very useful for comparing alternative refuge or resistance management strategies. In addition, via sensitivity analysis, they continue to be valuable tools for estimating the relative impact of key variables such as rs survival rates or initial gene frequencies for resistance (US-EPA, 1998). Modelling efforts to date have illustrated the following: a) early-planted *Bt* corn, along with regional suppression effects on European corn borer, can result in lower damage levels in the non-*Bt* corn refuge area, thus reducing the risk of economic losses to the grower (Alstad and Andow, 1995), b) minor recessive "modifier" genes for resistance, most likely to be more common based on laboratory selection studies (Huang et al. 1997), can interact with a dominant gene to accelerate evolution of resistance (Andow and Hutchison, 1998), c) *Bt* titer decline in an event such as "176" could accelerate evolution of resistance (Onstad and Gould, 1998), d) addition of an on-farm revenue analysis to a European corn borer ecology model resulted in refuge recommendations of 20-30% non-*Bt* corn because the long-term (15+ years) revenue gain far outweighed the short-term cost of maintaining a minimum 20% refuge (Hurley et al. submitted), and e) the addition of an inbreeding coefficient of only 0.04-0.40 has a major impact of accelerating resistance development (Table 1; Caprio 1998). Caprio's model is the first to incorporate the impact of inbreeding, a consequence of non-random mating between refuge and *Bt* corn areas. Moreover, if refuge size is too small (5%), the results indicate time to resistance is nearly 3-4 fold faster (see also Table 1). Sensitivity analyses of the models show that inbreeding coefficient and survival rate of RS individuals are two of the most important parameters driving resistance development and should therefore be the focus of more concentrated research.

Component 2: Multiple values/objectives and risk

As alluded to previously, values and objectives can vary by stakeholder, including differences of opinion between scientists on some facets of the available experimental data. Given the backdrop of industry and farmer interests, there are several key elements of resistance management that must be defined before thinking about how to best develop practical resistance management plans. A critical first question is what is a reasonable "long-term" planning horizon for a new transgenic crop (2, 10, 25 years)?

A grower, with considerable debt or close to retirement, may only have a one- to two-year planning horizon; in this situation it is very difficult to adopt longer-term resistance plans if there is a high risk of going out of business in the short-term. In the USA, the decision regarding how much *Bt* corn to plant, might actually be made by the bank controlling loans to the farm, and thus the banker becomes a critical but often overlooked stakeholder in resistance management. Similarly, any current questions about the

validity of a refuge/high-dose approach will be irrelevant to a grower or banker in this situation. Alternatively, if a grower is interested in long-term sustainability of the farm, has more manageable debt loads and thus more autonomy in planning, the variables germane to the use of models to study the development of resistance, along with current field data via ongoing resistance monitoring will likely be of more value.

A recent survey of 799 Iowa corn growers (Pilcher and Rice, 1998) shows that many are open to the idea of refuge areas of non-*Bt* corn, either within fields (30%), among fields at different planting dates (32%), or alternating *Bt* and non-*Bt* corn between years (45%), as various tactics to delay resistance. Only 17.3% of the growers had no interest in delaying the development of European corn borer resistance to *Bt*. Alternatively, some industry representatives have argued for low refuge areas (5%) (Sachs, 1998). In part, this approach is based upon their analysis of the most recent research. However, a low refuge approach also places more value on short-term revenue gains that would accrue from early, high market penetration rates, versus longer-term revenue gain at less market penetration in the short-term. Many scientists tend to support the longer-term planning horizon, and thus *Bt* corn refuge levels of 20-30%, as more *Bt* corn is deployed (e.g., Ostlie et al. 1997).

Given all the above information, as well as recent developments with the impact of inbreeding and possibly less gene exchange among European corn borer populations, the current NC-205 refuge recommendations continue to suggest that refuges of non-*Bt* corn be maintained at 20-30% of the total corn hectares on a given farm (40% if sprayed) (Ostlie et al. 1997). These results also support the possibility of the slightly more precautionary approach of 25% non-*Bt* corn (50% if sprayed) by Andow and Hutchison (1998). Following these outcomes, additional meetings were convened, with additional scientists and stakeholders to continue to refine resistance management recommendations. The results of these previous discussions were useful to the US-EPA sub-panel which reviewed current resistance management plans for *Bt* corn, cotton and potato (US-EPA, 1998). The NC-205 recommendations have also been reviewed and are being considered by Canadian regulatory authorities to set policy for the 1999 field season (G. Watson, Canadian Food Inspection Agency, personal communication). Currently, a joint industry/academic team, sponsored by the International Life Sciences Institute (ILSI) is finalizing their review of *Bt* corn resistance management, and how this should be delivered to growers (report not yet available). Most recently, the US-EPA, in registering *Bt* popcorn and the new *Bt* Cry9c event, specifically mandated that the registrants follow recommendations that were very similar to those of the NC-205 committee.

Benefits of resistance management dialogue among stakeholders

In addition to final outcomes useful for making recommendations, there were many advantages of having joint industry, academic and government based meetings. Scientists and EPA gain early insights into different industry perspectives including forces within a company driving technology development, and continue to be aware of rapidly changing alliances within the corn seed industry. Companies, regulatory agencies gain faster access to new data and unpublished results from scientists. Because of the fast pace in changes in technology, it has been necessary to average at least two meetings each year to exchange information.

Additionally, industry has made several contributions in meeting both the research needs and the educational needs of growers. Specifically, industry has contributed to the process by: providing research support, the development of several excellent grower-oriented resistance management publications (Novartis, 1998b), supporting the purchase and distribution of the NC-205 publication, "*Bt* Corn and European Corn Borer" (45,000 copies printed to date; 2nd printing), and most recently the advent of economic incentives to growers who purchase 20% non-*Bt* seed units with *Bt* seed (e.g., Novartis, 1998a). One additional development that may be attractive to growers wishing to reduce their risk exposure to losses in the refuge corn, is an interest among some crop insurance (and seed) companies in the Midwestern USA to provide "refuge" insurance. At the time this article was prepared we estimated that the first policy may still be a year away, but several companies are interested in pursuing the concept.

In summary, all registrations for *Bt* corn, since 1995, have been conditional, contingent upon new research and final resistance management plans. Final EPA registrations are due by 2000. In the

meantime, several critical research needs remain. In light of continued needs for basic information on European corn borer movement and mating behavior, we continue to see major needs for research that addresses the following: estimates of inbreeding (non-random mating) among populations (D.N. Alstad and D.A. Andow, unpublished data), initial estimates of frequency of resistance alleles (Andow and Alstad, 1998), mechanisms of resistance (Tabashnik et al. 1997), mating behavior and dispersal studies, development of new and complementary methods for monitoring for resistance (Siegfried et al. 1995; Pierce et al. 1997; Bolin et al. 1998; Andow and Alstad, 1998; Andow et al. 1998), and continued economic analyses to assess various refuge management recommendations on grower revenue (e.g., T. Hurley, unpublished data), both locally and regionally. Furthermore, similar efforts are needed for the south-western corn borer and corn earthworm, *Helicoverpa zea* (Boddie), both of which can be devastating pests of corn, with corn earthworm also being a severe pest of *Bt* cotton.

Epilogue

With over 500 arthropod species with confirmed resistance to one or more insecticides/acaricides the possibility of insect pests of corn developing resistance to *Bt* is not a new concern (Tabashnik, 1994; Gould, 1998). However, for several reasons, many *Bt* crops have many unique features that have generated heightened interest in pursuing proactive plans to delay the onset of resistance. Specific factors include: a) previous historical use of *Bt* foliar sprays on a variety of crops, e.g., over the past 15-30 years (Mellon and Rissler, 1998), b) recent confirmation of the genetic potential for several insects to develop resistance to *Bt* (Tabashnik, 1994), and c) a renewed interest by many stakeholders, including the US-EPA, to actively encourage industry and public-sector discussion to generate more long-term, proactive resistance management plans (Ostlie et al. 1997; US-EPA, 1998).

The alternative to a long-term plan is essentially what the US policy has been for the past 40 years, i.e., a registration approach that encourages overuse of new products, resulting in numerous resistance events and short-term life of insecticidal chemistries. With several insecticides, on crops such as cotton and potato, growers often experience the "pesticide treadmill" approach to pest management. The treadmill approach of introducing a new insecticide just as the previous product becomes ineffective, has sometimes worked surprisingly well in the USA, as long as the new insecticide is introduced just in time. The phenomenon quickly reverts to a reactive form of resistant pest management, rather than a more proactive resistance management (Andow and Hutchison, 1998). Although the new material is usually more expensive than the previous chemistry, the new insecticide may often be essential for a grower to stay in business. However, as the expense of developing new toxins continues to increase, there are mutual economic benefits to all stakeholders (developers and users) to begin to consider longer-term resistance management plans, which in turn would reflect a significant policy shift within the US-EPA. To date, previous registrations for conventional insecticides, such as pyrethroids, have not required submission of resistance management plans.

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Development of resistance management strategies for commercial cultivation of *Bt* cotton in China

Shi-Rong, J.

Biotechnology Research Center, Chinese Academy of Agricultural Sciences, Beijing, China

Abstract

Large-scale field trials of *Bt* cotton, developed by Chinese scientists, have been conducted in different locations since 1996. In 1997, the Biosafety Committee, Ministry of Agriculture, approved commercialization of *Bt* cotton in five provinces in China. Field performances show it has strong insecticidal acitivity to cotton bollworm. In this article, the development of resistance management strategies for *Bt* cotton is discussed in detail. Based on experimental data, we predict *Bt* cotton may at least be used for eight to nine years. Data also show transgenic tobacco with *Bt*/CpTI gene may delay cotton bollworm to develop a resistant population.

Introduction

Cotton is the most important cash crop in China with a cultivated area of 4.72 million hectares and a total output of 4.2 million metric tons in 1996. In the history, the maximum acreage of cotton reached 6.7 million hectares. However, it has been dramatically reduced in recent years which makes China a cotton import country instead of an exporter. One of the major factors that result in the reduction of cotton production is the severe damage caused by cotton bollworm (*Helicoverpa armigera* Hübner) in the recent seven years. For example, in 1992 alone, the yield loss caused by cotton bollworm valued 10 billion RMB. Therefore, it is urgently needed to develop an efficient method to control this insect.

Development of cotton cultivars resistant to bollworm is the best way in this respect, and an economic one. In 1991, the National High-Tech Program (the so-called 863 program since started in March, 1986) initiated a project on the research and development of insect-resistant cotton by genetic manipulation. The whole *Bt* gene was completely synthesized by Dr. Sandui Guo's group in the Biotechnology Research Center, Chinese Academy of Agricultural Sciences (BRC, CAAS) in 1992, according to the codon usage preferred by dicotyledonary plants. Meanwhile, a highly efficient plant expression vector containing a *Bt* CryIA gene was constructed. In the following year, the *Bt* gene was transformed into different cotton cultivars either by *Agrobacterium*-mediated gene transfer method or by pollen-tube passway. The integration and expression of *Bt* gene was confirmed by PCR, Southern, Northern, as well as Western analysis. In the case of pollen-tube passway, the plasmid, purified by CsCl gradient centrifuge, was injected into ovules of cotton plants 24 h after anthesis. By gradual improvement of injection methodology, the transformation frequency has now reached 1-2% that is practically applicable to cotton transformation. Particularly, the advantage of this method is not only that it is simple and rapid, but also without genotype-dependency. Now this method is routinely used in cotton transformation, and transgenic plants have been generated from more than ten cultivars dominantly cultivated in the Valleys of Changjiang River and Yellow River, and in the North inland region such as Xinjiang Autonomous Region.

During the period of 1994 to 1998, the bioassay of *Bt* transgenic cotton has been continuously conducted both in the laboratory, greenhouse, plastic house, and in field trials at different locations. Results show that it has excellent resistance to cotton bollworm and other *Lepidoptera* insects existing on cotton plants. In 1996, the transgenic *Bt* cotton was tested in 17 sites of nine provinces with a total acreage of 650 hectares. The acreage is further extended to 10,000 hectares this year. Farmers warmly welcome and accept *Bt* cotton and they state that the number of insecticide spray and the cost of labour can be dramatically reduced (15-20 times versus one to two times during the whole growing season) which results in savings of 1,200-1,500 RMB per hectare.

For biosafety assessment of *Bt* cotton and its products, a series of experiments have been conducted, and the results show that there is no harm to human and animal health and to the environment. In accordance, since 1997, the Biosafety Committee of the Ministry of Agriculture has approved commercialization of *Bt* cotton in five provinces (Anhui, Shandong, Shanxi and Hubei; Monsanto's *Bt* cotton has also been

approved for commercialization in Hebei province). In addition, a patent certification has been issued by the China National Patent Agency, and two *Bt* cotton cultivars have so far been registered.

For a large-scale commercialization of *Bt* cotton, it is needed to develop an efficient management strategy so as to allow us monitoring the field performance of *Bt* cotton, the expression of *Bt* gene in different organs and developmental stages, the geographic variation in sensitivity of cotton bollworm to *Bt* insecticidal protein, and the potential development of insect population resistant to *Bt* etc. All of these factors are considered as a part of pest management within the 863 program. Therefore, scientists involved in different disciplines have collaborated to gain experiences in different aspects. Following are the data accumulated to date.

Performance of *Bt* transgenic cotton

Field performance

In China, generally four generations of cotton bollworm occur each year. The first generation is raised on wheat or other crops because cotton is not sown at that time. The second to fourth generations usually cause great damage to cotton crop. In this regard, samples are collected each five days, starting from the second generation in the cotton field. It is evident that there is no difference in terms of egg density of bollworm (number of eggs per hundred plants) on non-transgenic and *Bt* cotton plants (data not shown). However, the larval density on *Bt* and control plants is significantly different during the second to the fourth generations (Figure 1). The percentage of apex and square damage in *Bt* cotton is usually less than 1%, while that in non-transgenic plants reaches 40-90% depending on the population size of bollworm in a particular location. Therefore, the insect control by *Bt* cotton is highly satisfied. The conclusion is that at least there is no need to spray pesticides during the second generation of the bollworm.

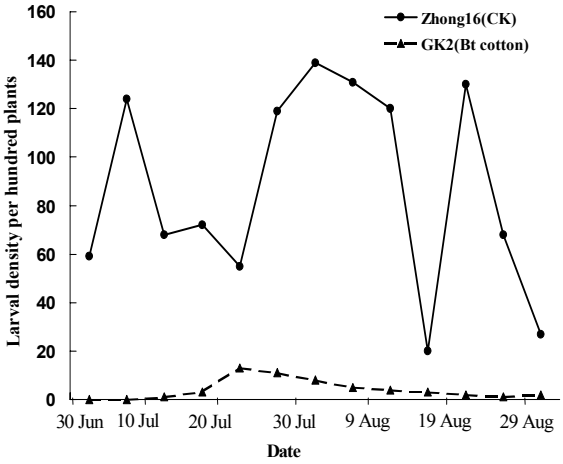

Fig. 1 Larval density of cotton bollworm in cotton field (Langfang, Hebei province, China, 1997)

Efficacy of *Bt* cotton in different developmental stages

In North China, cotton bollworm larvae of the second, third, and fourth generations generally occur in the last ten-day period of June, July and August. For determination of the efficacy of *Bt* cotton in different developmental stages, the first instar larvae were fed in the laboratory with squares or with top third expanded leaves collected during these three periods of time. Three days later, the mortality of insects was calculated. Results showed that the corrected mortality was 100%, 75% and 39.4%, respectively for the treatment with leaves taken from June, July and August, while the data for treatment with squares was 76.9%, 57.7% and 29.7%, respectively. It indicated that the mortality rate was reduced

as plants are gradually developed and the ability of squares to kill insects was lower than that of the leaves.

Efficacy of different organs of *Bt* cotton

As mentioned above, the leaves of *Bt* cotton showed highest insecticidal activity. However, during August 11-31, that correlated to the third and fourth bollworm generations, the insecticidal activities of different organs of *Bt* cotton against first instar larvae were significantly different. The order was bolls > leaves ≥ squares ≥ flowers (Table 1). However, for the second to fifth instar larvae the order of insecticidal activity was leaves > squares > bolls > flowers (data not shown).

Table 1 Insecticidal activity of different organs of *Bt* cotton against first instar larvae of the third and fourth generations of *H. armigera*

Organ	Mortality of CK (%)	*Bt* cotton Mortality (%)	Corrected mortality (%)
Leaves	12.0 ± 3.7	46.7 ± 9.7	39.4 b
Squares	10.0 ± 4.1	36.7 ± 3.3	29.7 bc
Flowers	22.0 ± 2.0	38.0 ± 4.9	20.5 c
Bolls	23.3 ± 3.3	80.0 ± 5.2	73.9 a

The values followed by the same letter are not significantly different ($p < 0.05$).

Effects of flowers of *Bt* cotton on the survival and development of neonate of *H. armigera*

As shown in Table 2, the mortality of the first instar larvae at 6 d, fed with flowers of *Bt* cotton, was 46%. It was significantly higher than that of the control (22%). Thereafter, the survival larvae were inoculated on young bolls. As a result, the weight of the survivors was dramatically reduced (51.0 mg versus 18.9 mg) and the duration of larval stage was prolonged (14.6 d versus 18.4 d). The percentage of larvae developed into pupae was 19.7%. If it was compared with that of the control (70%), the corrected data was 28.1% that means 28% of the neonate fed on flowers would develop into pupae. Therefore, *Bt* cotton indeed has a selective pressure on the third and fourth generations of *H. armigera* that may raise a concern of resistance development in insect population.

Table 2 Effects of flowers of *Bt* cotton on the survival and development of neonate of *H. armigera*

Description	CK	*Bt* cotton
Mortality at 6 d	22.0 ± 3.7 b	46.0 ± 5.1 a
Weight of survivors (mg)	51.0 ± 4.5 a	18.9 ± 1.2 b
Duration of larval stage (days)	14.6 ± 0.1 b	18.4 ± 0.3 a
Percent larvae into pupae (%)	70.0 ± 10 a	19.7 ± 2.5 b
Percent pupae into adults (%)	82.5 ± 2.5 a	87.5 ± 2.5 a

Values in a line followed by the same letter are not significantly different ($p < 0.05$).

As far as different parts of the flower are concerned, the highest insecticidal activity is shown in petal, lowest in anther, while there is no significant difference between sepal, stigma and ovary (Table 3).

Geographic susceptibility of *H. armigera* to *Bt* protein

In China, the cotton cultivation has been categorized into 5 ecological regions: the South China (SC), the Changjiang River Valley (CRV), the Yellow River Valley (YRV), the Liaohe River Valley (LRV) and the Northwest Inland (NWI) regions, among which the CRV, YRV and NWI are the largest ones for cotton production.

Table 3 Insecticidal activity of different parts of flower of *Bt* cotton against neonate of *H. armigera*

Part	Mortality of CK (%)	*Bt* cotton Mortality (%)	Corrected mortality (%)
Stigma	16.7 ± 5.3	73.3 ± 6.7 ab	66.6 ab
Anther	20.0 ± 6.2	70.1 ± 8.2 b	62.6 b
Petal	26.7 ± 4.1	90.0 ± 10.0 a	86.4 a
Ovary	23.3 ± 4.1	75.3 ± 5.3 ab	67.8 ab
Sepal	26.7 ± 6.7	83.3 ± 0.0 ab	77.2 ab

The values followed by the same letter are not significantly different ($p < 0.05$).

For study of the geographic variability in sensitivity of cotton bollworm to *Bt* CryIAc protein, samples of bollworm population were collected from 23 sites of the above five cotton ecological regions. The dose responses to CryIAc in terms of larval mortality or growth inhibition were evaluated. Results showed that the range of LC_{50} (larval mortality) and IC_{50} (inhibiting larval growth to third instar) among different populations were 0.09-9.07 μg/ml and 0.01-0.06 μg/ml, respectively. It demonstrated that the mortality data were highly variable, while the number of larvae reaching third instar was less variable, suggesting that the latter one was desired for evaluating or monitoring the susceptibility or resistance of bollworm to *Bt* protein. Based on the IC_{50} data obtained from 23 populations, we conclude that the resistance of bollworm to *Bt* protein has not been developed in all the populations collected within China. This baseline information is very important and critical to the development of an effective monitoring program and the implementation of resistance management strategies for *Bt* cotton commercialization.

Resistance development of *H. armigera* to CryIAc after generations of selection with *Bt* cotton and *Bt* powder

The first instar larvae were fed in the laboratory with a mixed artificial feed containing either *Bt* powder or top leaves of *Bt* cotton. A 50% mortality, corrected with the mortality of non-transgenic control, was chosen as a selective pressure. The selection was continuously performed generation after generation, and the 2nd instar larvae of selected population were cultured with a mixed feed containing 20% MVP solution of *Bt* CryIAc protein purchased from Mycogen company. Two weeks after culture, the mortality of insects, the lethal concentration of CryIAc (LC_{50}), and the resistance ratio (RR, LC_{50} of selected resistant population divided by the LC_{50} of original sensitive population) were calculated. Comparing LC_{50} of the original population, the RR after 6, 11, 17 generations of selection with *Bt* cotton was increased to 1.5, 4.0 and 7.1 fold, respectively. The resistance development was more rapid than that of the selection with *Bt* powder (RR was 4.4 after 17 generations of selection) (Figure 2). Correspondingly, the resistance grade of *Bt* cotton to bollworm was reduced from HR (highly resistant) to R (resistant) and MR (medium resistant) after 11 and 17 generations of selection (Table 4).

As mentioned above, the first generation of cotton bollworm exists on wheat or other crops, and most of the second generation's larvae are killed that indicates there is no selection pressure on the first and second generations. The selection only occurs in the third and fourth generations since 28% larvae would develop into adults and produce offspring. Therefore, two generations of selection may occur each year. Based on this consideration, we predict that the *Bt* cotton may at least be used for eight to nine years. The reasons for "at least" are as follows:

In natural fields, the selection pressure may not be as rigorous as continuous selection in the laboratory, since multiple non-*Bt* crops such as corn, soybean, peanut as well as many *Solanacea* species exist in the same growing area. In all cotton cultivation areas of China, multiple crop system is adopted. Therefore, although we do not use "cotton refuge" models (for example, in the USA 25% of non-*Bt* cotton is planted together with *Bt* cotton to minimize resistance development in insect populations), the multiple crops are indeed natural "refuges".

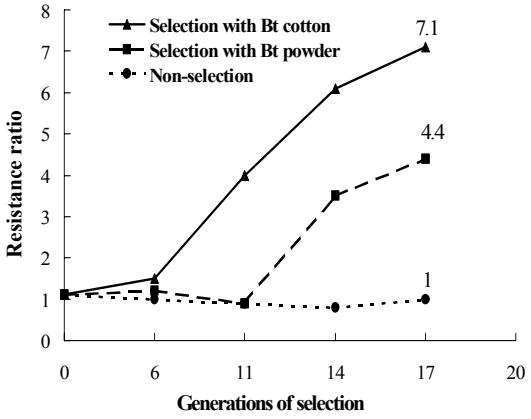

Fig. 2 Resistance ratio of *H. armigera* to CrylAc after generations of selection with Bt cotton or Bt powder

Table 4 Resistance development of *H. armigera* selected with CrylAc protein and *Bt* cotton

Generations of selection	Population	CrylAc LC_{50} (mg/l)	RR	*Bt* cotton leaves Corrected mortality (%)	Resistance
6	Non-selected		1.0	51.9	HR
	Selected	16.3	2.9	35.7	R
11	Non-selected		1.0	60.0	HR
	Selected	51.0	4.0	20.0	R
17	Non-selected	9.3	1.0	42.9	HR
	Selected	65.6	7.1	10.7	MR

RR: Resistance ratio, LC_{50} of selected population divided by LC_{50} of original population without any selection

It is known that the gene controlling resistance to *Bt* in most insects is incomplete recessive (degree of dominance: 0~-1). Therefore, the resistant population crossing with the sensitive population will still produce offspring closer to the sensitive parent.

In China, entomologists have studied the migration of cotton bollworm for decades and pointed out that this insect has a behavior of long distance migration thousands of kilometers far away from South to North. Therefore, the sensitive population from the growing area of non-*Bt* crops may migrate to the *Bt* cotton area, and natural crossing of these two populations will produce offspring resembling to the sensitive parent.

Resistance development of cotton bollworm on transgenic tobacco with single *Bt* or double *Bt*/CpTI gene

In order to minimize or delay the development of resistance in insect populations, transgenic tobacco lines with either single *Bt* gene or double *Bt*/CpTI gene were generated. The leaves of these two transgenic lines were used for selection of cotton bollworm by using the same method as indicated above. After 17 generations of repeated selection by artificial feed containing leaves of either *Bt*- or *Bt*/CpTI-tobacco, the RR to CryIAc was 13.1 and 3.0 respectively (Figure 3). To the best of our knowledge, this is the first time demonstrating by experimental data that transgenic crop with double gene *Bt*/CpTI may delay the insects to develop a resistant population.

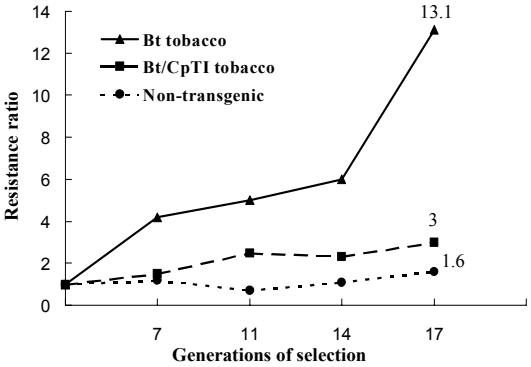

Fig. 3 Resistance ratio of *H. armigera* against CryIAc protein after generations of selection with Bt, Bt/CpTI and non-transgenic tobacco plants

Conclusion

According to the data and experience accumulated to date, a pest management strategy for commercial cultivation of *Bt* cotton can be drawn as follows:

Field performance of *Bt* cotton shows excellent ability to kill bollworm larvae of the second generation hence there is no need to apply pesticides during this period of time (May to July).

The requirement of pesticide spray in the third or fourth generation is fully dependent on the insect density in a particular location or a given year. In a common practice in some provinces, entomologists suggest 40 eggs per hundred plants as an index for pesticide spray. Apparently, egg density is not suitable for *Bt* cotton since it does not consider the larval mortality on *Bt* cotton plants. Therefore, we prefer to use larval density as an index. The exact figures (number of larvae per hundred plants) are currently under investigation and to be determined.

After survey of susceptibility of 23 populations of *H. armigera* to CryIAc protein, it is concluded that the resistance of bollworm to *Bt* has not been developed in all five cotton growing areas within China.

The multiple crop system adopted in all cotton growing regions of China may serve as a natural "refuge" to minimize potential development of resistant bollworm population.

Since the resistance grade of *Bt* cotton is decreased from HR to MR after 17 generations of repeated selection, and two generations of selection may occur each year, we predict *Bt* cotton may at least be used for eight to nine years. However, the validity and feasibility of this model established in the laboratory should be further tested under natural conditions.

The *Bt*/CpTI double gene is more efficient than single *Bt* gene both for controlling cotton bollworm and delaying the development of resistant insect population.

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Transgenic potatoes: Resistance management of the Colorado potato beetle

Ferro, D.N., Alyokhin, A.

Department of Entomology, University of Massachusetts, Amherst, MA 01003, USA

Abstract

It is important to have an appreciation of the reason that there is a niche for transgenic potato plants that express high concentrations of the Cry3A toxin produced by *Bacillus thuringiensis tenebrionis (Btt)*. This toxin is highly toxic to the larval stages of the Colorado potato beetle, the primary defoliator of potato worldwide. The beetle has developed resistance to all synthetic, broad-spectrum insecticides registered by USEPA for its control. Because of resistance problems, growers were forced to make multiple applications of these insecticides to control the beetle, until there was total failure. This situation made it cost prohibitive to grow potatoes in areas of high resistance. Transgenic potato plants that express high levels of the Cry3A toxin are virtually immune to the beetle, and can easily be integrated into IPM programs. However, the beetle is likely to develop resistance to these plants if populations of beetles are under continuous selective pressure. The selection for Cry3A resistant beetles can be delayed if a mandated resistance management plan is in place. This plan should be based on:

(1) high levels of expression of the Cry3A toxin,
(2) strategically placed refugia of susceptible beetles,
(3) monitoring by growers,
(4) incentive to growers to monitor for resistant populations,
(5) destruction of potentially resistant populations before reproduction occurs.

Biotechnology has ushered in a new era of insect pest management. Currently, the cornerstone of this technology for insect control is based on toxins produced by *Bacillus thuringiensis* (*Bt*) that have been formulated to be applied as foliar sprays, or the genes that encode for producing the toxins have been incorporated into the genome of plants. Potato, *Solanum tuberosum* L., was the first transgenic crop to be approved by the USEPA for commercial use. Before EPA approved commercial use of transgenic potato, there was an extensive review conducted to address concerns about effects of the toxin on non-target organisms, persistence of the toxin in the environment, movement of genetic material outside of crop, resistance development, etc. The primary concern at the final meeting of the EPA Scientific Advisory Panel in 1995 dealt with issues on the potential for the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), to develop resistance to the *Bt tenebrionis* (*Btt*)-toxin in transgenic potato plants. Fruitful discussions about the risks of the beetle developing resistance to the *Btt* toxin need to first include an appreciation of how populations of the beetle were being controlled before foliar *Btt* products and transgenic potato cultivars became available. And, it is important to have a thorough understanding of the reproductive biology of the beetle before the deployment of resistance management tactics. Also, it is important to compare the toxicity of *Btt* products and transgenic potatoes that express the *Btt* toxin with the broad-spectrum, synthetic insecticides registered for controlling the beetle on potato (Table 1).

In the USA, potato has a comparatively simple pest complex, including several species of pathogens and insects. The Colorado potato beetle is the most destructive insect pest of potato in North America (Ferro and Boiteau, 1993). The beetle has developed resistance to most categories of insecticides currently registered for its control (Forgash, 1985). Resistance is widespread but is most pronounced in the northeastern USA (Forgash, 1985; Roush et al. 1990) and to a lesser extent in Virginia (Tisler and Zehnder, 1990) and North Carolina (Heim et al. 1990), with isolated cases as far west as Michigan (Ioannidis et al. 1991), and in the Maritime Provinces of Canada, New Brunswick (Boiteau et al. 1987) and Prince Edward Island (Stewart et al. 1997). Because of widespread resistance by the beetle to a wide range of insecticides (Table 2), it became very costly for growers to control this pest (Table 3). This situation was the impetus for a bevy of activity to develop innovative approaches to managing the beetle. These included the use of plastic-lined trenches for capturing beetles as they colonized potato fields from overwintering sites, propane flamer to kill beetle life stages within the potato crop, vacuum collector to remove beetles and larvae from potato foliage, and trap cropping to control colonizing adults (Ferro, 1996). Although some of these tactics were very effective at reducing beetle populations, each tactic

required the growers to invest in new equipment and educate themselves in a new way to control this important pest.

Table 1 Toxicity of insecticides registered for control of the Colorado potato beetle

Insecticide	Oral LD50 mg/kg	Dermal LD50 mg/kg	Environmental Notes
azinphosmethyl	4	175	toxic to fish, birds
Bacillus thuringiensis	Toxicity Class III	Toxicity Class III	nontoxic to fish, birds
Cryolite	Toxicity Class III	Toxicity Class III	nontoxic to birds
endosulfan	23	359	toxic to fish, birds
esfenvalerate	458	>2,000	highly toxic to fish
imidacloprid	450	>5,000	
oxamyl	5	2,960	highly toxic to fish
parathion	2	50	highly toxic to fish, birds
permethrin	430-4,000	>4,000	toxic to fish
phosmet	230	>4,600	toxic to fish
rotenone	132-1,500	na	highly toxic to fish

Table 2 Abbreviated chronology of Colorado potato beetle resistance to insecticides on Long Island, New York

Insecticides	Year Introduced	Year 1st Failure	Chemical Group
carbaryl	1957	1958	carbamate
azinphosmethyl	1959	1964	organophosphate
phosmet	1973	1973	organophosphate
phorate	1973	1973	organophosphate
carbofuran	1974	1976	organophosphate
oxamyl	1978	1978	carbamate
fenvalerate	1979	1981	pyrethroid
permethrin	1979	1981	pyrethroid
fenvalerate + PBO	1982	1983	pyrethroid, synergist
imidacloprid	1995	1998	nitroquanidine

After Forgash 1985, Dively (personal communication)

The University of Massachusetts Potato IPM program implemented a Biointensive IPM program for managing the beetle that was dependent on crop rotation, delayed planting, and judicious use of foliar *Btt* products. Because of insecticide resistance and inability to control the beetle, growers were no longer able to use the broad-spectrum, synthetic insecticides to control the beetle, and they quickly embraced the Biointensive IPM Program. However, this program required growers to change the way they used insecticides, i.e. *Btt*-products had to be applied during a smaller window of time (Ferro and Lyon, 1991) than conventional insecticides. So, when imidacloprid became available as a soil-applied, systemic insecticide, growers quickly abandoned the Biointensive IPM Program. Imidacloprid could be applied once at planting and provide control of the beetle through most of the growing season. This greatly simplified beetle management but did not help in maintaining a refuge of *Btt*-susceptible beetles on farms.

Table 3 Number of insecticide applications and costs for controlling the Colorado potato beetle in the northeastern USA, 1991

Massachusetts	**Total $ US**
Biointensive IPM	
5 appl *Btt*	$102.74
IPM Cooperators (nonrotated fields)	
1 appl esfenvalerate + PBO	
6 appl *Btt*	
1 appl oxamyl + endosulfan	$171.33
Conventional Growers	
3 appl esfenvalerate + PBO	
4 appl oxamyl + endosulfan	
1 appl *Btt*	$207.00
Maine	
Southern Maine	
1 appl esfenvalerate + PBO	
1 appl permethrin + PBO	
2 appl cryolite	
2 appl oxamyl	
1 appl azinphosmethyl + endosulfan	$140.86
Aroostock County (northern Maine)	
2 appl esfenvalerate	
1 appl azinphosmethyl + endosulfan	
1 appl oxamyl	$51.37
New Jersey	
1 appl esfenvalerate	
2 appl cryolite	
2 appl oxamyl	
1 appl rotenone	$149.19
New York	
Long Island	
1 esfenvalerate + PBO + endosulfan	
1 esfenvalerate + PBO + permethrin	
1 esfenvalerate + PBO + parathion	
1 esfenvalerate + PBO + cryolite	
1 esfenvalerate + PBO + azinphosmethyl	
1 esfenvalerate + PBO + azinphosmethyl + endosulfan	
1 *Btt*	
2 cryolite	
1 rotenone + PBO + endosulfan	
1 rotenone + PBO + parathion + permethrin	
1 phosmet + permethrin + PBO + azinphosmethyl	$399.69

Ferro (1991, unpublished survey), in 1991 $ US

The introduction of transgenic potato plants that express high doses of the *Btt* toxin presented a very effective and simple means for growers to control the Colorado potato beetle; historically growers have quickly embraced new technology that is effective and easy to implement. However, the probability of rapid resistance development will increase drastically with the increased persistence and coverage of the Cry3A toxin, a condition typical for transgenic plants (Ferro, 1993). Gould (1988) and Ferro (1993) presented theoretical evidence that the beetle is likely to develop resistance to transgenic plants expressing high levels of the Cry3A toxin within six generations if the use of these plants is mismanaged. As transgenic seed becomes available, potato growers will quickly integrate this seed into their

operations. For this reason, it is imperative that we have in place, as detailed as possible, resistance management plan before transgenic potatoes gain a major share of potato production. This plan must be based on, as much as is feasible, a knowledge of the reproductive biology of the beetle and potential for gene flow between resistant and susceptible populations.

Before discussing the existing resistance management plans, we will present a summary of the seasonal biology of the beetle and recent findings on its reproductive biology. The beetle has developed resistance to all insecticides registered in the USA for its control, including the recently registered material imidacloprid. Dively (personal communication) showed beetles collected from Long Island to be 24x more tolerant to imidacloprid than a susceptible population, and Alyokhin and Ferro (unpublished) showed a field-collected Massachusetts' strain to be 30-40x more tolerant.

Colorado potato beetle life history

The beetle's success as a pest of potato is largely determined by its remarkably diverse and flexible life history (Weber and Ferro, 1994). Migrations, closely connected with diapause, feeding, and reproduction, allow this insect to employ "bet-hedging" reproductive strategies (Voss and Ferro, 1990), distributing its offspring in both space (within and between fields) and time (within and between seasons). As a result, the risk of catastrophic losses of offspring due to insecticides or crop rotation is diminished (Solbreck, 1978; Voss and Ferro, 1990). Furthermore, the beetle's mating behavior is strongly directed towards maximizing genetic variability of its progeny. After summer generation beetles accumulate at least 34 degree-days (DD) after eclosion (Alyokhin and Ferro, 1999b), both males and females perform multiple copulations with different partners (Szentesi, 1985). Multiple matings are necessary for females to realize their full reproductive potential (Boiteau, 1988). When a female mates to two different males, sperm precedence is incomplete, with the first male fertilizing 33-48% of the eggs (Boiteau, 1988; Alyokhin and Ferro, 1998). Female beetles do not usually start ovipositing until they accumulate at least 51 DD after eclosion (Alyokhin and Ferro, 1999b).

Reproductive activity is an important factor affecting insect dispersal (Johnson, 1969). Colorado potato beetle is a highly mobile insect, which is capable of moving both by flight and by walking. Adult beetles start walking soon after they emerge from the soil (Voss and Ferro, 1990). Within the first several days of their lives, the beetles generally follow two distinctly different dispersal strategies (Alyokhin and Ferro, 1999b). Approximately 75% of the newly emerged beetles walk away from the site of larval development before their reproductive system is fully mature. The other 25% of the population remain in the vicinity of emergence sites, accumulating enough degree-days to initiate reproduction. These beetles likely mate with each other, and then either move away, or stay near the place of their larval development. If these beetles developed on the same plant, they are likely to be siblings, especially within a field planted.

Colorado potato beetles overwinter in the soil as adults, with the majority of them aggregating in woody areas adjacent to fields where they have spent the previous summer (Weber and Ferro, 1993). After diapause is induced by a short-day photoperiod (de Kort, 1990), the beetles engage in a low altitude flight directed towards tall vegetation. Upon arrival to overwintering sites, they immediately burrow into the soil to diapause (Voss and Ferro, 1990), and their flight muscles undergo significant degeneration (Stegwee et al. 1963). Refractory phase of diapause, during which the beetles do not react to the change in environmental conditions, lasts for approximately three months. After that, the beetles respond to elevation of temperature above 10°C by emerging from the soil (de Kort, 1990). The beetles usually accumulate 50-250 DD before they appear on the soil surface (Yang, 1994; Ferro et al. 1999). Males and females terminate diapause simultaneously (Yang, 1994; Ferro et al. 1999), and require only 60-80 DD before they are able to mate (Ferro et al. 1999).

After emergence from the soil, overwintered Colorado potato beetles colonize potato fields both by flight and by walking (Voss and Ferro, 1990). The beetles do not start flying until they accumulate 150-200 DD (amount required for flight muscle regeneration; Yang, 1994), and beetle flight is strongly encouraged by the absence of food (Caprio and Grafius, 1990; Ferro et al. 1991; Weber and Ferro, 1996). If the fields are rotated, the beetles are able to fly up to several kilometers to find a new host habitat (Ferro et al. 1991; Weber and Ferro, 1996). Mating starts before beetles leave for the host habitat, with at least half of the population mating within the overwintering sites (Ferro et al. 1999). However, post-

diapause females need not mate in the spring to produce viable offspring; they overwinter enough sperm from the previous fall/summer matings to produce 80% of potential offspring (Ferro et al. 1991). Preliminary results showed complete sperm precedence for male sperm from spring matings (Ferro, unpublished). From a resistance management perspective, this means that a female that mated to a resistant male could produce 300-400 homozygous resistant offspring if no mating occurs with homozygous susceptible males in overwintering sites the following spring. This is one reason that it is critical that there is a high proportion of susceptible beetles in overwintering sites of resistant beetles.

Approaches to resistance management

The currently adopted Colorado potato beetle resistance management plan for transgenic potato fields relies on the use of refugia to support a population of susceptible individuals sufficient to curtail mating between resistant individuals or nullify the effects (Whalon and Ferro, 1998). Insecticide resistance is incompletely dominant in the majority of insects (Roush and Daly, 1990), including laboratory-selected Colorado potato beetles resistant to Cry 3A toxin (Rahardja and Whalon, 1995). Therefore, it is improbable that individuals heterozygous at the resistant allele (progeny of resistant x susceptible crosses) will survive high toxin concentrations expressed in transgenic plants. In recognition of this approach, planting a 20% refuge adjacent to or as close as possible to a main transgenic crop is currently required by a license agreement between commercial growers and the manufacturer of genetically engineered potatoes, NatureMark Corporation, Boise, Idaho, USA (Whalon and Ferro, 1998).

Success of the refugia/high dose approach depends on the existence of significant gene flow between resistant and susceptible Colorado potato beetle populations. Mating between resistant and susceptible beetles is likely to be encouraged by high beetle mobility and an extended period of sexual activity characteristic for this species. Increase in the flight activity of resistant beetles exposed to the Cry 3A toxin in transgenic foliage is going to increase an outflow of resistant alleles from the main crop into refugia of susceptible beetles (Alyokhin and Ferro, 1999a), where a homozygously resistant population is unlikely to establish because of its reduced relative fitness (Trisyono and Whalon, 1997; Alyokhin and Ferro, 1999c). Although homozygously susceptible populations will not be able to survive and reproduce on transgenic plants, those beetles that complete development to sexual maturity on regular plants in refugia remain reproductive even after moving to transgenic stands (Alyokhin and Ferro, 1999a). These beetles will mate with the local resistant beetles, and the resulting heterozygous offspring will not survive high doses of the Cry 3A toxin expressed in the transgenic foliage. Beetle tendency to aggregate within overwintering sites outside of the field (Weber and Ferro, 1993) will also contribute to gene flow between resistant and susceptible populations. After diapause termination, a significant proportion of post-diapause beetles mate within or near overwintering sites (Ferro et al. 1999) and sperm from spring matings take precedence over fall/summer matings (Ferro, unpublished), which should then result in heterozygous individuals. Offspring from these individuals would not survive in transgenic crops, and would mate with susceptible beetles in nontransgenic crops. Gene flow between resistant and susceptible populations could be further enhanced by the close integration of non-transgenic and transgenic potatoes. The most efficient refugia configuration could be created by planting blocks of non-transgenic plants adjacent to the transgenic fields, or by planting strips of non-transgenic plants within transgenic fields (Whalon and Ferro, 1998). The non-transgenic strips should be oriented parallel to the wooded field borders, so that susceptible beetles from refugia and resistant beetles from the main crop overwinter at the same sites, and then mate with each other in the spring (Ferro et al. 1999).

Despite being a useful tool in delaying Colorado potato beetle resistance to transgenic plants, refugia alone will not provide a “silver-bullet” solution to the problem. About 25% of recently emerged summer generation beetles stay close to the place of their larval development until their reproductive system is mature (Alyokhin and Ferro, 1999b). Therefore, a significant proportion of resistant beetles developing to reproductive adults on transgenic plants will mate with each other and leave homozygously resistant offspring. During the summer generation, even when resistant females previously mated to resistant males mate to susceptible males, up to a half of their offspring will still be homozygously resistant (Boiteau, 1988; Alyokhin and Ferro, 1998). As a result, only complete cessation of mating between resistant beetles will remove all resistant homozygotes from the population. Clearly, that is not likely to happen with the transgenic crop.

Overall efficiency of a resistant management program could be dramatically improved if spatial refugia, previously discussed, are complemented with temporal refugia in the form of crop rotation. Roush et al. (1990) demonstrated that frequency of resistant Colorado potato beetle alleles are usually lower in rotated fields when compared to non-rotated fields. However, simple rotation of purely transgenic fields to non-host vegetation will encourage long-distance flight of post-diapause adults (Caprio and Grafius, 1990; Ferro et al. 1991; Weber and Ferro, 1996). Since only resistant beetles survive highly toxic transgenic foliage, this could lead to exporting resistance to distant potato fields (Caprio and Tabashnik, 1992; Grafius, 1995), and if these homozygous resistant beetles should colonize transgenic fields, further selection will occur. Furthermore, annual crop rotations are often rejected by commercial growers for economic reasons. However, we believe that the most acceptable resistance management system should rely on combining refugia of susceptible beetles with planting fields to transgenic plants no more frequently than every third year.

Therefore, it may be beneficial to plant non-transgenic potatoes in the same field or adjacent fields (for fields rotated out of potato) during the following season. The majority, if not all, of the overwintering survivors will be susceptible beetles preserved in the refugia. Reduced general fitness of the few beetles resistant to Cry 3A toxin will place them in a severe competitive disadvantage within conventional fields (Trisyono and Whalon, 1997; Alyokhin and Ferro, 1999c), and the frequency of resistant alleles will decrease even further in comparison with the previous season. The beetle population on non-transgenic plants is likely to build up throughout the summer. However, commercial availability of imidacloprid-based foliar insecticides (Boiteau et al. 1997), or other highly efficacious insecticides, will allow growers to keep beetle densities below the economic injury level. However, it is critical that growers maintain a high proportion of susceptible beetles within these refugia; this will best be accomplished by using established IPM practices.

There are geographical variations in the Colorado potato beetle life history (Weber and Ferro, 1994), resistance development patterns (Ioannidis et al. 1991; Grafius, 1997), and cultural practices for potato production. Therefore, it is important that individual resistance management plans are adapted to local conditions. Nevertheless, we believe that the general approach to managing against resistance development to combine the relaxation of selection pressure through spatial and temporal refugia with high toxin dose expressed by transgenic plants, and early detection of resistant populations. Because resistance is likely to be a rare event, the likelihood of a monitoring program to detect such is unrealistic. It is more likely that growers, who are in their fields routinely, will be the first to notice surviving populations within the field. For this reason, it is imperative that the growers become an integral part of a monitoring program, and be provided with some incentive for closely scrutinizing their fields (Whalon and Ferro, 1998). A wide-scale resistance management program is a difficult task, and it will require a high degree of cooperation between commercial growers, biotechnology industry, and scientific/extension communities (Table 4). However, the only alternative to such a program is losing transgenic plants and foliar *Btt* products to the beetle resistance.

Table 4 Resistance management plan for delaying resistance development by the Colorado potato beetle to transgenic potatoes that express the Cry3A toxin

Recommendations	Notes
Plant refugia of susceptible plants (20% of acreage)	
adjacent to transgenic crop	between crop and overwintering sites, encourages mating between R[1] & S[2]
or, within transgenic crop	plant as manageable strips (spray width), encourages mating between R & S
Manage refugia using IPM practices	maintain high proportion of S beetles
Rotate field out of potato	force beetles to fly to new host habitat
Do not plant field 2 years in a row to transgenics	reduce selective pressure
Monitor for beetle survival in transgenic crop	grower detection
grower incentive	monetary incentive
ship larvae to be bioassayed	to determine if resistant
biochemical detection	to determine if plant (s) expresses toxin
destroy resident population	to prevent survivorship of R beetles

[1]R = resistant beetles, [2] S = susceptible beetles

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Implications of transgenic plants expressing *Bacillus thuringiensis* protein on insect natural enemies

Hilbeck, A., Bigler, F.

Swiss Federal Research Station for Agroecology and Agriculture, 8046 Zurich, Switzerland

The large-scale use of various transgenic crop plants expressing *Bacillus thuringiensis* (Berliner) (*Bt*) proteins is resulting in the increase in spatial and temporal availability of *Bt* proteins in the agroecosystems of many countries throughout the world. In current commercially available transgenic crop plants, *Bt* proteins are present throughout most of the plant during essentially the entire growing period. Further, *Bt* is expressed in relatively high concentrations and, in contrast to *Bt* insecticides, in a truncated, active form. The current and future trend in plant molecular biology is to dramatically increase expression levels of *Bt* proteins in plants with the best example being the expression of Cry1Ac in tobacco chloroplasts (McBride et al. 1995). Consequently, all herbivores (target and non-target) colonizing transgenic *Bt* plants will ingest plant tissue containing *Bt* protein which they may pass on to their natural enemies in a more or less processed form. Therefore it is necessary to verify and monitor the long-term compatibility of this new pest management strategy with natural enemies.

In this presentation, we show that Cry1Ab adversely affected *Chrysoperla carnea* (Stephens) larvae, a worldwide important biological control agent (New, 1975). In tritrophic laboratory studies using transgenic *Bt* corn plants (Cry1Ab) and the corresponding untransformed, *Bt*-free corn hybrid (both varieties were kindly provided by formerly Ciba Seeds, now Novartis Seeds, Basle, Switzerland) we studied prey-mediated effects of *Bt* corn on the mortality of *C. carnea*. Two different prey species were used in the experiments, the Egyptian cotton leafworm, *Spodoptera littoralis* (Boisduval), a lepidopterous, non-target pest (not or slightly sublethally affected by *Bt* corn) and the European corn borer, *Ostrinia nubilalis* (Hübner), the lepidopterous, target pest (lethally sensitive to *Bt* corn). Small instar *S. littoralis* and *O. nubilalis* larvae were allowed to feed on *Bt* plant leaves for a maximum of one day before they were provided as prey to *C. carnea* larvae. By then *O. nubilalis* larvae exhibited early symptoms of disease but did not die until another one to three days later. *S. littoralis* larvae did not exhibit any noticeable effects. Prey larvae were replaced every day. Total larval and pupal mortality of *C. carnea* raised on *O. nubilalis*(+) or *S. littoralis*(+) was 59% and 66%, respectively, compared to 37% when raised on *Bt* free prey of both species (Figure 1). There was no significant difference in mortality between chrysopid larvae reared on *O. nubilalis*(+) or *S. littoralis*(+) (Hilbeck et al. 1998a). Similarly, no significant difference in mortality was detected when chrysopid larvae were raised either on *O. nubilalis* (-) or *S. littoralis*(-). This suggested a direct *Bt*-induced effect.

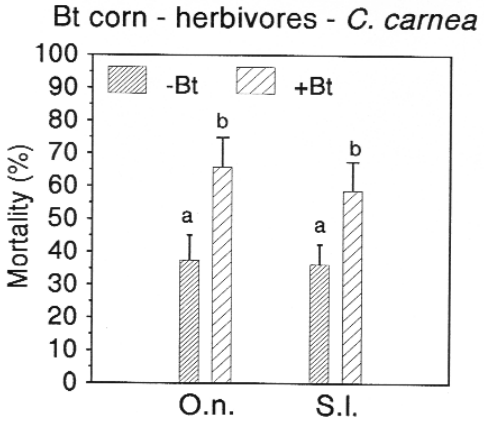

Figure 1 Total mean larval and pupal mortality [%] and standard error of *Chrysoperla carnea* larvae feeding on *Bacillus thuringiensis* corn-fed (+*Bt*) and *Bacillus thuringiensis*-free (-*Bt*) larvae of *Ostrinia nubilalis* (O.n.) and *Spodoptera littoralis* (S.l.). (Columns with different letters represent treatment means that are significantly different at p=0.05 (LSMEANS)).

To further investigate a direct *Bt*-induced effect, we fed the respective *Bt*-toxin directly to *C. carnea* larvae using a novel bioassay technique which allowed for incorporation of the activated Cry1Ab toxin into a liquid diet specifically developed for optimal nutrition of *C. carnea*. This media was encapsulated within small paraffin spheres. Because only second and third instars can penetrate the skins of the paraffin spheres, two different methods were used to rear chrysopid larvae through first instar. The first method used 0.5 cm^3 foam cubes soaked in non-encapsulated, liquid diet. For one treatment, activated Cry1Ab toxin (100 µg/ml diet) was mixed into the non-encapsulated diet whereas only an equivalent amount of water was added to the diet for the corresponding control (second treatment). The second method used *Ephestia kuehniella* (Hübner) eggs as prey during first instar. After reaching second instar, all larvae received encapsulated, artificial diet with (third treatment) or without Cry1Ab (fourth treatment), respectively. In a fifth treatment, chrysopid larvae were raised on *E. kuehniella* eggs only. When reared only on artificial diet containing Cry1Ab toxin, total larval and pupal mortality was significantly higher (56%) than in the respective untreated control (30%) (Figure 2; Hilbeck et al. 1998b). Also, significantly more chrysopid larvae died (29%) that received Cry1Ab later during their larval development compared to the respective control (17%). Only 8% of the *C. carnea* died when reared exclusively on *E. kuehniella* eggs (Figure 2). These results demonstrate that activated Cry1Ab toxin is toxic to *C. carnea* at 100 µg/ml diet. However, when considering the differences between concentrations of Cry1Ab expressed in transgenic corn and the concentration used for the direct feeding study, this result was somewhat surprising. In *Bt* corn, the concentration of Cry1Ab protein per gram fresh weight does not exceed 4 µg in leaves and was always lower in all other tissues analyzed (Fearing et al. 1997). Consequently, it is reasonable to assume that the amount of Cry1Ab toxin passed on to *C. carnea* by its herbivorous prey in the study using transgenic *Bt* corn was much smaller than the amount present in the artificial diet containing Cry1Ab toxin at a concentration of 100 µg/ml, yet mortality was similar. This suggests that interactions between the herbivores and *Bt* plants occur that either cause unnoticed secondary effects in the herbivore or further process the *Bt* protein rendering the *Bt* corn-fed prey more toxic to *C. carnea*.

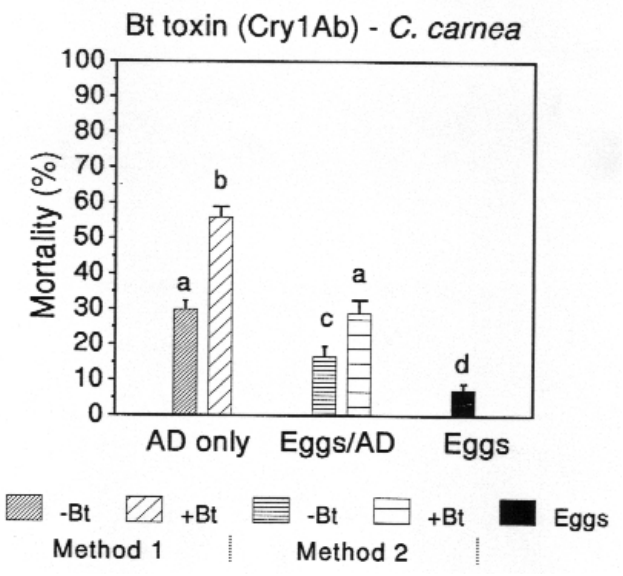

Figure 2 Total mean larval and pupal mortality [%] and standard error of *Chrysoperla carnea* larvae feeding on different types of Cry1Ab toxin-containing and untreated diets. (Means with different letters are significantly different at p = 0.05 significance level (LSMEANS); AD = artificial diet only incl. first instar; Eggs/AD = *E. kuehniella* eggs during first instar, artificial diet during second and third instar; Eggs = *E. kuehniella* eggs only).

To further explore these interactions, tritrophic studies were conducted again where different concentrations of Cry1Ab toxin (100 µg/g, 50 µg/g and 25 µg/g diet) and Cry1Ab protoxin (200 µg/g, 100 µg/g and 50 µg/g diet) and one concentration of Cry2A protoxin (100 µg/g diet) were incorporated into a meridic diet for *S. littoralis*. Raising *C. carnea* on *Bt* diet-fed prey led always to significantly higher total immature mortality (first instar to adult) (47%-78%) compared to the control (26%) (Hilbeck et al., unpublished data; Figure 3), while *S. littoralis* did not exhibit increased mortality, except at the highest Cry1Ab toxin concentration (100 µg/g) (44%). At 100 µg Cry1Ab toxin/g diet, 78% of *C. carnea* died during their development to adult. Mortality declined as Cry1Ab toxin concentration decreased. In the Cry1Ab protoxin treatments, mortality was also overall significantly higher than in the control but on average lower than in the Cry1Ab toxin treatments and no dose response could be observed. This finding suggests that either *S. littoralis* or *C. carnea* itself can process the Cry1Ab protoxin to a stage where it adversely affects *C. carnea*. These results suggest that *Bt* by herbivore (i.e. prey) interactions exist where *S. littoralis* mediates *Bt* related effects that lethally affect *C. carnea* but not itself.

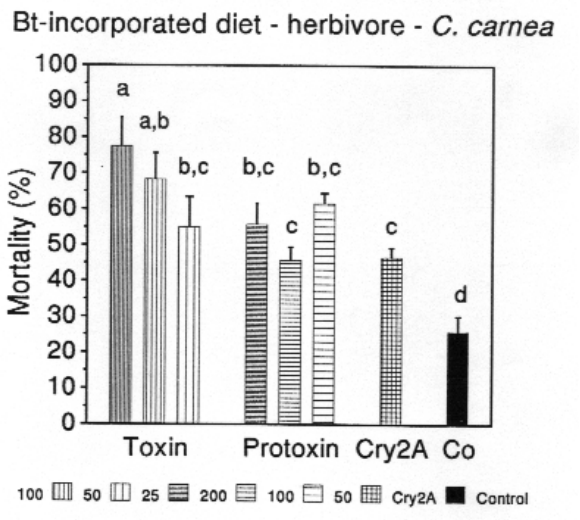

Figure 3 Total mean larval and pupal mortality [%] and standard error of *Chrysoperla carnea* larvae raised on *Spodoptera littoralis* larvae fed different types of *Bacillus thuringiensis* protein-containing (Cry1Ab toxin, Cry1Ab protoxin and Cry2A protoxin) and untreated diets. (Means with different letters are significantly different at p = 0.05 significance level (LSMEANS)).

These findings illustrate that different approaches are necessary to determine reliably the long-term agroecological consequences of transgenic *Bt* plants. Both tritrophic studies and direct feeding studies are necessary to explore insecticidal proteins expressed in plants on third trophic level organisms.

Field studies must be conducted to determine the ecological consequences of laboratory findings because performance and fitness of natural enemies in *Bt* crop fields also may affect pest resistance development (Gould et al. 1991; Johnson et al. 1997). Our laboratory results may translate into chronic long-term effects rather than acute short-term effects in the field which are difficult or impossible to detect in two to three-year field-plot trials. Some potential agroecological long-term implications are discussed. Depending on parameters such as interspecific competition among nontarget herbivores, impact of the *Bt* on nontarget pests and natural enemies, and the extent of control previously exerted by routine insecticide treatments, very different population-ecological scenarios are possible. These can range from an increase in natural enemy abundance and simultaneous decline of nontarget pests, to the contrary, a decline in natural enemy abundance and an increase in nontarget pests. Therefore, monitoring programs should be established to assess the long-term compatibility of naturally-occurring biological control with

the utilization of transgenic crop plants expressing insecticidal proteins, such as *Bt*, in order to secure sustainable use of these transgenic crop plants.

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Monitoring of microbial communities

Smalla, K. (Moderator)

Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), 38104 Braunschweig, Germany

The scope of the workshop

An adequate assessment of potential effects of genetically modified organisms on indigenous microorganisms or monitoring of the fate of genetically modified microorganisms or their DNA requires the application of new monitoring techniques. The dilemma that only a small portion of bacteria (less than 1%) are accessible by traditional standard cultivation techniques ("Great plate count anomaly") and that bacterial cells might lose the ability to grow on solid media in response to environmental stress (viable but nonculturable) can only be overcome by analyzing community DNA or RNA directly extracted from environmental samples or by the application of in situ techniques. During the past decade the application of new molecular methods has greatly improved our ability to analyze microbial communities. Biosafety researchers addressing potential effects of genetically modified organisms on the indigenous microbial community have greatly contributed to develop appropriate monitoring methods. These tools should allow cultivation-independent analysis of many samples which is essential for studying spatial and temporal variations of microbial community structures in relation to environmental factors and shifts due to perturbations. The scope of the workshop was to examine the monitoring methods that are currently available for the assessment of the environmental fate of microbial inoculants as well as the impact of genetically modified organisms on soil microbial communities with respect to their potentials and limitations. Furthermore, the workshop aimed to present examples for how these monitoring techniques had been applied in field or microcosm studies. The following talks were given:

- Marker and reporter genes in microbial ecology (J.K. Jansson, Stockholm University)
- Potentials and limitations of T-RFLP of 16S rDNA for microbial community analysis (L. Forney, University of Groningen)
- Field release, gene flux and monitoring the impact of GMMs in the phytosphere (A. Lilley, IVEM, Oxford)
- Fate and effects of genetically modified plant growth promoting bacteria in rice in China (J.D. van Elsas, IPO-DLO, Wageningen)
- Polyphasic approach to field risk assessments: alfalfa inoculated with recombinant *Sinorhizobium* with enhanced N2 fixing abilities (R. Seidler, EPA, Corvallis)
- Monitoring effects of released transgenic *Sinorhizobium meliloti* strains on rhizosphere and bulk soil microbial communities (C.C. Tebbe, FAL, Braunschweig)
- Monitoring effects of transgenic T4-lysozyme expressing potatoes on the bacterial rhizosphere population (K. Smalla, BBA, Braunschweig).

Rapporteur's comments

The unequivocal detection of microbial inoculants in different environmental habitats requires the presence of reporter genes. The choice of the reporter gene depends on the strain, the environment studied, and the question addressed. Potentials and applications of marker and reporter genes were discussed in the talk given by J. Jansson. Marker genes were applied for tracking the fate of released genetically modified *Sinorhizobium meliloti* strains in the rhizosphere of *Medicago sativa* (C.C. Tebbe), or *Pseudomonas fluorescens* in the phytosphere of sugar beets (A. Lilley).

To adequately study microbes in complex microbial communities, new techniques allowing to analyze microbes at a community level and without prior isolation rather than the traditional single-cell, pure culture analyses are required. PCR-fingerprinting based on 16S rDNA fragments amplified from DNA extracted directly from soil was the most frequently used technique in the different studies which were presented during the workshop. While the cloning and sequencing strategies are rather labour and cost intensive the recently developed fingerprinting techniques, such as denaturing gradient gel

electrophoresis (J.D. van Elsas, K. Smalla), single strand conformation polymorphism (C.C. Tebbe), terminal restriction analysis (L. Forney) or restriction analysis (R. Seidler) of PCR-amplified ribosomal DNA 16S rDNA have been applied to analyze potential effects of genetically modified organisms on indigenous soil microbial communities. Changes have to be related to microbial community shifts which are due to seasonal effects, to the field site, due to plant exudates or normal agricultural practice. Thus baseline information is needed for a sound assessment of potential effects of genetically modified organisms on soil microbial communities. In general, these fingerprinting techniques generate a profile of the most dominant bacterial populations. To study less abundant populations group-specific primers can be applied. As in all PCR-based approaches, selective amplification of genes from mixed communities by PCR may bias the analysis.

Methods available to analyze the functional diversity and potential of complex microbial communities are clearly less developed than tools to analyze their structural diversity. The easily produced substrate utilization profiles using BIOLOG microtiter plates allow for intensive spatial and temporal analysis of microbial communities. However, the approach is biased towards fast-growing bacteria and thus the metabolic fingerprints are unlikely to resemble the in situ metabolic potential. Substrate utilization patterns assayed by using the commercially available BIOLOG microtiter plates have been used to detect changes due to inoculation of *Sinorhizobium meliloti* strains (R. Seidler; C.C. Tebbe) under field conditions or due to the introduction of *Alcaligenes faecalis* into a soil microcosms (J.D. van Elsas).

All techniques have their own limitations, in particular when they are applied to complex microbial communities. Biases and limitations need to be carefully checked. Multiphasic approaches should be used to study microbial communities.

Field release, gene flux and monitoring the impact of GMMs in the phytosphere

Lilley, A.K., Bailey, M.J.

Institute of Virology and Environmental Microbiology, Laboratory for Molecular Microbial Ecology, Mansfield Road, Oxford, OX1 3SR, UK

Abstract

A plasmid-free, non-pathogenic, ribosomal RNA group I fluorescent pseudomonad, *Pseudomonas fluorescens* SBW25, was selected and chromosomally marked with two gene cassettes to allow selection on media containing kanamycin (kanr, aminoglycoside 3'-phosphotransferase) in combination with a colorimetric assay (catechol 2,3 dioxygenase, xylE), or to allow the isolation of pseudomonads uniquely able to utilize lactose and convert X-gal (5 chloro-4-bromo-3-indolyl ß-D galactopyranoside) to a blue product (lactose permease and ß-galactosidase, lacZY). The genetically modified microorganism (GMM), *P. fluorescens* SBW25EeZY6KX, was released as a seed inoculum (ca. $1x10^7$ cfu/seed) in two consecutive years to field-grown sugar beet. The GMM successfully colonised the roots and leaves of the developing crops and persisted in the phytosphere throughout growing seasons of more than 270 days. We review these results with regard to colonisation, persistence and dispersal. These studies also confirmed the genetic stability of the GMM and found, under all conditions tested, that no loss of phenotype or transfer of the marker genes could be detected. The released GMM, *P. fluorescens* SBW25EeZY6KX (lacZY, aph, xylE), was also monitored throughout the two release experiments to evaluate whether other gene transfer events to the GMM could be observed. Acquisition of mercuric resistance plasmids by the GMM was confirmed in roots and leaves in the two releases of the GMM in adjacent field sites in 1993 and 1994. In both experiments transconjugants were isolated within a mid-season window of eight weeks. These results indicate plasmid transfer is an active process in the phytosphere. The implications of this are discussed.

Introduction

Assessments of the risks from genetically modified microorganisms (GMMs) have been made *in situ* in field releases where inocula have been released into the natural environment (Wilson and Lindow, 1993; Cory et al. 1994; Lindow et al. 1988; Drahos et al. 1992; Thompson et al. 1995c; Bailey et al. 1996). Particular consideration has been given to the potential impact GMMs may have on ecosystems by disturbing natural communities or by exchanging genetic material (Tiedje et al. 1989). The survival, colonisation and dispersal of GMMs have been studied to assess the performance of inocula with biotechnological potential, to estimate risks and to better understand the microbial ecology of the natural environment. The plant surface and rhizosphere are the target of a number of releases (Wilson and Lindow, 1993) and have been the focus of many studies, including those of fluorescent pseudomonads in the rhizosphere of cereal crops such as wheat (Drahos et al. 1992; Kluepfel et al. 1991; Ryder, 1994; Parke et al. 1992; DeLeij et al. 1995).

In this report we provide a brief background summary of our studies regarding the release of a genetically modified fluorescent pseudomonad and then report and consider those results relevant to our focus at this conference: gene flux and monitoring the impact of GMMs in the phytosphere. For more detailed background to our release studies the reader may wish to consult Thompson et al. (1995c), Bailey et al. (1996); Lilley and Bailey (1997a,b), and Bailey et al. (1997).

Background to the release experiments

Our basic approach to the development of inocula with which to study the autecology of a GMM in the natural environment has been that the candidate bacteria should be selected from indigenous populations adapted to the target habitat. Over consecutive seasons we have investigated the microbiology of the sugar beet phytosphere grown at the same field site proposed for the release of a GMM (Thompson et al. 1993a,b; 1995a,b; Rainey et al. 1994). These investigations demonstrated the abundance of a group of related fluorescent pseudomonads which proliferated over consecutive seasons on root and leaf tissue. Analysis of component populations was undertaken following the identification of isolates by fatty acid methyl ester gas chromatography using commercial apparatus, Microbial Identification System, FAME-MIS (MIS, Delaware, USA) as described by Thompson et al. (1993b) and confirmed by RFLP-

ribotyping methods (Ellis et al., submitted). This work highlighted the niche adaptation of isolates where a rapid turnover of particular genotypes was observed as plant tissues developed within a season and the same genotype can be isolated from plants over consecutive seasons.

A group of related fluorescent pseudomonads which proliferated over consecutive seasons on root and leaf tissue were identified (Thompson et al. 1993a). From this population, a plasmid-free, non-pathogenic, ribosomal RNA group I fluorescent pseudomonad, *P. fluorescens* SBW25, was selected and chromosomally marked (Bailey et al. 1995). Marker gene cassettes were constructed to allow selection of bacteria on media containing kanamycin (kanr, aminoglycoside 3'-phosphotransferase) in combination with a colorimetric assay (catechol 2,3 dioxygenase, xylE), or to allow the isolation of pseudomonads uniquely able to utilize lactose (Drahos et al. 1986; Barry, 1988) and convert X-gal (5 chloro-4-bromo-3-indolyl ß-D galactopyranoside) to a blue product (lactose permease and ß-galactosidase, lacZY). The kanr-xylE cassette was inserted into one chromosomal site and the lacZY cassette inserted into another site approximately 1 Mbp distant on the physically mapped 6.6 Mbp bacterial chromosome (Rainey and Bailey, 1995), to provide a method for assessing genome stability (Bailey et al. 1995). Simple assays were developed for the sensitive detection of the GMM on defined media (De Leij et al. 1993) or by PCR amplification (Bramwell et al. 1995).

The GMM *P. fluorescens* SBW25 (lacZY and kanr-xylE) was released as a seed inoculum (ca. $1x10^7$ cfu/ seed) in two consecutive years to field-grown sugar beet. The GMM successfully colonised the roots and leaves of the developing crops and persisted in the phytosphere throughout the growing seasons of more than 270 days. When data from the two separate releases, 1993 and 1994 were compared, it was apparent that the inocula became established at significantly higher densities in 1994 in both the rhizo-sphere and phyllosphere (Bailey et al. 1996). In 1993 the GMM constituted a maximum of 6% of pseudomonads isolated from the phyllosphere of seedlings. In 1994 population densities for the GMM were up to 10,000-fold greater, and on average they represented 62% of the total pseudomonads isolated from emerging leaves sampled 100 days after planting. The differences in population densities observed were attributed to a reduction in the initial development rate of the seedlings in 1994, resulting in pre-emptive colonisation by inocula.

The GMM could not be detected on the leaves or roots (<20 cfu g-1) of overwintering plant tissue or in the soil (<1 cfu g-1). When sugar beet was resown the following season, colonies of GMM bacteria were detected by selective plating, indicating that the GMM survived at low levels in the soil to colonise germinating and developing seedlings. Detection, however, was sporadic and has not occurred in subsequent seasons.

Limited dispersal of the GMM from sugar beet to other plant species was observed and lateral dispersal through the soil could not be demonstrated. The detection of GMM bacteria that had dispersed to the foliar parts of indigenous weed species and to leaves of uninoculated sugar beet in the guard rows was rare and at the limits of detection. Field experiments were also conducted to investigate dispersal of the GMM by the phytophagous caterpillar *Mamestra bassicae* (Lilley et al. 1997). The GMM was transferred to neighbouring plants where it survived and colonised. Forty-eight days after the pupation of the introduced larvae, the GMM population had increased on the leaves and was similar to that determined for plants that had been seed-inoculated.

Transfer of the lacZY or kanr-xylE gene cassettes to indigenous microbial populations

Gene transfer was assessed by selective plating, exploiting the fact that the GMM had been constructed with two sets of marker genes inserted at separate and distant sites in the bacterial chromosome. Previous studies had shown that the chromosomal DNA flanking the kanr-xylE insert was highly conserved within population of phyllosphere pseudomonads (Bailey et al. 1995). It was assumed that the markers would be more likely to transfer to recipients which were closely related to SBW25 and would be able to recombine with homologous DNA flanking the marker sites. Using GMM selective agar (containing kanamycin and X-gal) naturally occurring kanamycin-resistant pseudomonads (white colonies) could be distinguished from recipients of the xylE-kanr gene cassette by treatment with catechol. No kanamycin resistant, catechol 2,3 dioxygenase active, ß-galactosidase negative bacteria were isolated.

Laboratory and field-based investigations with SBW25EeZY6KX confirmed the genetic stability of the GMM and under all conditions tested, no loss of phenotype or transfer of the marker genes could be

detected. The absence of direct evidence for marker gene transfer, or loss of marker gene phenotype, confirmed the genetic stability of *P. fluorescens* SBW25EeZY6KX and the suitability of locating introduced genes on the chromosome of bacteria destined for deliberate release.

Gene transfer to the released GMM

The released GMM, *P. fluorescens* SBW25EeZY6KX (lacZY, aph, xylE), was monitored throughout the two release experiments to evaluate whether gene transfer to the GMM could be observed in the changing and varied habitats described by a plant crop from germination to maturation and harvest. Acquisition of mercuric resistance was selected as a suitable natural phenotype with which to monitor gene transfer to the GMM. Mercuric resistant bacteria have been reported in many natural environments and these genes are commonly associated with mobile genetic elements (plasmids, transposons) (Silver and Walderhaug, 1992). In previous investigations we had also demonstrated that at least five groups of genetically distinct, large, conjugative mercury resistance plasmids are present as typical genetic components of the sugar beet microflora at our field site (Lilley et al. 1996). These studies therefore investigated the acquisition of mobile genetic elements from the indigenous microflora.

Transconjugant GMM (mercuric resistant *P. fluorescens* SBW25EeZY6KX) were isolated from roots and leaves in the two releases of the GMM in adjacent field sites in 1993 and 1994. In both experiments transconjugants were isolated within a mid-season window of eight weeks. In 1993 plant samples were pooled and transconjugants were isolated from two out of three plots, from mature leaves, immature leaves, rhizoplane, rhizosphere and beet core tissues. Transconjugants were found in these samples at frequencies (mercuric resistant SBW25EeZY6KX-cfu/g/ SBW25EeZY6KX-cfu/g) ranging from one in 75-100%. In 1994 transconjugants were detected in 11 out of 12 plants at frequencies ranging from one in $5x10^7$ to one in $2x10^3$ isolated GMM. Transconjugants from each sample were analysed and found to have acquired conjugative plasmids carrying mercuric resistance genes. These results demonstrate that conjugative gene transfer between bacterial populations in the phytosphere may be an event common to specific environmental conditions. The plasmids acquired *in situ* by the colonising inocula were identified as natural variants of restriction digest pattern group I, III or IV plasmids, from five genetically distinct groups of large, conjugative plasmids known to persist in the phytosphere of sugar beet at our field site (Lilley et al. 1996).

Conclusions

Plasmid transfer between natural communities of bacteria has been demonstrated in two independent, replicated experiments conducted in the phytosphere of sugar beet crops on two successive years. This observation clearly demonstrates the genetic plasticity of natural bacterial communities in a unique, "real time" field study. The transfer of plasmids to these colonisers results from an active interaction between the inocula and the indigenous microflora which normally maintain the mercury resistance plasmids. It is also apparent that distinct genetic groups of mercury resistance plasmids exist which transfer between populations of bacteria colonising sugar beet plants. Because of the different plasmid types isolated, on different sampling occasions, from root and leaf habitats, it was concluded that a large number of individual transfer events had occurred.

In this study we have restricted our analysis to the isolation and description of a transferable phenotype, i.e. plasmids conferring mercuric resistance. Given the low levels of mercury found in soil at the field site and that a single phenotype has been considered, it is reasonable to assume that our results substantially underestimate the extent of plasmid acquisition by bacteria, and by populations of *P. fluorescens* SBW25EeZY6KX in the phytosphere.

This is highly relevant to considerations regarding the open release of GMMs as considerable interest has been shown in the potential of manipulated genes to transfer from the donor and create novel phenotypes in indigenous populations. Many researches have sought (unsuccessfully) to detect introduced genes being lost from the inocula or transferred to indigenous members of the bacterial community. However, it should be taken into account that the acquisition of genes by a GMM would probably be the initial and perhaps major source of genetic novelty mediated by the mobilisation of conjugative plasmids or (as reported in other studies) the activity of transducing bacteriophage (Kidambi et al. 1994).

Tests of plasmid segregation frequencies *in situ* revealed that the failure of *P. fluorescens* SBW25EeZY6KX transconjugants to persist in older plants is not related to plasmid loss and indicates a loss of fitness. An important question is raised here as to the conditions required for the spread of mobile genetic elements or introduced genes. For example, why were these plasmids able to become established in the GMM population in the first place, and why did they then fail to persist into the late season or winter? Indications of the reasons for this may be found in an experiment where the GMM, both with and without one of the plasmids acquired in a release experiment, was released into the field in replicated plots (Lilley and Bailey, 1997). The phytosphere population densities of *P. fluorescens* SBW25EeZY6KX (lacZX, aph, xylE) carrying pQBR103 (Hgr, tra+, 330 kbp) declined significantly after seed inoculation compared with the plasmid-free variant. Throughout a mid-season period, ca. 100 days after planting, a simultaneous selection for plasmid-carrying hosts was observed in the phyllosphere and rhizosphere of field-grown plants. The recovery of these populations, to densities indistinguishable from plasmid-free inocula ($4x10^5$ cfu/g in the rhizosphere), demonstrated that phytosphere-associated plasmids conferred specific fitness advantages on host bacteria. The key question here concerns the basis for the horizontal spread and establishment of genes. The relative significance of selection and parasitism (selfish) in bacterial diversity and adaptation is discussed further in Lilley et al. (1998).

With respect to conferring fitness advantages, the potential genetic reservoir constituted by these plasmids should not be underestimated with sizes mostly between 230 kbp and 380 kbp and much of this coding capacity (and that of many other uncharacterised plasmids) remaining unresolved. Plasmids studied in phytosphere bacteria are often found to confer traits of specific ecological significance, as indicated in the experiment reported above. Plasmids may therefore confer habitat-specific, fitness-enhancing traits on introduced bacteria. The elucidation of the functions of these plasmids, and of the mechanisms and factors that stimulate transfer at the plant surface are objectives of our ongoing research.

Genetic recombination, and more specifically the horizontal transfer of genes within and between bacterial populations, is commonly regarded as an important mechanism in the selective adaptation of bacteria to changes in the local environment. Our results indicate that horizontal transfer is an active process which is, however, likely to be strongly limited by any lack of selection.

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Fate and effects of genetically modified and wild type plant growth promoting rhizobacteria in rice in China

Elsas, J.D. van [1)], Lin, M. [1, 2)], Smalla, K.[3)]

[1)] Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, The Netherlands;
[2)] Institute for Application of Atomic Energy, CAAS, Beijing, 100094, P.R China;
[3)] Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Messeweg 11-12, 38104 Braunschweig, Germany

Introduction

There are currently very few examples in the Western world of promising genetically modified (or unmodified) bacteria that have made or will make it to the market, and for which a thorough assessment of biosafety aspects has been successfully performed (Scupham et al. 1996; Van Veen et al. 1997). This apparent difficulty for commercialization has on the one hand been caused by a general lack of consistency in the efficacy of many applications, and on the other hand by regulations with respect to biosafety (Van Elsas et al. 1991; Smit et al. 1992). In fact, the risk assessment conundrum has spurred both intense debate and research as well as proposals for frameworks for better guidance of decision makers (Smit et al. 1992; Käppeli and Auberson, 1997; 1998; Covello and Fiksel, 1985). It has also temporarily delayed the exploitation of the enormous possibilities that lie ahead of us with respect to application of genetically modified microorganisms (GMMs). On the other hand, genetically modified bacterial inoculants developed in China in the late 1980's, that were initially intended to boost nitrogen fixation rates in rice cropping (Lin, 1997; You et al. 1995), have already been released for several years. Specifically, *Alcaligenes faecalis*, *Enterobacter cloacae* and *Klebsiella oxytoca* isolated from the rice rhizosphere have been proposed for application as general plant growth promoting bacteria. In a later stage, they have been modified via the introduction of an exogenous, constitutively expressed, *nifA* gene (You and Zhou, 1989, 1991; Lin, 1998, unpublished results). The modification caused wild-type strains to fix nitrogen in high ammonia-containing media, thus relieving the repression of the system by high concentrations of bound nitrogen (You and Zhou, 1989; You et al. 1995). Experiments with rice and soybean performed in fields in South China showed about 5-12% yield increases with the modified strains as compared to 3-7% yield increases when the unmodified control strains were used (You et al. 1995). Unfortunately, the fate and putative effects of the inoculants were not studied, notwithstanding the recognition that monitoring of inoculant fate and effect in soil is primordial as a prelude to large-scale commercial releases (You et al. 1995). As from 1995, Chinese rice fields that had reportedly received inoculants, were monitored to assess the prevalence of inoculant organisms. Also, a thorough assessment of fate and effects of one inoculant strain, *Alcaligenes faecalis* A1501, in soil/rice microcosms was made.

This chapter will briefly review key aspects of the latter studies which pertain to the biosafety of the released *A. faecalis* and other diazotrophic strains. First, the methods needed for monitoring the genetically modified and wild-type forms of the inoculant will be discussed. Subsequently, the inoculant's fate and effects in rice (field and microcosms) will be highlighted.

Monitoring methods for assessing the fate of rice inoculant strains in soil

General considerations

As outlined recently, a polyphasic approach to monitoring the fate and effect of inoculant strains in a soil habitat is highly recommendable (Van Elsas et al. 1998). The different strategies used for monitoring inoculant fate and effects are outlined in Table 1. For a sound appraisal of the fate of introduced inoculants, these should be monitored by a cultivation-independent method next to a cultivation method, since the use of either method alone can yield incomplete data with respect to the different subpopulations assessed (culturable versus total cell counts). In particular for biosafety reasons, the putative occurrence of viable-but-nonculturable (VBNC) cells is a concern that needs to be addressed. Cultivation methods are commonly based on selective plating, whereas methods that enumerate total cells can be based on either immunological principles (e.g. immunofluorescence) or on nucleic acids (e.g. quantitative specific PCR, whole cell hybridization, or direct dot blotting). Secondly, the effects of inoculants on the indigenous microflora can be assessed by a range of traditional and advanced methods

that assess, for instance, the soil microbial biomass size, the quantity of soil enzymes, soil functionality and microbial community structure (Smit et al. 1992; Naseby and Lynch, 1997; Van Elsas et al. 1998). As outlined in Table 1, some methods provide overall information on the microbiota in the systems studied, others specifically dissect the microbial populations into their component groups. It has been suggested that in any monitoring programme, at least one functionality-based, e.g. Biolog community-level physiological profiling (CLPP; Garland and Mills, 1991), and one population structure-based method, e.g. PCR-DGGE, should be used.

Table 1 Methods for studying the fate and effect of GMMs released into soil

Method	Explanation	References/Comments
Cultivation-based:		
Unselective plating	Overall (total) cfu counts	Van Elsas et al. 1998
Selective plating	Use of selectable markers, e.g. resistance or chromogenic markers	Van Elsas et al. 1986; 1998
Immunofluorescence colony staining (IFC)	Plating followed by staining of colonies with specific antiserum	Van Vuurde and Van der Wolf, 1995
Most probable number	Growth of dilution series & determination of extinction point	
Cultivation-independent:		
Microscopy using aspecific stains	Assessment of total microbial cells	Naseby and Lynch, 1997
Immunofluorescence (IF)	Assessment of total cells of target organism using antisera	Van Overbeek et al. 1997
Whole cell hybridization	Idem using phylogenetic probes	Hahn et al. 1992
Community DNA/Specific PCR	Detection of specific targets in total community DNA	Van Overbeek et al. 1997
Function-based:		
Soil microbial biomass	Black box approaches	Naseby and Lynch, 1997
Soil respiration	Assessing total response of community	Van Elsas et al. 1998
Soil enzymes		
Biolog CLPP	Community-level physiological profiling	Garland and Mills, 1991; Winding, 1994
Community-based:		
PLFA profiles	Community-level phospholipid fatty acid profiles: low resolution dissection of communities	Frostegard et al. 1997
PCR-DGGE/TGGE	Melting-based dissection of mixed amplicons representative of phylotypes in the community	Heuer and Smalla, 1997a; Muyzer et al. 1996
T-RFLP	Restriction-based dissection of community amplicons	
PCR-SSCP	Single-strand chain polymorphism based separation of community amplicons	Tebbe, pers. comm.

Monitoring genetically modified inoculants in Chinese rice fields

The strains used in the GMM releases in the Chinese rice fields all contained an insert composed of an intact *nptII* gene (conferring resistance to kanamycin to a wide range of hosts) adjacent to a constitutively expressed *nifA* gene, both carried on an intact copy of transposon Tn5. As both *nptII* and Tn5 are rare in soils, and the adjacent *nifA* provided an extra tag, monitoring of the genetically modified inoculants was based on an initial screening for the presence of the *nptII* gene within the population of kanamycin resistant bacteria, as well as in total microbial community DNA extracted from soil (Smalla et al. 1993; Van Elsas and Smalla, 1995). For that purpose, both an *npt*II-specific PCR/probe method and direct colony hybridization were employed, using standard molecular techniques in accordance with literature (Sambrook et al. 1989; Smalla et al. 1993; Smalla and Van Elsas, 1995; Van Elsas and Wolters, 1995).

Monitoring of fate and effect of strain A1501R in soil microcosms

To assess the fate of *Alcaligenes faecalis* strain A1501R in soil microcosms, selective plating was employed. For that purpose, a rifampicin-resistant derivative of inoculant strain *A. faecalis* 1501, denoted 1501R, was obtained by selection of mutants on rifampicin-containing agar plates. The strain selected had a stable resistance phenotype and grew at wild-type rate. Furthermore, it was easily detectable on rifampicin-containing agar plates, with a very low background (Lin et al. 1999).

To allow the detection of inoculant cells on the basis of their DNA, a probe was produced from the variable region V6 of the 16S rDNA, following the procedure of Heuer and Smalla (1997a). The V6 region of the 16S rDNA of *A. faecalis* A1501R was amplified using primers to the regions around positions 971 and 1057 (*E. coli* numbering system of Brosius et al. 1978). To validate probe specificity, about 90 strains prevalent in the soil were used to produce amplicons using primers 968f and 1401r (Heuer and Smalla, 1997a), and applied to a membrane. Hybridization with the V6 probe revealed that only two strains showed weakly positive signals, whereas all others were negative. Hence, the V6 probe was employed for the detection of *A. faecalis* A1501R targets in soil DNA in dot blot assays.

The putative effect of the inoculant strain on the soil microbiota was further assessed using two different approaches, i.e. (1) Soil DNA extraction followed by 16S rDNA based PCR and separation of fragments on DGGE (Heuer and Smalla, 1997a; Muyzer et al. 1996), and (2) Biolog CLPP (Garland and Mills, 1991; Garland, 1997; Winding, 1994). The two methods aimed to detect shifts in the microbial community population structures over time, at the phylogenetic (PCR-DGGE) and substrate utilization (CLPP) levels, brought about by the introduction of the inoculant.

Fate of rice inoculants in soil and rhizosphere

Monitoring of genetically modified inoculants in the field

In 1995, fields with mature rice plants in South China (Jiaxing and Guangzhou areas) that had received genetically modified strains containing the constructs outlined in Figure 1, were sampled (rice plants plus adhering rhizosphere soil), and the samples were processed for molecular analysis with respect to the presence of the inoculants. Colonies grown on the selective (kanamycin-containing) plates were assessed for the presence of the insert, i.e. *nifA-nptII*, via *nptII*-specific colony hybridization and PCR. Furthermore, total community DNA was obtained from these samples in order to directly detect the target sequences, without dependancy on cultivation.

The results showed that none of the samples taken in the two areas contained any vestiges of the *nptII*-containing inoculant bacteria. Several explanations can be forwarded to explain these data, i.e. (1) poor survival of the inoculant strains, or their dilution along growing rice roots, (2) loss or rearrangement of the insert, leading to an inefficient detection method, and (3) a conversion of the possibly low numbers of inoculant cells present into VBNC forms, by which these escaped the cultivation-based as well as direct detection methodology applied. In order to resolve the inconclusiveness brought about by these incidental data, further experiments on inoculant fate were performed under more controlled conditions in soil microcosms.

Monitoring of the *A. faecalis* inoculant in soil/rice microcosms

A study, fully described elsewhere (Lin et al. 1999), was set up to assess the fate and effects of *A. faecalis* strain A1501R under conditions in microcosms which mimicked the conditions of the field releases. Thus, inoculated (washed freshly-grown cells in water) as well as uninoculated flooded Flevo silt loam (FSL) soil microcosms were cropped with rice plants and kept at 28°C under a light/dark regimen. The inoculant population density was followed over time by plating on rifampicin-containing plates. It increased at the beginning of the experiment, and reached a maximum of about 10^8 CFU g^{-1} dry soil after two days in bulk soil and after 15 days in rhizosphere soil. A gradual slow decline of population size was seen thereafter. The inoculant finally kept a roughly stable population, of between 10^6 and 10^7 CFU g^{-1} dry soil, until the end of a 60-day incubation period. The numbers of inoculant CFU in the rhizosphere soil were generally similar to those in corresponding bulk soil samples. They were significantly higher only at one timepoint, i.e. 15 days after inoculation.

To monitor the inoculants total cell numbers via an assessment of the specific DNA targets, DNA obtained from uninoculated and inoculated soils from the microcosm study was subjected to hybridization with the V6 probe in a quantitative dot blot approach. The results revealed weak background signals in DNA obtained from uninoculated soils only on days zero and 40, but an absence of background at other timepoints. On the other hand, the inoculated soil samples consistently showed strong (at least tenfold the background) signals up to day 40. The intensities of the background signals equalled those from the inoculated soil only in the day-40 samples. On days zero and 15 about 10^7 copies of the target g^{-1} soil were estimated to be present, whereas these declined to about 10^6 cfu g^{-1} soil after 30-40 days. These estimations corroborated the population size determinations obtained by selective plating. Hence, the data obtained did not provide evidence for the occurrence of substantial numbers of VBNC inoculant cells. Rather, they suggested that the majority of *A. faecalis* cells were trackable on selective rifampicin plates under the conditions used.

Monitoring of effects of the *A. faecalis* inoculant on microbial numbers, community structure and metabolic potential

The total counts of culturable bacteria in bulk and rhizosphere soil samples (10^8-10^9 CFU g^{-1} dry soil) remained roughly stable over time, between bulk and rhizosphere soil and between inoculated and uninoculated soil microcosms (Lin et al. 1999). In spite of this stability of the total microbial numbers, there might have been shifts in the population structure not detectable by the enumeration method employed. Moreover, only a fraction of the total bacterial population (0.1-10%) is often detected on common agar plates (Bakken, 1997). Hence, we employed 16S rDNA based PCR with soil microbial community DNA as the target followed by DGGE community profiling to assess putative changes in bacterial community structure due to the release. The DGGE analysis first showed that a strong band, absent from profiles of uninoculated soil, was consistently visible in profiles derived from inoculated soils, up to day 15 in bulk soil and up to day 30-40 in rhizosphere soil samples (Lin et al. 1999). The band migrated to the same position as the PCR product generated with strain A1501R. Use of the *A. faecalis* strain A1501R specific V6 probe consistently hybridized to these bands.

As outlined elsewhere (Lin et al. 1999), dissection of the DGGE patterns showed that a limited number (6-7) of dominant bands and about 30 weak bands were present in virtually all samples. The dominant bands were similar between the profiles obtained for control and inoculated samples, whereas the weak bands were variable. Clustering of the bulk soil-derived profiles via the unweighted pair group method with mathematical averages (UPGMA; Dice coefficient of similarity) confirmed the resemblance (>90% similarity) between all profiles, suggesting the absence of a major effect of the inoculation. The rhizospheric DGGE profiles clustered together at about 80% similarity, and again no effect of inoculation was found. There was an effect of incubation time (root age), as at about 92% of similarity, three clusters could be formed, i.e. the day-40 samples, the day-30 samples and the day-zero plus day-15 samples, irrespective of the presence or absence of the inoculant (Lin et al. 1999).

To assess the effect of strain A1501R on the metabolic potential of the soil microbial commmunity, CLPP was analyzed over time using the Biolog GN system (Garland and Mills, 1991; Garland, 1997; Heuer and Smalla, 1997b). The potential for utilization of the 95 substrates by the soil microbial communities remained, with a few, varying, exceptions, largely unchanged due to the release. Overall, only 10 of the 95 substrates could not be utilized at all by the microbial communities of the FSL soil. Up to about 60 other substrates were utilized to a similar extent between inoculated and uninoculated soils, whereas the remaining substrates were used differentially. An analysis performed after 23 days of incubation indicated that the (integrated) responses to about 70 of the 95 BIOLOG substrates were similar between the microbial communities from inoculated or uninoculated bulk or rhizosphere soils. Among the substrates that revealed differences, three groups of substrates could be distinguished, (1) a group that suggested an effect of the rhizosphere, (2) a group indicating an effect of the inoculant, and (3) a group that indicated an effect of the inoculant in conjunction with the rhizosphere (Table 2). Whereas the effect of the inoculant in some cases might be attributed to its metabolic capacity, in other cases it was probably indirect (Table 2).

Table 2 Contribution of strain A1501R to CLPP as evidenced by Biolog*

Substrates	Effect	Explanation/comments (number of BIOLOG gn plate* or name)
Analysis of total substrates:		
Group I:		
02, 15, 20, 93	Rhizosphere	Substrates that reveal different responses between rhizosphere and bulk soil communities
Group II:		
17, 66, 70, 74, 79	Inoculant	Substrates that reveal different responses between soil communities with versus without the inoculant strain
Group III:		
06, 08, 22, 28, 30, 32, 39,47, 48	Rhizosphere/inoculant	Substrates that reveal greater responses by 52, 54, 55, 58, 73 communities from inoculated soil in the presence of the rhizosphere
Analysis of six selected substrates:		
Lactic acid	Strongly selective	Inoculant highly competitive in the presence of lactic acid
Glycogen, dextrin, citric acid, p-HPAC**	Indifferent	Inoculant develops roughly at rate of selected competitors
Asparagine	Deselective	Inoculant is poor competitor in the presence of asparagine

*Biolog (1993); **p-HPAC: p-hydroxy phenylacetic acid. Upper data are from an analysis on day 23, the lactic acid and asparagine data from that on day 40.

To assess the effect of the inoculant on the responsiveness of the communities present to selected Biolog substrates, PCR-DGGE as well as selective plating were applied to communities present in wells containing dextrin, glycogen, citric acid, p-hydroxy phenylacetic acid, lactic acid and asparagine. Following incubation, the number of bacteria was about 10^9 CFU ml^{-1} in all wells. The density of strain A1501R in the lactic acid well was also about 10^9 CFU ml^{-1}, whereas it was around 10^6 CFU ml^{-1} in wells containing dextrin, glycogen, citric acid and p-hydroxy phenylacetic acid. In the asparagine-containing well, strain A1501R was not found. PCR-DGGE confirmed the effect of all substrates in selecting communities of low complexity from soil. The data further corroborated the observations made by plating, i.e. positive selection of the inoculant by lactic acid, its counterselection by asparagine, and its apparent indifference in the presence of the other substrates. A1501R was therefore a dominant strain in the lactic acid-utilizing bacterial community, whereas it was a minor player in those degrading the other substrates. Thus, CLPP served to ascertain the conditions under which the inoculant has a competitive edge in the soil microbial community (viz lactic acid), as well as the conditions under which it might be inhibited.

Concluding remarks

The inoculants described in this study probably affected rice crop yield by a combination of two effects, i.e. nitrogen fixation and plant growth promotion via plant growth hormones (Ueckert et al. 1990; Lin and You, 1989; Lin et al. 1992). However, in spite of the fact that the modified strains were reported to result in higher crop yields than the respective wild types, the relative contribution of the two effects is an open question. Moreover, the lack of data on inoculant fate in the field still precludes a sound assessment of its ecology with respect to the mechanism of the effects on crop yield. Given the potential of these strains, future work in this area should be strongly encouraged.

The data from the microcosm study indicated that *A. faecalis* A1501R was probably able to maintain a high population level in the rice rhizosphere for an extended period of time. As this assumption is supported by data from platings, dot blot hybridization of soil DNA with the V6 probe and from the

DGGE patterns, these methods detected primarily culturable and, by exclusion, no VBNC cells, of strain A1501R. Similar slow declines of inoculant bacteria in FSL soil have been found before for fluorescent pseudomonads (Van Elsas et al. 1986; Van Overbeek et al. 1997). Also, Compeau et al. (1988) described this behaviour for a range of rifampicin resistant *P. fluorescens* mutants. The silt loam soil can confer protection to inoculant strains by its clay content, however, progressive predation by protozoa is the likely cause of the declines observed (Van Veen et al. 1997). Strain A1501R showed a response to the rice roots, which was significant at one time point, i.e. 15 days. It is likely that the exudates that become increasingly available in the vicinity of the rice roots (Lin and You, 1989) peaked after 15 days, activating the inoculant. However, more work is needed to fully understand the time course of liberation and specificity of these nutrients, as this will promote our understanding of the rhizospheric plant growth promotion activity.

The PCR-DGGE profiles generated with soil DNA were in principle useful to detect bacterial community shifts, as a result of the release, including communities that have hitherto been unseen (Duineveld et al. 1998; Heuer and Smalla, 1997a; Muyzer et al. 1996). However, the DGGE profiles obtained revealed a picture of gross stability in the dominating bands, which presumably represent numerically dominant species. This stability was seen over time, as well as when rhizospheres or bulk soils of inoculated versus uninoculated soils were compared. Therefore, the data were indicative of the stable presence of these major contributors to the patterns, irrespective of the presence or absence of strain A1501R. Stable PCR-DGGE profiles have also been observed by Duineveld et al. (1998) for bacterial populations in the Chrysanthemum rhizosphere. The presence of inoculant strain A1501R thus did not drastically affect the dominant members of the microbial communities in flooded FSL soil. Microbial commmunities in active ecosystems may have the capacity to maintain stability in structure and consequently to blur effects of introduction of bacterial inoculant strains. Reichardt et al. (1997) also reported that a comparison between bulk and rhizosphere soil revealed no significant differences in microbial community structure in rice fields, as evidenced by comparing groups and ratios of directly extracted phospholipid fatty acids. In our study, however, an effect of time was noted in the rhizosphere samples, and this was indicative of population changes as a result of root growth. It is thus likely that changes in root exudation affect microbial community structure and activity more strongly than the addition of an inoculant strain.

The changes of the CLPP patterns observed between control and inoculated soils were intriguing, and further analysis should point out how significant effects are over time. The presence of strain A1501R mainly affected the functional diversity by changing the magnitude of the response to some substrates. On the other hand, the analysis of the community responses to selected substrates by plating and PCR-DGGE (Smalla et al. 1998) indicated the potential for a drastic effect of the presence of strain A1501R in the community under, e.g., lactic acid selection. It was, however, clear that only a few bacterial types out of the total diversity in soil could thrive in the Biolog wells. Obviously, strong selection for quick adaptors to the copious new substrate takes place in these wells (Smalla et al. 1998), which makes Biolog a poor method for monitoring the *in situ* microbial activity. The *A. faecalis* inoculant was clearly well suited to thrive on lactic acid in the presence of indigenous competitors.

Overall, the association of strain A1501R with rice may have affected the potential functional diversity of the bacterial community more than the structural diversity. However, in this study no evidence was obtained that could relate these or other effects of the inoculant to effects on the plant. Such effects are of great importance if the rice growth promotion is to be fully understood and exploited (e.g. Fujii et al. 1991). The current research on inoculant fate and effects will ultimately provide the basis for an environmentally sound strategy for the application of modified or wild-type *Alcaligenes faecalis* in commercial rice cropping in China.

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Polyphasic approach to field risk assessments: Application to transgenic Alfalfa inoculated with recombinant *Sinorhizobium meliloti* with enhanced N2-fixing abilities

Widmer, F.[1], Porteous, L.A.[2], Donegan, K.K.[3], Doyle, J.D.[4], Seidler, R.J.[2]

[1)] National Research Council;
[2)] US EPA, Western Ecology Division, National Health and Environmental Effects Research Laboratory;
[3)] Dynamac Corp., 200 S.W. 35th Street, Corvallis, OR 97333, USA;
[4)] Oregon Dept. of Human Resources, Salem, OR 97310, USA;

Abstract

A field test with transgenic alfalfa and recombinant *Sinorhizobium meliloti* was performed in 1995/96 in Corvallis, Oregon. Parental and two transgenic lines (alpha amylase or lignin peroxidase producing) of alfalfa were either not inoculated or inoculated with parental *S. meliloti* strain RMBPC, antibiotic-resistant strain RMB7201, or antibiotic-resistant and enhanced nitrogen-fixing strain RMBPC-2. Over two growing seasons plant and rhizosphere soil samples were obtained from triplicate plots of the 12 treatments. The polyphasic approach involved population counts of indigenous soil bacteria, fungi, nematodes, protozoa and microarthropods; identification of nematodes and microarthropods; soil microbial respiration; metabolic and DNA fingerprinting of soil microbial communities; plant biomass and chemical analyses; soil chemical and enzyme activity analyses; and monitoring of the recombinant inocula in the field. The lignin peroxidase producing plants had a significantly lower biomass, and higher nitrogen and phosphorous contents than the other plant types. Significant differences for other parameters were also detected for this transgenic line and included distinct metabolic fingerprints (Biolog™ GN plates) of the microbial rhizosphere communities; higher population levels of culturable, aerobic spore forming and cellulose-utilizing bacteria; lower soil dehydrogenase and alkaline phosphatase activities; and higher rhizosphere soil pH. Selective plating, colony hybridization, and PCR methodologies were used to identify recombinant inocula recovered from the field. During the first year, only inoculated rows tested positive for recombinant *S. meliloti* with counts up to 5.6×10^4 CFU/g dry wt soil(gdws). In the second year, counts increased in inoculated rows by about an order of magnitude to up to 8.7×10^5 CFU/gdws. After unusual flooding of the plot in the winter of 1996, the recombinant *S. meliloti* also became detectable in rhizospheres of uninoculated alfalfa plants across the field plot. The portion of positively tested uninoculated locations increased during the second year and counts were up to 1.2×10^5 CFU/gdws. Off-plot monitoring at the end of the experiment in the fall of 1996 revealed one positive location at 3 m distance from the inoculated field. In the fall of 1996 the plot was sprayed with herbicide and disced in 1997. Two additional post experiment monitorings in the summers of 1997 and 1998 revealed persistence of the recombinant inocula in the field with counts up to 2.5×10^4 CFU/gdws in July 1998. Off-plot monitoring in 1998 revealed two positive locations at 3 m distance from the inoculated field. The results of this study demonstrated that genetically engineered organisms are capable of causing distinct and detectable changes in soil ecosystem components. Furthermore, recombinant sinorhizobia established in the field, spread to nearby uninoculated alfalfa rhizospheres and still persist in the field.

Introduction

Sinorhizobium meliloti [formerly *Rhizobium meliloti* (De Lajudie et. al. 1995)] is a symbiotic nitrogen-fixing bacterium which induces root nodule formation on leguminous plants. Inoculation of plant seeds or seedling roots with *S. meliloti* may increase the number of root nodules and the plant biomass (Athar and Johnson, 1996; Horikawa and Ohtsuka, 1996; Rice et al.1995). In order to increase the plant biomass of agronomically important plants such as alfalfa, efforts have been made to genetically engineer these microorganisms for improved nitrogen-fixation capabilities (Bosworth et al. 1994; Scupham et al. 1996).

The environmental release of genetically engineered microorganisms (GEMs) is controversial. Several issues have to be considered when assessing potential risks (Seidler et al. 1998). Gene transfer to indigenous microorganisms with a different host plant range may result in unintended effects on nontarget plants. Survival, dissemination, and competitiveness of the recombinant organisms also represent critical factors that are best addressed in small-scale field studies (McClung and Sayre, 1994).

Results of previous studies indicated that little dispersal of the *S. meliloti* strains occurred and that no increased competitiveness was found as compared to parental strains (McClung and Sayre, 1994).

In a four-year field study with parental and transgenic alfalfa plants inoculated or not inoculated with parental or recombinant *S. meliloti* strains, various endpoints of possible differences in effects of parental versus genetically engineered organisms on an agro-ecosystem were investigated (Donegan et al. 1998). The objectives of the study were to evaluate measurable parameters indicating ecologically different effects of genetically modified versus parental organisms and to develop and apply traditional and molecular tools for identification and monitoring of recombinant *S. meliloti* strains in the field. Here we report on the results obtained from monitoring the field plot over two growing seasons and then for two years after the field test was completed.

Material and Methods

Plants and Microorganisms

Alfalfa plants (*Medicago sativa* L.) parental line RSY27 and two genetically engineered lines expressing α-amylase or lignin peroxidase (Austin et al. 1995) were kindly provided by Dr. Sandra Austin (University of Wisconsin, Madison, WI). *S. meliloti* parental strain RMBPC and two recombinant strains RMB7201 (antibiotic resistance tagged) and RMBPC-2 (engineered for improved nitrogen fixation) (Bosworth et al. 1994) were kindly provided by Dr. Eric Triplett (University of Wisconsin, Madison, WI).

Alfalfa propagation, inoculation, and planting

The experimental design of this field study has been described before (Donegan et al. 1998). Briefly, alfalfa was grown from cuttings in the greenhouse. After nine weeks, on the day of planting in the field, plants were recovered and trimmed. Inocula were prepared and provided by Dr. E. Triplett. Cells were stored and transported at –70°C from the University of Wisconsin. For inocula preparation, cells were washed by centrifugation and adjusted to 10^7 cells/ml. Plants were placed for ca. 30 min in the inoculum and planted by hand in the field with 30 cm spacing within rows and 1 m spacing between rows. Special care was taken in order to not cross contaminate treatment rows by keeping planting crews separate, wearing gloves and booties and using aseptic tools.

Design and maintenance of experimental field plot

The 60 x 20 m experimental field plot, located at Oregon State University's Hyslop Experimental Field Laboratory in Corvallis, Oregon, has also been described previously (Donegan et al. 1998). The soil of the plot was classified as Woodburn silt loam (6.3% sand, 72.2% silt, 21.5% clay). During the study, total soil nitrogen content ranged between 0.12 and 0.17 % and the soil pH ranged from 6.1 to 6.9. Twelve experimental treatments were established: the three genotypes of alfalfa were either not inoculated or inoculated with one of the three *S. meliloti* strains RMBPC, RMB7201, and RMBPC-2. The experiment was organized in a randomized block design where each row represented a separate block (Figure 1). Each treatment was prepared in triplicate resulting in a randomized arrangement of 36 rows. Each row contained 36 plants designed for nine samplings with four plants each. The experimental plot was surrounded by two rows of buffer parental plants.

Sampling, extracting, and selective plating for recombinant *S. meliloti*

At each sampling day 3x10 cm soil cores were recovered from all four sides around each of the four plants in a sampling station. The 16 cores from one station were pooled resulting in a total of 36 soil pooled samples per sampling day (three replicates of 12 treatments). A sub-sample was used for dry weight determination. Ten grams from each sample were used for microbial extraction with a Multi Wrist Shaker (Lab-Line Instruments, Inc., Melrose Park, IL) in sterile water. Serial 10 fold dilutions were plated on M/RDM-N agar plates (Bosworth et al. 1994). For selective cultivation of the recombinant *S. meliloti* strains RMB7201 and RMBPC-2 resistances to spectinomycin (500 µg/ml), streptomycin 200 µg/ml), and trimethoprim (10 µg/ml) were used. Cycloheximide (100 µg/ml), and benomyl (100 µg/ml) were added to prevent fungal growth.

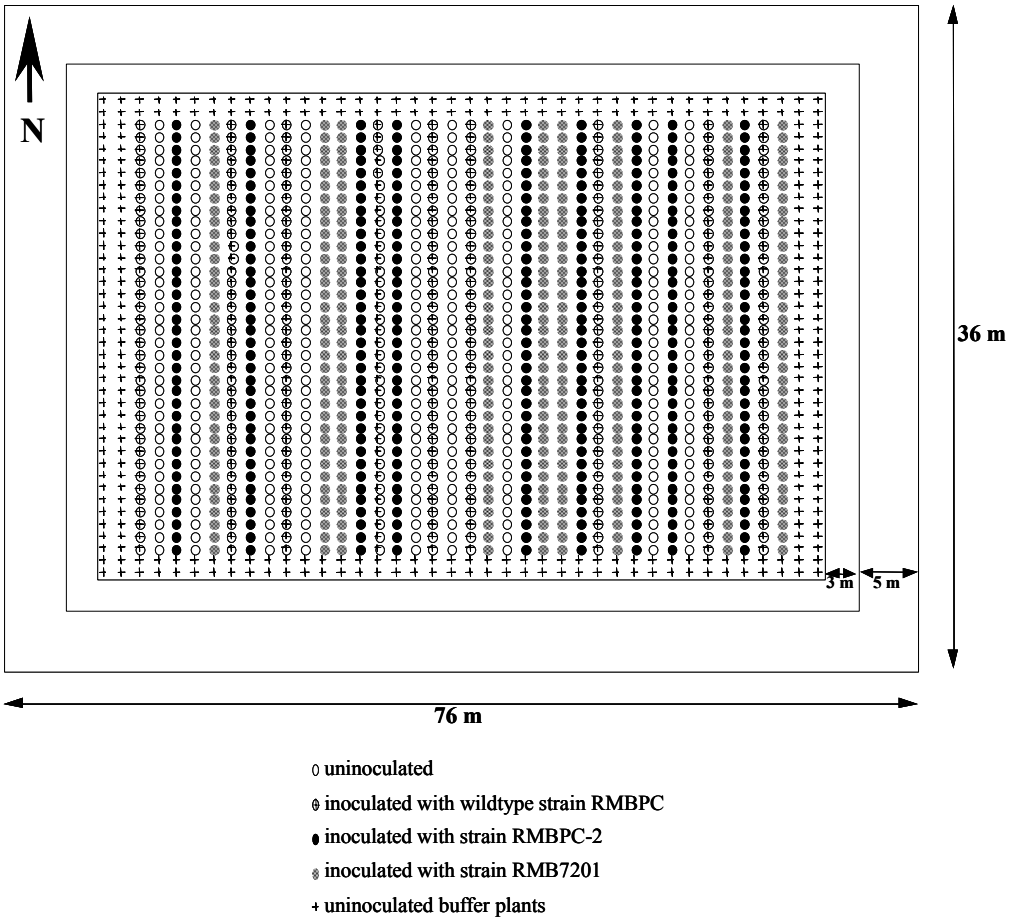

Figure 1 Map of the field plot with the inoculation mode of the alfalfa plants. The area shown off the plot was monitored for inoculum dispersal at distances of 0 m, 3 m, and 8 m

Molecular Analyses

For *S. meliloti* strain identification two PCR primer sets were developed based on specific sequences present in the recombinant strains (Bosworth et al. 1994). Primer set 1 yielded differently sized PCR products for each of the two recombinant *S. meliloti* strains RMB7201 and RMBPC-2. Primer set 2 was specific for strain RMBPC-2. PCR was performed according to standard protocols (Widmer et al. 1996) on cells from colonies or on nodule homogenates added directly to the PCR.

For colony hybridization two probes were prepared. Amplification products with primer set 1 and primer set 2 were obtained from pure cultures of *S. meliloti* strains RMB7201 and RMBPC-2 respectively. The products were labeled with digoxigenin using random priming with the Genius System according to the manufacturer's recommendation (Boehringer Mannheim, Indianapolis, IN). The resulting hybridization probes were the REC-Probe (PCR primer set 1 product) and PC2-Probe (PCR primer set 2 product). Filter lifts from either control or experimental treatment plates were performed using standard methods (Boehringer Mannheim Biochemicals, 1992; Sambrook et al. 1989) and were probed with the REC-Probe and the PC2-Probe.

Results and Discussion

A summary of the results on effects of genetically modified organisms on the field ecology

Alfalfa plants and rhizosphere soil were analyzed and results published and described elsewhere (Donegan et al. 1998). These analyses included metabolic and DNA fingerprinting of soil microbial communities; soil microbial respiration; population counts of indigenous soil bacteria, fungi, nematodes, protozoa and microarthropods; identification of nematodes and microarthropods; plant biomass and chemical analyses; and soil chemical and enzyme activity analyses. The lignin peroxidase producing plants had a significantly lower biomass, and higher nitrogen and phosphorous contents than the other plant types. Distinct metabolic fingerprints, based on substrate utilization patterns on Biolog™ GN plates, were exhibited by the soil bacterial communities associated with the three alfalfa genotypes and those for the lignin peroxidase producing plants were the most unique. Significantly higher population levels of culturable, aerobic spore forming and cellulose-utilizing bacteria, lower soil dehydrogenase and alkaline phosphatase activities, and higher pH, were also associated with the lignin peroxidase producing plants. These results suggest that the release of genetically engineered organisms can cause distinct and detectable changes in some components of the soil ecosystem.

Identification of the recombinant *S. meliloti* strains RMB7201 and RMBPC-2

For differentiation of the recombinant *S. meliloti* strains, DNA based tools had to be developed and validated. PCR with the specific primer sets 1 and 2 was shown to produce characteristic and specific amplification products for the recombinant *S. meliloti* strains. The parental strain RMBPC did not yield amplification products with either primer set while each of the two recombinant strains yielded differently sized products with primer set 1. Strain RMBPC-2 only gave a product with primer set 2. The size of this product did not correspond to the one expected from the sequence analysis. Since primer set 2 reproducibly gave the specific result and allowed identification of RMBPC-2, amplification with primer set 2 was maintained. Based on these PCR analyses a clear differentiation of the three genotypes of *S. meliloti* was possible. For efficient monitoring of the alfalfa field a sole PCR approach was not suitable. Because the recombinant *S. meliloti* strains were tagged with antibiotic markers, selective plating was chosen as a first step for selectively recovering presumptive inocula from the field. In order to avoid labor intensive PCR analysis of colonies from the large number of samples, an additional, more rapid assay was sought. The PCR products obtained with primer sets 1 and 2 from pure cultures of *S. meliloti* strains RMB7201 and RMBPC-2, respectively, were labeled and used as hybridization probes for colony hybridizations. The theoretically predicted sequence amplified by the two primer sets would not allow us to distinguish the strains if used as hybridization probes. Since primer set 2 gave an unexpected product size, it was labeled and tested in colony hybridizations. The hybridization experiments revealed that the product obtained with primer set 1 hybridized to colonies of both recombinant strains RMB7201 and RMBPC-2. The probe was therefore labeled REC-probe. The product of primer set 2 hybridized to strain RMBPC-2 only and was labeled PC2-probe. Neither probe hybridized to the wildtype strain RMBPC. However, because the sequences encoded by the hybridization probes were naturally occurring sequences, and it was found that the antibiotic plates were not perfectly selective for the inocula only, these probes did not allow an unambiguous identification of field isolates. Rather, they allowed a rapid screening of filters lifted from the selective plates for colonies containing homologous sequences. For positive identification, the colonies with hybridization signals had to be tested by PCR and sizing of the products. Furthermore no tool for positively identifying the wildtype strain RMBPC could be found, preventing a direct monitoring of this inoculum. Therefore the monitoring scheme that was established for use in the field consisted of the three steps shown in Table 1. Colonies positively identified by this procedure were counted and numbers were normalized with the dry weight equivalents of the soils they were extracted from.

Table 1 Regime developed for monitoring the recombinant *S. meliloti* strains

I	Selective plating based on recombinant genomic antibiotic resistance markers.
II	Colony hybridization for identification of colonies encoding the genes used for engineering the *S. meliloti* strains.
III	PCR for confirmation of the recombinant nature of the colonies with positive hybridization signals, and sizing of PCR products for positive identification of the *S. meliloti* strains.

Monitoring the recombinant *S. meliloti* strains in the field

The field experiment was started on June 12, 1995. During planting and maintenance of the field extreme care was taken in order to prevent cross contamination of rows with inocula. Monitoring of the recombinant *S. meliloti* strains was performed two months after planting on August 15 and again on October 10, 1995. It was found that all sampling locations contained the expected inoculum type indicating that no cross contamination or spread of the inoculum had occurred (Figure 1 and Table 2). Furthermore an increase by one order of magnitude was detected between August and October indicating that the *S. meliloti* strains had established in the alfalfa rhizospheres. In the winter of 1995/96 unusually heavy rainfall caused severe flooding of the area including the experimental field. On April 26, 1996 root nodules and the soil associated with the roots of plants on the field and in the east and west buffer zone were monitored. Pools of 10 nodules were screened from either inoculated or uninoculated plants. All nodule samples for RMB7201 inoculated plants, and a third of the RMBPC-2 inoculated plants were positive for the inoculum only whereas all uninoculated plants were negative. In one soil sample from the east buffer zone, one colony was found that tested positive indicating minimal spread beyond the field borders. Monitoring of the field was also done on June 4, 1996. Counts detected in inoculated rows were comparable to those found in October of the previous year. However, in contrast to the previous year, four of 18 uninoculated rows were found to be positive for recombinant inocula, and four of the 18 specifically inoculated rows were positive for the other recombinant inoculum. On July 30, 1996, the numbers of counts increased again for both recombinant strains in inoculated and uninoculated rows. Eleven uninoculated rows were found to be positive for the recombinant strains and five inoculated rows were cross contaminated. At the end of the field trial the field was again sampled on October 1, 1996. A slight decrease in the counts was detected in most cases. Only uninoculated rows showed a dramatic increase in average counts for strain RMBPC-2. On October 30, 1996, 30 locations at 0 m (buffer), 3 m, and 8 m off the field, and also nodules from inoculated and uninoculated alfalfa plants were monitored. In the buffer zone, five of 10 locations were positive. At 3 m distance, one of 10 locations was positive and at eight m all 10 locations were negative. All nodule samples for inoculated plants were positive for the inoculum only whereas all uninoculated plants were negative.

Table 2 Molecularly confirmed counts[a] of recombinant *S. meliloti* within the field plot

Date		Strain RMB7201		Strain RMBPC-2	
		uninoculated	inoculated	uninoculated	inoculated
1995	August 15	ND	3.54 ± 0.118	ND	3.28 ± 0.10
	October 10	ND	4.37 ± 0.082	ND	4.20 ± 0.15
1996	June 4	0.40 ± 0.19	4.19 ± 0.19	0.58 ± 0.23	4.07 ± 0.19
	July 30	1.76 ± 0.37	5.49 ± 0.09	0.73 ± 0.30	5.42 ± 0.15
	October 1	1.63 ± 0.30	5.27 ± 0.10	2.82 ± 0.25	5.32 ± 0.06
1997	July 10	1.03 ± 0.51	3.64 ± 0.49	0.97 ± 0.49	2.76 ± 0.25
1998	July 7[b]	2.08 ± 0.14		1.69 ± 0.28	

[a] Log CFU/gram dry weight soil ± standard error of the mean or none detected (ND)

[b] After discing the field in the fall of 1997 it was not possible to distinguish uninoculated and inoculated locations.

After the field experiment was terminated in fall 1996, the field plot was sprayed with herbicide and left uncultivated. Two post experimental monitoring surveys were performed: 1) On July 10, 1997 when 11 locations on the field plot were monitored a decrease of average count numbers was found as compared to the previous sampling. However, counts were still about at the level of the first sampling. Three of eight uninoculated rows were positive for recombinant inocula, and none of three specifically inoculated rows was positive for the other recombinant inoculum; 2) After the field was tilled in 1997 and remained fallow for one year, the area was monitored again on July 7, 1998. Soil samples from six locations on the field plot and the 30 previously sampled locations off the field were analyzed. Of the six field plot samples, all tested positive for strain RMB7201 and four were positive for strain RMBPC-2. Counts ranged between 63 CFU and $2.5x10^4$ CFU/gdws. In the buffer zone, seven of 10 samples tested positive. At a 3 m distance, two of 10 locations were positive and at 8 m all 10 locations were negative. In addition, clover nodules were collected and pools of 10 nodules were screened with PCR for the presence of the recombinant genes. None of the clover nodule pools from the six locations within the field plot or four locations off the plot were positive.

Conclusions

A polyphasic approach was used in this study to assess the biosafety of genetically modified alfalfa and *S. meliloti* in a field release. It was revealed that engineered organisms can cause distinct and detectable changes in soil ecosystem components compared to the corresponding wildtype controls. Gene probes were designed for specific and sensitive identification of the recombinant *S. meliloti* strains recovered from the field allowing for specific enumeration when combined with selective plating. This enumeration method revealed that recombinant strains became established at the site and spread throughout the plot even in the absence of alfalfa plants. We speculate that the *S. meliloti* strains have now become a permanent part of the indigenous flora at the field site. In the present experiment, the changes in microbial community structures and activities seem to have been driven by transgenic alfalfa rather than by recombinant *S. meliloti.* Advances in environmental analyses techniques have made it experimentally simpler to design appropriate measurements to detect changes in soil microbial populations and processes following exposures to genetically engineered organisms but it is still challenging to evaluate the intensity, longevity, and ecological significances of such changes.

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Monitoring effects of released transgenic *Sinorhizobium meliloti* strains on rhizosphere and bulk soil microbial communities

Tebbe, C., Miethling, R., Schwieger, F.

Bundesforschungsanstalt für Landwirtschaft (FAL), Institut für Agrarökologie, Bundesallee 50, 38116 Braunschweig, Germany

Abstract

Survival and rhizosphere colonization of two luciferase marker gene-tagged *Sinorhizobium meliloti* strains (*luc*$^+$) were studied after a field release. Both strains were capable to persist on field plots (five replicates of 9 m^2 for each strain) grown with alfalfa (*Medicago sativa*), the symbiontic partner of *S. meliloti*, for more than three years at population sizes of approx. 10^4 cfu g soil^{-1}. Non-inoculated control plots, which were located between inoculated plots, were already colonized by *luc*$^+$ cells 16 weeks after the field release, most likely as a result of aerosol spread during inoculation and subsequent growth in the rhizosphere of alfalfa. The diversity of the bacterial community in the rhizosphere of alfalfa was higher on inoculated plots than on non-inoculated control plots, as determined 12 weeks after the field release. Rhizospheres of weed plants which grew nearby alfalfa plants were also colonized by *luc*$^+$, however, population sizes were two orders of magnitude lower. Greenhouse experiments with two different soil types, one with no detectable indigenous *S. meliloti* population and one with an *S. meliloti* population of approx. 10^4 cfu g soil^{-1} and with two plant species, namely alfalfa and rye, indicated that microbial communities in rhizospheres were mainly selected by the plant species but also modified by the soil environment. Effects of *S. meliloti* inoculation onto the microbial community structure in rhizospheres were rather small or not detectable at all, as measured by fatty acid patterns (FAME) and the immediate metabolic response ("Biolog") of extracted microbial consortia. Generally, the lack of indigenous *S. meliloti* populations increased the capacity of inoculated cells to colonize the rhizosphere of alfalfa and thereby modify the structure of such communities.

Field release of luciferase marker gene-tagged *Sinorhizobium meliloti* strains

In order to assess the fate and ecological impact of genetically engineered microorganisms in their environment it is necessary to be able to monitor their presence and analyze their effect on natural microbial communities with which they would compete in the field. In collaboration with the University of Bielefeld (A. Pühler, M. Keller, W. Selbitschka), we have conducted a field release experiment with two luciferase marker gene-tagged soil microorganism strains at the FAL Braunschweig in April 1995 (Tebbe et al. 1998). Both strains belonged to the species *Sinorhizobium meliloti* (former name: *Rhizobium meliloti*). The parental strain of both strains was *S. meliloti* 2011. The strains were genetically modified by the chromosomal insertion of the luciferase reporter gene (*luc*) either into the *recA* gene (strain L1, *luc*$^+$, *recA*-; Selbitschka et al. 1992) or next to this gene (strain L33, *luc*$^+$, *recA*$^+$; Dammann-Kalinowski et al. 1996). By means of the marker gene, the recombinant cells were detectable after growth on agar plates and addition of luciferin by their bioluminescence. A single colony can be detected among approximately 1,000 grown colonies on an agar surface. In combination with selective media ("Nutrient poor", Bromfield et al. 1994) amended with streptomycin, detection thresholds of approx. 10 cells of bioluminescent *S. meliloti* (colony forming units, cfu) g soil^{-1} can be achieved (Selbitschka et al. 1992).

A total of 10 different square field plots, each 9 m^2 in size, were inoculated with either *Sinorhizobium meliloti* strain L33 (recA$^+$) or *S. meliloti* strain L1 (recA-). Alfalfa plants (*Medicago sativa*), which can be nodulated by *S. meliloti* for symbiontic nitrogen (N_2) fixation, were seeded one day before *S. meliloti* field inoculation. The field site also included five plots inoculated with the wild-type strain *S. meliloti* 2011 and another five plots which were not inoculated. The plots were located in randomized order and separated from each other by 3 m grass strips. Our field site was selected and pretreated to allow a study of released *S. meliloti* cells over several periods of vegetation. Two factors were considered to enhance the survival of *S. meliloti* at the field site: low levels of nitrogen and a lack of an indigenous *S. meliloti* population. In order to reduce the nitrogen concentration in the field, wheat, a plant with high nitrogen demands, was cultivated without additional fertilizers during the year before the field release. Due to this treatment the nitrogen concentration in the field was below 30 mg total nitrogen kg soil^{-1}. The presence

of alfalfa was assumed to selectively support growth of indigenous *S. meliloti* populations, since nodulation requires the bacterial cell divisions during the infection process. Therefore we selected a field where alfalfa had not been cultivated during the last ten years before the field release.

Inoculation of the field plots was achieved by spraying cell suspensions of the respective *S. meliloti* strains with a mobile applicator. A total of $3.5x10^{12}$ *S. meliloti* cells was sprayed onto the surface soil of each field plot. Water was sprayed after the application to allow dilution of the inoculated cells in the surface soil. The wind during the field inoculation ranged between 0.5-3 m s^{-1}, which can be considered medium strength. Sedimentation plates placed next to the plots during inoculation indicated that single marker gene-tagged cells were present in aerosols during the application. Analysis of the recombinant cells in surface soil, one day after the field release, indicated that the cells were homogeneously distributed on the inoculated plots in concentrations varying from 10^5-10^6 cfu g $soil^{-1}$. No marker gene-tagged cells were detectable outside of these plots.

Bulk soil: Survival, spread and effects of the recombinant strains

In bulk soil the population sizes of the both inoculated *S. meliloti* (*luc*$^+$) strains dropped dramatically by three orders of magnitude within the first three months after inoculation. This decrease was accompanied by growth of alfalfa plants and warmer soil temperatures. In September, 21 weeks after the field release, an increase to approx. 10^4 cfu g $soil^{-1}$ was observed. Throughout the following years until the last analysis included in this report (May 1998), the population size of *S. meliloti* (*luc*$^+$) remained constant with slight seasonal variations in the range of 10^3-10^5 cfu g $soil^{-1}$. In our laboratory analyses, we could not detect any significant differences between the survival rates of strain L1 and L33.

Fourteen weeks after inoculation, *luc*$^+$ cells were also detectable on three of five non-inoculated control plots. After 20 weeks all non-inoculated control plots contained *luc*$^+$ cells. The population of *S. meliloti* (*luc*$^+$) increased to numbers as high as on the inoculated plots in November (32 weeks). As observed for the inoculated plots, a stable *S. meliloti* (*luc*$^+$) population is still detectable until the most recent analysis.

Colonization of rhizospheres of host and "non-host" plants by *S. meliloti*

Alfalfa plants from field plots inoculated with *S. meliloti* L33, and from non-inoculated control plots were collected with their roots 12 weeks after field inoculation. At this point in time, no *luc*$^+$ cells were detectable in bulk soil of non-inoculated control plots. However, when bacterial cells extracted from rhizospheres of the collected plants from control plots were analyzed, we found $1.3x10^5$ *luc*$^+$ cells g fresh weight root $material^{-1}$.This population size was in the same order of magnitude as the size of *S. meliloti* L33 found in rhizospheres from plants collected from inoculated plots ($4.8x10^5$ cfu g^{-1}). The results clearly indicated that the colonization of bulk soil, which was two weeks later on the control plots, started from the rhizosphere of alfalfa. Most likely, few of the *luc*$^+$ cells sprayed during inoculation were transported as aerosols outside of the inoculation area and such cells were sufficient to colonize the rhizosphere of alfalfa on the non-inoculated plots.

Since no herbicide treatment was applied, a large variety of weed plants grew within the seeded field plots. In fact, a total of 19 different weed species could be detected during the first period of vegetation. Among them, predominant representative species during the beginning of the growth season (June 1995) were *Chenopodium album*, *Capsella bursa-pastoris*, and *Lolium perenne*. Rhizospheres of weed plants collected from inoculated plots, 12 weeks after inoculation, also contained significant populations of *luc*$^+$ *S. meliloti* cells. The population sizes were, based on cells per g root fresh weight, two orders of magnitude below those detected with alfalfa. Few *luc*$^+$ cells were also detected in rhizospheres of *C. album* and *L. perenne* collected from non-inoculated control plots (approx. 2x10 cfu g^{-1}) but no cells were detected in rhizospheres of *C. bursa-pastoris*. Our results suggest that weed rhizospheres may provide more suitable conditions for survival and growth of *S. meliloti* than bulk soil and, thus, have to be considered as niches for persistence after a field release. The attractiveness of rhizospheres of different plants for colonization by *S. meliloti* may vary dramatically between different species. It would therefore be desirable to understand in more detail the general principles which enhance the success of colonization of rhizospheres by *S. meliloti*. Nevertheless, as indicated by the different population sizes in rhizospheres of alfalfa and weed plants, "host" plants, i.e. plants which can be nodulated by *S. meliloti*, probably provide more specific conditions for growth and colonization of *S. meliloti* than non-host plants.

Impact of *S. meliloti* inoculation on microbial community structure in rhizospheres

Even though the cell numbers of *S. meliloti* (*luc*$^+$) in rhizospheres of alfalfa collected from inoculated and non-inoculated field plots were similar 12 weeks after the field release, the question remained whether the inoculation resulted in selection of a different rhizosphere microbial community and thus created a biological effect at all. To address this question we isolated bacterial pure cultures by cultivation of cells extracted from the rhizosphere on a growth agar. We selected a growth agar which contained four different amino acids, namely L-proline, L-leucine, L-histidine and L-ornithine, as sole carbon and nitrogen sources (AA-medium). *S. meliloti*, which is a good colonizer of the alfalfa rhizosphere was found to be capable to utilize all of these amino acids and thus, we considered, that other rhizosphere bacteria would have similar metabolic properties. In fact, cell numbers (cfu) obtained on AA-medium were similar compared to R2A agar, the latter being a medium known to be suitable for cultivating and isolating bacteria from rhizospheres. We selected the AA-medium instead of R2A for this study since we anticipated that competition for the same carbon sources would result in interactions between the released and the indigenous bacterial population in the rhizosphere.

Total numbers of cultivated cells on AA-medium from the rhizosphere of alfalfa were approx. $5x10^7$ cfu g fresh weight root material^{-1}. Thus, *S. meliloti*, as determined on selective growth agar, only represented 2% of the cultivated microbial community. It is known that a considerable proportion of environmental microorganisms is not culturable on standard growth media and, thus, the proportion of *S. meliloti* might even be smaller.

The diversity of these cultivated bacterial strains from rhizospheres was assessed by means of the ARDRA (amplified ribosomal DNA restriction fragment analysis) technique. The almost complete 16S rRNA operon of each bacterial isolate was amplified by PCR using primers complementary to highly conservative regions at the beginning and at the end of this gene. From a total of 1,119 colonies suitable PCR products were obtained from 1,054 isolates. These isolates were derived from either rhizospheres of alfalfa or *C. album*, and collected from either inoculated or non-inoculated field plots. Restriction fragment length polymorphism was analyzed after digesting the PCR products with *Cfo*I, *Hae*III, or *Alu*I. The results of this analysis were obtained with digital image analysis and similarity calculations using Pearson correlation (WinCam 2.0, Cybertech, Berlin). Isolates with identical ARDRA patterns were considered to be the same "phenon". In summary, groups of bacterial isolates were found which only colonized the rhizosphere of alfalfa and not of *C. album* and vice versa. Also, the "phenon richness" of alfalfa rhizosphere communities isolated from inoculated plots was higher than from non-inoculated plots. A similar effect of inoculation was not found in the rhizosphere of *C. album*. Currently, the PCR products of the 16S rRNA genes of representative isolates are sequenced in order to allow identification at the phylogenetic level.

The limitations of the ARDRA approach described here are twofold: (1) major representatives of a specific microbial community may not be included in the analysis due to the failure of cultivation, and (2) the cultivation and analysis of a large number of isolates is rather laborious. Despite the previously mentioned reasons which justify the use of rhizosphere adapted growth media for cultivation of a microbial community, cultivation inevitably excludes organisms which are not capable of growth at all or unable to compete with other, fast growing bacteria. A cultivation-independent approach to characterize the bacterial communities was selected by extracting total DNA from bacterial cells derived from the rhizosphere. From this "community-DNA" fragments of the 16S rRNA genes were amplified and the products were analyzed by their single-strand DNA conformational polymorphism (SSCP) after gel electrophoresis (Schwieger and Tebbe, 1998). With this approach we currently identify major components of the microbial communities. The selected approach is in terms of time and material requirements superior to the cultivation-dependent approach. Thus, for future analysis larger numbers of samples which are generally required for environmental analysis, can be analyzed to study the ecological impact of a genetically engineered microorganism.

Calibrating the impact: Comparison of selective forces imposed by "host" plants, soil type and *S. meliloti* inoculation (Greenhouse study)

Since it was shown in our field release that inoculation with *S. meliloti* in fact resulted in alterations of the microbial community structure in the rhizosphere of alfalfa, we were interested to know more about

the factors affecting the structure of microbial communities in rhizospheres. A greenhouse experiment was designed in order to analyze the impact of three different factors, i.e. plant species, soil type and *S. meliloti* inoculation. Two plants were included in this study, alfalfa as the host plant of *S. meliloti*, and rye as a widely used cereal cultivated in crop rotations throughout Germany. Two soils, one from the FAL field release site and one from the prospective second field release site Straß-Moos (STM) were compared. Both soils were relatively similar in many of their physical and chemical properties (pH, sandy soils, brown earth) but different in two respects: The nitrate concentration of the FAL soil was only 4.4 mg N kg soil^{-1} (176 kg ha^{-1}) and that of STM was 61.9 mg N kg soil^{-1} (2.476 kg ha^{-1}). Both plants were cultivated in separate polypropylene boxes (size: 40 cm x 25 cm x 20 cm) filled with soil from Ap-horizons collected at the FAL or STM site, respectively. Half of these containers were inoculated with *S. meliloti* after seeding and the other half was not inoculated. All treatments were analyzed in four replicates. The experiments were stopped after 10 weeks (alfalfa) and 11 weeks (rye), respectively. The soil was carefully removed from the roots and the bacteria were extracted from the rhizosphere by washing.

Two methods were used to characterize the rhizosphere microbial communities in this investigation: the immediate metabolic response and the patterns of fatty acids. The immediate metabolic response is a modification of the substrate utilization assays for microbial community analysis developed by Garland and Mills (1991). Microtiter plates with 95 different carbon sources (BiologGN, Biolog, Hayworth, CA, USA) were inoculated with microbial consortia extracted from rhizosphere. Plates were incubated and the substrate utilization was recorded. Patterns and speed of substrate utilization were used to characterize the metabolic potentials of such communities. In our laboratory we found it useful to only read the plates within the first 24 h after inoculation in order to not allow strong enrichment of specific carbon source degrading bacteria during the microtiter plate incubation. We therefore determine the "immediate metabolic response" of the extracted consortia rather than metabolic potentials. This approach was successfully applied to detect effects of soil inoculation with *Corynebacterium glutamicum* or the addition of a protease inhibitor to soil (Vahjen et al. 1995). The other method used to analyze the same rhizosphere extracted microbial consortia was fatty acid methyl ester analysis (FAME). This method for microbial community analysis aims at detecting the composition of fatty acids, mainly from microbial cell membrane phospholipids (Frostegård et al. 1996). In order to compare the metabolic response ("Biolog") or microbial community structure (FAME), both data sets were analyzed by principle component analysis (PCA; SAS 6.1, SAS Institute Inc., Cary, NC, USA).

With both techniques, FAME and the immediate metabolic response, communities could be differentiated by principle component 1 (PC 1) according to plant species which provided the rhizosphere. Using a combination of PC 1 and PC 2, in one case PC 1 and PC 3, it was also possible to distinguish rhizosphere communities from the same plant species, alfalfa or rye, grown in the two different soil types. This indicated that both factors, soil and plant species influenced the structure and metabolic activity of the rhizosphere communities. An effect of *S. meliloti* inoculation onto the rhizosphere structure of *C. album* was not detectable with both methods. The microbial community extracted from rhizospheres of alfalfa was altered (PC 2) as a result of *S. meliloti* inoculation when plants grew in FAL soil (low indigenous *S. meliloti* population), as detected by FAME. In STM soil, using a combination of PC2 and PC3, communities from inoculated and non-inoculated alfalfa plants were different in their immediate metabolic response. The interpretation of the results obtained from principle component analysis is rather preliminary due to the small amount of replicates analyzed. However, in summary, both FAME and "Biolog" indicate that the microbial communities in rhizospheres are greatly influenced by the plant species and also by the soil type whereas bacterial inoculation is a rather weak factor of selection.

Conclusions

Marker gene-tagged strains of *Sinorhizobium meliloti* were capable of persisting in soil for more than three years after a field release. Released cells were capable of spreading into non-inoculated field plots, growing in the rhizosphere of alfalfa, and colonizing bulk soil. Probably low nitrogen concentrations in soil and the lack of indigenous *S. meliloti* explained these results. Field inoculation with *S. meliloti* resulted in increased bacterial diversity in the rhizosphere of its host plant alfalfa but not of the weed

Chenopodium album. Comparison of factors selecting rhizosphere bacterial communities indicated that the strongest impact was imposed by the plant, followed by soil, followed by bacterial inoculation.

The biosafety data collected in this study demonstrate that, as expected, no ecological risk was associated with the release of these marker gene-tagged soil bacteria. A combination of environmental factors, such as nitrogen concentration, presence of competing indigenous soil bacteria of the same species or the non-controlled occurrence of potential habitats provided by weed rhizospheres or soil insects (not reported here), influenced the fate of the released cells. Since these factors are difficult to simulate in greenhouse studies, small-scale field releases are indispensable in the development of safe environmental applications of bacterial inoculants. Due to the spread of bacterial cells by wind or transport by biological vectors, future field studies should also consider interactions with non-target ecosystems, if their colonization by released bacterial cells would imply potential environmental risks.

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Monitoring effects of transgenic T4-lysozyme expressing potatoes on the bacterial rhizosphere population

Smalla, K., Heuer, H.

Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Messeweg 11-12, 38104 Braunschweig, Germany

Abstract

Bacterial rhizosphere communities of T4-lysozyme expressing potato plants (lines DL4, DL5) were compared to those of a transgenic control without lysozyme gene and the wildtype. To assess potential effects of the T4-lysozyme in relation to natural variability, studies were done on three growth stages of the plants, in two subsequent years and on two field sites. Bacterial communities were analysed in a polyphasic approach. Genetic fingerprints of the total community were done by PCR amplification of a 16S rDNA fragment and electrophoretic separation in a denaturing gradient (PCR-DGGE). At both field sites, the fingerprints from the rhizosphere of young as well as of flowering plants hardly varied. Differences in plant lines became detectable at the beginning of senescence. Some major bands appeared which could be assigned to *Enterobacteria*. In the rhizosphere of DL4, a band representing *Erwinia carotovora* became much more dominant than for the other plant types at one field site. Common differences of both T4-lysozyme potato lines compared to the control plants were not observed. The plant type had less effects on the fingerprints than the season or the field site. Stability of the rhizosphere communities was indicated by many bands which were present regardless of plant type, season, or field site. To compare the rhizosphere communities on a more functional level, the potential to utilize 95 different carbon sources was analysed in Biolog plates. Communities from DL4 differed significantly in the pattern of catabolic rates from one or both control lines at some samplings. Nevertheless, the utilized substrates were the same, and PCR-DGGE fingerprints of wells with substrates which were utilized at significantly different rates differed only in relative intensities of some bands. No significant differences were found for DL5. Finally, random selections of bacterial isolates from each plant line were characterized by fatty acid profiling. A high diversity of species was recovered and a lot of them were only found once or twice. The more frequent species were present in the rhizosphere of all plant lines. Again, DL4 was different from the other plant lines as more *Pseudomonas* and less *Actinomycetales* were found. Additionally, senescent DL4 had significantly higher CFU and reduced diversity in the rhizosphere at one field site. Common to both T4-lysozyme producing plant lines was a reduced number of *Agrobacterium* and a higher number of *Comamonas* compared to the controls. Differences of the rhizosphere community of DL4 to the other plant lines, which were found by all the methods applied, coincided with condensed growth and significantly reduced root mass, probably due to somaclonal variation.

Introduction

Conventional breeding strategies could not sufficiently improve resistance of potatoes against bacterial pathogens like *Erwinia carotovora*. Thus, the group of Dr. Düring (BAZ, Quedlinburg) used genetically modified potato plants to express bacteriophage T4-lysozyme and secrete it into intercellular spaces (Düring et al. 1993). Field testing of the plants was accompanied by investigations on the biosafety of the new plant lines. It focused on effects of the plants on the associated bacterial community because T4-lysozyme does not specifically target pathogens but a broad spectrum of different species. Microbes are important factors in plant development and soil quality. The T4-lysozyme producing plants provide a model to investigate the stability of the balance between beneficial and deleterious microorganisms in agroecosystems. In this study, microbiological and molecular methods were applied which allowed to characterize and compare the plant-associated bacterial communities as completely as possible. The main focus was on molecular tools as the major part of naturally occurring bacteria could not yet be cultured. The objective was to test for effects of T4-lysozyme released by the transgenic plants on associated bacterial communities. To allow an evaluation of potential changes in relation to the natural variability of the community structure, the influence of season and field site was investigated.

Field experiment, sampling and extraction of bacteria from the potato rhizospheres

Two T4-lysozyme producing potato lines (DL4, DL5), a transgenic control (DC1), and the wild-type cultivar (Désí) were planted in a completely randomized block design with eight replicate plots per plant line, at two field sites near Quedlinburg and Rostock in 1996 and 1997. Roots with adhering soil were sampled from plants of principal growth stages 1, 6, and 9 (BBCH scale according to Hack et al. 1993), corresponding to early leaf development, flowering, and beginning of senescence. Bacteria were mechanically separated from plant and soil material by Stomacher blending and differential centrifugation (Smalla et al. 1998).

Molecular analysis of potato-associated bacterial communities

Genetic fingerprints of bacterial communities based on 16S rRNA gene sequences allow for a rapid comparison of multiple environmental samples and detection of bacteria including those not readily culturable or those in a non-culturable state. Fragments of 16S rRNA genes can be amplified by PCR from environmental DNA and separated in denaturing or temperature gradient gel electrophoresis (DGGE or TGGE; Heuer et al. 1997, 1997a), resulting in fingerprints of the bacterial communities (Muyzer et al. 1993). Genomic DNA was extracted from rhizosphere bacterial fractions (Smalla et al. 1993) and 16S rRNA genes were PCR amplified between *E. coli* positions 968-1401, and separated in DGGE as previously described (Smalla et al. 1998). The fingerprints from rhizosphere communities of T4-lysozyme producing plants and control plants were compared. Using primers specific for bacteria, PCR-DGGE resulted in genetic fingerprints reflecting the most prominent bacterial species of the potato rhizosphere samples as the detection limit for a 16S rRNA gene is ca. 1% of the total amount of 16S rRNA genes extracted from the sample analysed. The fingerprints of the bacterial communities from the rhizosphere of young and flowering potato plants revealed no differences between T4-lysozyme producing plants and controls. The pattern variability between samples from different field plots was low. Thus, the structure of the bacterial community was stable against influences due to differences between the plant lines but also against influences of field heterogeneity or differences in plant development. A higher variability of the fingerprints was observed with rhizosphere samples from plants in the early stages of senescence. The plant line DL4, which had the highest expression level of T4-lysozyme, differed significantly in the rhizosphere bacterial community patterns to the other plant lines. Mainly one dominant band in the DL4 patterns derived from the field site in Quedlinburg was much stronger in seven of eight patterns from replicate plots compared to the patterns from the other plant lines. This band corresponded to the species *Erwinia carotovora* as revealed by sequence analysis of the 16S rDNA fragment. The rhizosphere patterns of the other T4-lysozyme producing plant line DL5 were similar to the control lines Désí and DC1. Although the higher expression level of T4-lysozyme in DL4 compared to DL5 might explain bacterial community differences, it is more likely that changes in the rhizosphere of DL4 were a consequence of clonal variation of this plant line which resulted in a shorter sprout and in less root and tuber mass compared to the other plant lines when grown in the field by clonal variation. More pronounced than any plant line effect were seasonal changes of the rhizosphere community patterns. The relative proportion of *Enterobacteriaceae* increased with plant age, and that of *Actinomycetales* decreased. Even more obvious than seasonal changes were differences in the patterns of the two field sites. Thus, potentially undetected effects of the T4-lysozyme on bacterial communities in the rhizosphere are small in comparison to the natural variability and might not be ecologically relevant. Moreover, many populations of bacterial species were present in the rhizosphere in more or less constant proportions of the total community regardless of plant line, season, field plot, or site. This gives evidence that the bacterial community of the rhizosphere is stable enough to buffer environmental changes like the modification of the potato plants.

The sole comparison of fingerprints remains unsatisfactory without additional information about the taxonomic structure of the underlying community and its function within the ecosystem. The DNA sequence of a 16S rRNA gene fragment excised from a community DGGE pattern is often not sufficient to identify the corresponding species, or even to deduce ecological traits of it. This is because, on the one hand, the DGGE fragments are for methodological reasons typically shorter than 500 bp, thus providing only limited sequence information, and relatively few well characterized bacterial species are included in the available 16S rRNA sequence databases, while on the other hand the physiological diversity of bacteria is hardly reflected by 16S rRNA sequence diversity. The properties of bacterial strains can best

be studied with pure cultures. Thus, a methodological approach was developed in this project to link bands of community DGGE-fingerprints with bacterial isolates from the same habitat by polynucleotide probes (Heuer et al. 1998). The hypervariable region V6 (*E. coli* positions 985-1046) was chosen as the probe target with respect to the phylogenetical conservation of the 16S rRNA sequences, which counteracts probe specificity. The V6-region is part of the DGGE-fragment used in this study and can be amplified by PCR with primers which target closely flanking conserved sequences. Therefore, the probes can be generated from excised DGGE-bands without sequence information. Several dominant bands from the potato rhizosphere patterns were analysed to gain insight into the taxonomic community structure (Table 1). The bands were excised, cloned and sequenced. Polynuclectide probes targeting the V6 region were generated from three of them (B1, B2, B3), and for each a corresponding bacterial rhizosphere isolate could be found which was positive in dot blot hybridization, and which had a 16S rRNA gene fragment of identical electrophoretic mobility in DGGE as the excised band. Band B1, which was significantly more intense in the rhizosphere patterns from old DL4 plants compared to the other plant lines, corresponded to an isolate which was identified by the fatty acid composition as *Erwinia carotovora* subsp. *carotovora* using the Microbial Identification System (MIDI Inc., Newark, NJ, USA). The isolates corresponding to bands B2 and B3 were identified as *Kluyvera cryocrescens* and *Enterobacter amnigenus*, respectively. This was in agreement with the sequence analysis of the DGGE bands, and correspondance was also confirmed by 16S rDNA sequencing of one isolate. Bands B1, B2, B3, and B6 all belonged to *Enterobacteriaceae* which largely increased in their relative abundance when the plants started to become senescent. Bands B4, B5, and B7 represented prominent populations which were stable in their relative abundance over the season and not affected by the plant line or T4-lysozyme.

The patterns obtained by PCR-DGGE reflect only the most prominent species as the detection limit is ca. 1% of the total community. Taxon specific primers for *Actinomycetales* and alpha-Proteobacteria were developed and tested to selectively amplify these ecologically important groups and monitor shifts in DGGE which might not be detectable in total bacterial patterns. Application of the *Actinomycetales* specific PCR-DGGE showed a clear seasonal decline of this group in the potato rhizosphere. Effects of T4-lysozyme on this group were not detectable, although Gram-positive bacteria may be seen as indicator bacteria for effects of lysozyme enzyme activity due to their cell wall composition. Also effects of the T4-lysozyme on alpha-Proteobacteria were not yet detected in the rhizosphere.

Table 1 Analyses of partial 16S rRNA gene sequenes (*E. coli* positions 985-1377) from prominent bands of potato rhizosphere DGGE patterns

Band	Acc. no.	Database reference (accession no.)		Similarity
B1	AF060530	*Erwinia carotovora* ATCC 15713	(M59149)	98.7 %
B2	AF060531	*Kluyvera cryocrescens*	(Y07652)	98.5 %
B3	AF060532	*Enterobacter amnigenus* JCM 1237	(AB004749)	100 %
		Potato rhizosphere isolate *E. amnigenus*	(AF060537)	100 %
B4	AF060533	*Flavobacterium succinicans* DSM 4002	(RDP)	100 %
B5	AF060534	*Pseudomonas corrugata* ATCC 29736	(D84012)	100 %
B6	AF060535	*Erwinia persicinus* ATCC 35998	(U80205)	98.7 %
B7	AF060536	*Cellulomonas cellulans* DSM 43879	(X83809)	95.3 %

Sole-carbon-source utilization profiles of rhizosphere communities

The bacterial communities from plant extracts were compared in their catabolic potential as functional units (Garland and Mills, 1991). This method complements the molecular and microbiological approaches which compare the community structure, as it reflects functional properties of the communities. Therefore, utilization of the 95 carbon sources in Biolog GN microplates (Biolog Inc., Hayward, CA, USA), as indicated by the concomitant reduction of tetrazolium violet to a dye, was photometrically measured. Carbon sources were mainly mono- or disaccharides, carboxylic or amino acids, as well as some polymers, amines, amides, and others. A statistical procedure was developed to

compare the patterns of substrate utilization and test for significant differences (Glimm et al. 1997). Application, sensitivity, reproducibility, and limitations of the method were previously described (Heuer and Smalla, 1997b; Smalla et al. 1998).

Until now, significant differences in the relative substrate utilization rates were only found between rhizosphere communities of DL4 and the control lines Dési and DC1 in some of the samplings, but not between the Biolog rhizosphere patterns of the second T4-lysozyme producing plant line DL5 and the other plant lines (Table 2). The substrate utilization patterns were qualitatively highly similar in all cases, i.e. the same substrates were oxidized. PCR-DGGE analysis of the community structure of those wells where the sole carbon source was differentially utilized showed that differences between DL4 and Dési/ DC1 were not based on the presence of different species but on slightly differing abundances of the same species. Only activities of culturable and fast-growing aerobic-heterotrophic populations were detected by the Biolog method (Smalla et al. 1998).

Table 2 Comparison of patterns of sole-carbon-source utilization rates of bacterial rhizosphere communities from T4-lysozyme producing potato plants (DL4, DL5) and control lines (Dési, DC1)

Field site/year	Principal growth stages of the potato plants at sampling		Pairs of plant lines with different Biolog patterns (P < 0.1)	
Groß Lüsewitz 1996	Leaf development	(MS 1)		DC1 - DL4
	Flowering	(MS 6)		
	Senescence	(MS 9)	Dési - DL4	DC1 - DL4
Quedlinburg 1996	Leaf development	(MS 1)		
	Flowering	(MS 6)		DC1 - DL4
	Senescence	(MS 9)	Dési - DL4	DC1 - DL4
Groß Lüsewitz 1997	Leaf development	(MS 1)	Dési - DL4	DC1 - DL4
	Flowering	(MS 6)		
	Senescence	(MS 9)		
Quedlinburg 1997	Senescence	(MS 9)	Dési - DL4	DC1 - DL4

Cultivation, isolation and characterization of bacteria from the rhizosphere

The species composition of readily culturable bacteria from the rhizosphere of the different plant lines was compared. The bacteria suspended from the plants were serially diluted and plated on R2A (Difco, Detroit, MI, USA). A random selection of colonies was picked for each sample. All isolates were characterized by their fatty acid profiles, and a majority was identified by means of the Microbial Identification System, as described in Heuer and Smalla (1997b). If identification was not possible for a strain, its affiliation to a taxon was determined using signature fatty acids and comparison to a database of 2,800 identified potato isolates. In a few cases (*Stenotrophomonas, Comamonas*) the result was confirmed by sequencing of 16S rRNA genes. This could only be done for the field site near Quedlinburg and for one season, because analysis of a sufficient number of strains is highly labour-intensive. A diverse spectrum of species from the alpha-, beta-, gamma- and delta-subclasses of the Proteobacteria, from the *Flexibacter-Bacteroides-Cytophaga*-group, and from Gram-positive bacteria of the low-G+C- and high-G+C-group was recovered from the rhizosphere of all potato lines. All species which were frequently isolated (>10 isolates) were present in the rhizosphere of T4-lysozyme producing as well as control lines. The relative number of Pseudomonads retrieved from the rhizosphere of DL4 was higher compared to the other plant lines, and that of Actinomycetes was lower (Table 3). This trend was consistent throughout the season. In the beginning of senescence, colony forming units in the rhizosphere of DL4 in Quedlinburg were significantly higher than for the other plants, and concomitantly the diversity of species declined. That means less species were recovered and some of them were largely enriched, namely *Pseudomonas syringae, P. chlororaphis, Flavobacterium* sp. and *Sphingobacterium* sp. A trend common for both T4-lysozyme producing plant lines was a lower percentage of *Agrobacterium* isolates and a higher percentage of *Comamonas* isolates compared to the control lines. These results are

supported by only a small number of isolates per species due to the high diversity found, and need to be confirmed by other methods.

Table 3 Taxonomic distribution of bacterial isolates from the rhizosphere of T4-lysozyme producing potato lines (DL4, DL5) and control lines (DC1, Désí) sampled from a field site near Quedlinburg in 1996

	Percentage of isolates from each plant line			
	Désí	**DC1**	**DL4**	**DL5**
Pseudomonas sp.	27	23	36	24
Enterobacteriaceae	7	3	3	4
Xanthomonas / *Acinetobacter*	7	8	8	6
ß-Proteobacteria	15	22	14	23
α-Proteobacteria	3	4	1	1
Gram-positive bacteria: High G+C	20	20	11	21
Gram-positive bacteria: Low G+C	12	12	12	10
FBC, *Sphingobacterium*	9	7	14	11
No. of analysed strains	**273**	**273**	**288**	**278**

Conclusions

The applied methods of community-level analysis are well-suited to detect shifts in bacterial rhizosphere communities. However, pronounced effects which could clearly be attributed to the expression of T4-lysozyme in the transgenic plants were not detectable. Some peculiarities of the rhizosphere communities associated with DL4 are probably due to the altered growth of these plants. Some evidence was found for minor effects of the T4-lysozyme on the genera *Agrobacterium* and *Comamonas*. But overall, the rhizosphere communities were shown to be rather stable, and potential effects of T4-lysozyme expression of the plants were negligible in relation to seasonal and other natural influences. There is no clear evidence for severe environmental impacts of the transgenic plants so far. Nevertheless, some uncertainty remains as the focus of the study was mostly on highly abundant species. Efforts to cover also less abundant bacteria by specific PCR-DGGE were initiated. In addition, the effects of the T4-lysozyme on other microbes within the rhizosphere should be studied. Especially fungi may be a target of T4-lysozyme in the rhizosphere as Düring et al. (1993) showed also a non-enzymatic effect of this protein on some fungal species.

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Communication of biosafety results

McGloughlin, M.

University of California, Davis, CA, USA

New technologies have been applied in agriculture and food production as they were developed. Despite assertion that any revolutionary technology will be disruptive at the socio-economic level most of the technologies that have been applied to production agriculture have come into common usage without much controversy or even knowledge by the average consumer. In the past we have not regulated perceived revolutionary changes in agriculture based on unpredictable socio-economic consequences. However, some recent innovative technologies, namely biotechnology and more specifically recombinant DNA technology have grabbed the public attention in a manner unlike any other previous technological development.

Over two decades of research have provided no evidence that rDNA techniques or rDNA modified organisms pose any unique or unforeseen environmental or health hazards. In fact, a National Research Council study found that: "As the molecular methods are more specific, users of these methods will be more certain about the traits they introduce into plants intimating that greater certainty means greater precision in safety assessments." They further state that no conceptual distinction exists between genetic modification of plants and microorganisms by classical methods or by molecular techniques that modify DNA and transfer genes, whether in the laboratory, in the field or in large-scale environmental introductions (NAS, 1989). However, certain groups who claim to have a mandate from the public have unilaterally condemned this technology.

Million acres of transgenic crops were grown commercially with 54% being planted for herbicide tolerance, 31% for insect resistance, 14% for viral resistance and approximately 1% for quality traits. This figure is a dramatic increase on the 7.0 million acres planted in the previous year. This massive increase occurred in spite of demand exceeding the supply of transgenic seed in many cases. With only two years of widespread use behind us, it already is clear that recombinant products offer tremendous benefit to the farmer, the consumer and the environment. How the application and potential of this technology is being perceived and communicated to the public will be presented.

In a world whose population is increasing at a rate that threatens to confirm Malthus' worst predictions, it is hard to envisage feeding and sustaining these numbers in a livable environment without the use of biotechnology. The FAO reports that global demand for food could easily double over the period 1990-2030, with two-and-a-half to threefold increases in the poorest countries (FAO, Rome, 1996). It is difficult to imagine what is a "promising alternative" to biotechnology and industrial agriculture that will sustain such numbers without catastrophic consequences.

What has made humans unique in the animal kingdom is the ability to manipulate our world. Many millennia B.C., people discovered that microorganisms could be used in fermentation processes, to make bread, brew alcohol, and produce cheese. Through mutation and selection processes, use of microorganisms as process tools became more and more sophisticated as time went by and this ability took another dimension with the advent of recombinant DNA technology in 1973. Likewise we have been modifying animals and crop plants through cross breeding, selection and evening culling those with undesirable characteristics for hundreds of years. The manipulation of living organisms is one of the principal tools of modern biotechnology. Although biotechnology in the broadest sense is not new, what is new, however, is the level of complexity and precision involved in scientists' current ability to manipulate living things, making such manipulation predictable, precise and controlled. This level of control is a tremendous asset in the quest to improve the quality of life.

While exploitation of the fundamental biological phenomenon of genetic change has been the principal wellspring of agrarian evolution and, in no small way, has assisted the advancement of civilization, it did not emerge as an issue of social concern until scientists attained the means to manipulate life at the molecular level. Although a word of caution was raised by an early researcher in this field. He stated that "We have recently advanced our knowledge of genetics to the point where we can manipulate life in a

way never intended by nature. We must proceed with the utmost caution in the application of this new found knowledge". This is a comment made by Luther Burbank in 1906. He suggested that the application of the tools that he had perfected in traditional cross breeding could be regarded as a radical step with unintended consequences. This probably is the first documented instance of a researcher in this field suggesting caution.

In essence, commercialization of the products of recombinant DNA technology is just another facet in a long history of human intervention in nature for agricultural and food production. And, as such, the same parameters of risk-based assessment should apply. Commercialization of all types of organisms must be undertaken within a regulatory framework that insures adequate protection of the consumer and the environment while not stymieing innovation. The latter may slow the entry of new products into the marketplace and such a delay can sustain reliance on less efficient, less precise, less predictable and sometimes more hazardous alternative technologies and products.

The gravity with which researchers hold these tenets is demonstrated by the fact that, following the development of the initial capabilities of recombinant DNA technology, Paul Berg and distinguished colleagues, "Science" (1974) published the findings of the NAS Committee on Recombinant DNA Molecules in which effectively called for a moratorium on genetic engineering research. This publication spurred the convening of scientists at Asilomar in 1975 where every facet and implication of recombinant DNA research was explored. The product of this milestone conference was a set of guidelines that outlined strict procedures for ensuring the safety of genetic engineering experiments. Over two decades of experimentation and a plethora of supporting data has demonstrated the safety of this technology and has resulted in a lessening of the restrictive nature of the guidelines.

Apart from the philosophical concerns with the technology which are beyond the scope of this paper, there are many other issues which apply equally to traditional methods of modification, selection and breeding but which have taken on a different complexion in the context of current technological capabilities. These capabilities offer tremendous potential for addressing many of the pressing human and environmental needs including increasing the efficiency and sustainability of production agriculture; assuring the abundance, variety, quality and safety of food; providing means to monitor and reduce pollution and offering versatile methods to combat infectious agents. Implicit in the effective utilization of these biotechnology applications is the requirement for public acceptance. Consumer acceptance can be influenced by effective consumer education. As Hoban pointed out consumers' willingness to buy biotechnology products will depend on biotechnology's willingness to educate consumers (Hoban, 1997).

The mechanisms by which this communication is undertaken and the media used in the process are critical to the effectiveness of the undertaking. Takashi Tachibana (1998) despaired that the current level of basic scientific knowledge is so low that it is difficult to interest even the brightest layman or non-science student in what modern science is doing. He went on to say "I border on despair at my inability to keep them interested long enough to correctly understand both a specific research project, and its aims. The chasm between scientist and nonscientist has widened to become a gulf. And it is the task of science and society to narrow that gulf through an intellectual shift of tectonic plates." It is a matter of some concern where in a survey when asked if tomatoes had genes before they were introduced through genetic engineering a significant number of individuals replied that they did not.

Implicit in Tachibana's assertion is the requirement for effective communication to allow the public to make informed decisions about scientific and technological issues that may impact their lives. And effective communication is vital to the development of rational oversight of technology. We must remember that laws are made by politicians not scientists, and politicians are easily swayed by their perceptions of public opinion. Political decisions are not always rational, since public emotions can easily be influenced by irrational arguments. As Hoban observes, vagueness, anxiety, fear, or abhorrence often prevail over rational judgement, and incorrect or even hostile commentary about certain kinds of research spreads quickly. Key to promoting wise political decisions about scientific matters is a sound understanding of science among the general population and the media. Having participated in many education outreach initiatives over the past ten years I have established a number of criteria that I have determined to be effective tools for providing various groups with information to insure that they can use factual data to make informed decisions.

The principal issues of concern to the public are:

- Ethical concerns about genetic modification.
- Safety concerns about food and introducing genetically engineered organisms into the environment.
- Concerns about the alleged radical novelty of biotechnology, or about its alleged unpredictability or irreversibility.
- Concerns about negative employment impacts.

It is also important to understand and anticipate differences among consumers from different regions and countries. There are worldwide differences in reaction to the products of biotechnology based on culture, history, economic conditions, and regulators' response to the issue. Hoban (1997) points out that these elements influence potential for activist opposition. Consumers' lack of acceptance of biotechnology may be most strongly correlated with the efforts of activist groups to oppose it. Many of those most vocal in opposition to biotechnology rely on emotive arguments to sway public opinion and it is inordinately difficult to counter emotion with well reasoned logic. Major obstacles to effective communication include the level of scientific literacy among the public; the lack of clear and tangible benefits of agricultural biotechnology from the perspective of the consumer; the lack of concern among the public for the integrity of agriculture (when asked many children will assert that milk comes from the store and not cows); regulatory complexities; slowly developed databases; opposition by groups philosophically opposed to the technology and public uncertainties which arise from normal scientific debate.

Often the credibility of those involved in the scientific debate is an issue in and of itself. For many consumers a scientist is a scientist and their pronouncements on any scientific subject carries equal weight no matter what his or her area of expertise. This is a matter of some contention as asserted in an editorial in "Nature Biotechnology" in January, 1998. The editor contends that "science-based" reviews, like those now being conducted serially in the European Union may convince troubled citizens that governments are acting in their best interests. But what they undoubtedly achieve, especially when they are blatantly pointless or speculative, is the undermining of real scientific assessment of benefit and risk. Scientists who want to wage political battle should be free to do so, but they should leave off the mantle of science when they take up the fight.

The method by which information is provided is of crucial importance. I have found the following mechanisms to provide a relatively effective approach to communication.

In illustrating the benefits and uses of agricultural biotechnology it is important to use examples that put biotech products or processes into a familiar context. For example, I describe how chymosin produced through genetic engineering is now used to make 80% of all cheese and in large part has replaced reliance on rennet, the enzyme that is isolated from the forestomachs of unweaned calves, in cheese production.

Placing issues in a historical context often helps with credible presentation of the facts. For example, noting that we have been modifying the world around us for centuries through plant and animal breeding and microbial fermentation processes is an effective approach.

It is imperative to address the interests of the target audience and tailor your presentation to meet these interests. For example, when addressing consumers, topics should be focused on issues relevant to this audience such as food, taste, price, nutrition, safety. Specific technical details on how, for example, the seed was developed will probably be of little interest to such an audience.

Audiences need to be reassured that effective watchdogs are in place. Using quantitative data and analogies that puts risks/benefits into perspective is an effective approach to efficiently explain the issues of risks and benefits. As Sandman stated the public does not respond to technology based on the rational calculation of actual hazards. He contends that perceived risk is a function of hazard plus outrage. He uses the formula Hazard = Death and Outrage = Annoyance. Risks that kill and those that annoy are very different. Far of the unknown is a major consideration for many when determining reaction to issues. In addition the perception that the individual is being subject to risks outside of his or her control is a consideration. As Thompson observed confidence may be more closely correlated with participation and consent or the structure under which dietary decisions are made, rather than the end state that is produced. For example, an individual will not be as outraged with the potentially greater risk of eating

Caesar's salad made from raw eggs than consuming products made from genetically engineered soybeans because the former risk is under their control while they perceive the latter to be forced upon them. This is why it is important that risks and benefit must be perceived to be placed within the control of the stakeholders and that all risks appear to be shared equally. Individuals need to feel that they are part of the decision process. At the other end of the spectrum, the corollary of this is that perceived benefit is a function of the magnitude of the gain plus euphoria. This may explain why individuals will feel good about consuming copious quantities of supplements rather than change a lifetime of eating habits by switching to a balanced diet.

To illustrate the issue of adequate labelling and the consumers' right to know I use the argument that the US food supply is generally regarded as the safest in world but what we sometimes forget is that many of the food crops we eat are inherently toxic and must be processed before consumption. Examples are kidney beans, cassava and potatoes. I show the audience a sign stating "This product contains genes from deadly nightshade" and ask if they have seen this displayed on their produce aisle. When they assert that they have never seen such a sign I ask the audience if the requirement to label was taken to its extreme, where should this sign be displayed. Many are amazed to hear that tomatoes and potatoes, as members of the Solanaceae family, qualify for this label since they contain lycopene and solanine respectively, both heat-stable glycoalkaloids. Most agree that, while this statement may be scientifically accurate, it contravenes one of the primary tenets of the labelling law that labels should inform and not mislead the consumer about the safety and efficacy of the product. So, in defense of the present regulations and labelling requirements, I argue that it should be the product not the process that is regulated. Therefore, stating on a label that, for example, a tomato contains a peanut protein is of importance as many individuals are allergic to peanuts but stating that the tomato was produced using recombinant DNA techniques is not informative. Communication methods I have found that do not generally work effectively include:

- Emphasizing the long-term benefits of basic research. Consumers are not interested in hearing about esoteric issues that may impact them in the distant future.
- Presenting information in a marketing-style format. If consumers feel that they are being sold a bill of goods they will not buy it.
- Highly technical presentations. It is easy to lose an audience's interest if you mire them in technical details. They may also think that you are trying to hide something in the details.
- Being overly generalized or overly specific. If your presentation glosses over the issues and is couched in broad generalities it will be unconvincing. Likewise focusing on minutiae will loose the interest of the audience.
- Inability of speaker to respond effectively to concerns. It is crucial that the concerns of the audience are not dismissed as being unimportant. The presenter must be careful not to appear to respond in a condescending or patronizing manner. If the presenter does this then he or she will lose the respect of the audience.
- Utilizing poorly delivered or prepared material. If individuals cannot easily follow what you are attempting to present they will not bother to try to understand the message. As an example of obscurity I often present the following:
  Would you buy this product? The apparatus consists of a radio frequency cavity (RFC) in which the food is enclosed. The power supply consists of a high-voltage transformer in the range of 4,000 to 5,000 volts. This electrical power is converted by means of a magnetron to short electromagnetic waves, moving at the speed of light with a frequency between 100,000 hertz and 100,000,000 hertz. Since the electromagnetic waves can be harmful the door which provides access to the radio frequency cavity must have energy sealing or trapping structures to prevent stray radiation. Experts assure us that this device is safe for consumers and that the benefits far outweigh the risks.

This is a clear example of obfuscation and illustrates how easy it is to lose the message by couching it in highly technical and unclear jargon. It also illustrates another point which can equally apply to biotechnology, that is the technology described in this instance, the microwave, also went through a stage of distrust by the consumer before its convenience overshadowed any lingering safety concerns. In addition, as with many novel technologies, familiarity bred content.

Overall there has been a tremendous level of support for the products of biotechnology in the USA. Of course medical biotechnology has received broad-based acceptance as it addresses life or death issues but individuals bring a rather different set of values to bear when deciding on supporting life sustaining medical advances than they do in deciding what corn cob to buy in the store. In response to an International Food Information Council Survey in March 1997 in which 78% of those surveyed predict biotechnology will benefit them in the next five years, Sylvia Rowe, President, IFIC, noted that "These results clearly underscore the willingness of US consumers to accept biotechnology as part of their lives. American consumers recognize the value of food biotechnology, because they appreciate environmental benefits such as protecting crops from insect damage while reducing pesticide use. They also like the potential for fresher, more healthful and better tasting foods and vegetables made possible through biotechnology." Positive messages from credible opinion leaders such as Sylvia Rowe carry considerable weight among consumers.

The level of public acceptance of biotechnology in the USA has been high due to consistent and proactive educational efforts. Partnerships between universities, industry, agencies, organizations work effectively. But one should remember that education must be based on assessment of public knowledge and attitudes. As illustrated above, it is important to reach consumers by educating opinion leaders such as scientists, health experts, government officials, the media, and food industry employees. The effectiveness of concerted public education campaigns was recently demonstrated by the overwhelming defeat of the Swiss referendum to ban genetic engineering. From initial support for the ban to its rejection by Swiss citizens a two-year education campaign conducted by consortia of academics, scientists and other leaders was instrumental in swaying public opinion.

And if communication is based on words carrying more than their weight in credibility perhaps the most appropriate conclusion is to leave the last word to someone with sufficient credibility to communicate any message effectively. "Responsible biotechnology is not the enemy; starvation is." President Jimmy Carter.

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Public opinion about biotechnology in Britain

Kinderlerer, J., Gunter, B., Beyleveld, D.

Sheffield Institute of Biotechnological Law and Ethics, University of Sheffield, Sheffield, UK

Abstract

This paper reports on surveys undertaken during 1997 and 1998 to gauge public opinion on the use of modern biotechnology in agriculture and food. In addition to face-to-face interviews with 2,185 individuals throughout Great Britain, focus groups were set up to explore the issues for both adults and for teenagers (16-19). A small survey of the opinions of scientists and of journalists writing about biotechnology was also undertaken.

The majority of the general public know little about biotechnology, and associate the science with food problems (for example, BSE and *E. coli* food poisoning). The survey raised the consciousness of modern biotechnology but also increased the proportion who believed that risk outweighs benefit. Few of those interviewed knew of the regulatory system, and most believe that it needs to be substantially strengthened. Education about risk and about technology may not assist in improving acceptance of biotechnology. An open but strict regulatory regime may be important in allaying fear or disquiet.

Introduction

The introduction of the products of modern biotechnology has proceeded in many countries with little public comment or objection. In evidence to the House of Lords in July Mr. T. Galvin of the US Department of Agriculture was asked the reason for the "tremendous confidence in the system that the US Government operates in terms of food". The reply stated that the transparency in the system in the USA is a primary reason for greater public acceptance of the technology (Galvin, 1998). Written evidence indicated significant support for the introduction of novel, genetically modified foods into the supermarkets in America.

In Europe, however, the impact of both test trials and commercialisation of genetically modified crop plants has, in many instances, created a storm. In Britain, there have been many newspaper articles over a number of years, most of which have opposed to the technology, or sought to sensationalise scientific studies. This has also been true of reporting on television and radio. An example is a "World in Action Programme" on ITV in Britain on 10 August 1998 that in particular addressed two issues. The first, trumpeted in all the media, suggested that all genetically modified products could result in problems for the human immune system as potato that *had* been modified to include a gene for a lectin known to suppress the immune system had done just that. The programme also showed great concern that genes could survive, intact, when eating a genetically modified product and be transferred in some way to the human! As far back as October 1996 *The Observer* Newspaper published a two-page spread entitled "Look what's coming to dinner… scramble gene cuisine" (Durham, 1996) which was extremely critical as to the implications of modern biotechnology for food. During December 1997 *The Independent* published an article by Nicholas Schoon (1996b), their Environment Correspondent, which reported that a 'synthetic' crop had been imported into the United Kingdom without a licence. "Genetically altered Maize has already arrived in Britain." The same author wrote "in Germany - where concern about the issue runs high, Unilever has promised it will not use GMO soya" (Schoon, 1996a).

The strong opposition expressed by organisations such as Greenpeace, Genewatch (1998) and the Consumers' Association (1998) perhaps best exemplifies the controversy over the introduction of genetically modified products in Europe and particularly into Britain. Perhaps the most telling article to be published in recent weeks was that of the Prince of Wales (1998). He argued that "We simply do not know the long-term consequences for human health and the wider environment of releasing plants bred in this way. We are assured that these new plants are vigorously tested and regulated, but the evaluation procedure seems to presume that unless a GM crop can be shown to be unsafe, there is no reason to stop its use. The lesson of BSE and other entirely man-made disasters in the cause of "cheap food" is surely that it is the unforeseen consequences which present the greatest cause for concern." He stated that "I happen to believe that this kind of genetic modification takes mankind into realms that belong to God, and to God alone." Later in the same article, he writes, "We are also told that GM techniques will help to

"feed the world". This is a fundamental concern to all of us. But will the companies controlling these techniques ever be able to achieve what they would regard as a sufficient return from selling their products to the world's poorest people? Nor do I believe that the basic problem is always so simple." In writing in this way he appeared to express the views of a very vocal group within British society. English Nature, the statutory government body responsible for nature conservation has argued for a moratorium on the introduction of herbicide tolerant crops into the UK until further research has been undertaken. They are not fundamentally opposed to either development or marketing of these crops, but believe there is a need to be more cautious (Joint Nature Conservation Committee, 1998). A British Organic Farmer, Guy Watson, has recently attempted to stop registration trials of genetically modified maize near his farm in Devon, England, because of the impact on his organic produce. He failed in the courts, which ruled that the implementation of the legislation on release of modified organisms had been followed correctly, although the Ministry of Agriculture had not properly followed the legislation on registration trials (Shaw, 1998).

It was with this background that we undertook a research project for the Ministry of Agriculture, Fisheries and Food during 1997 and 1998 to examine consumer awareness and attitudes towards biotechnology. This paper represents a brief report on some of the results reported to MAFF from the surveys.

There is clearly a need for better public understanding of Biotechnology (Durant, 1992). How this might be achieved is more difficult. To what extent can or should "the public" be involved in shaping biotechnology policy development, what conceptions of "risk" should inform a public assessment of biotechnology? (Bradbury, 1989; Nelkin, 1985).

During June 1998 GeneWatch surveyed some 950 individuals in face-to-face interviews in their homes. Mori conducted the survey on their behalf. Four questions were used to attempt to gauge the public view of biotechnology. The first presented to the respondent a densely argued full-page text about Genetic Engineering. The question that followed asked for the level of support of the development and introduction of genetically engineered food. 31% of those questioned supported the technology. 51% opposed it, with only 2% without an opinion. 77% of the public believe there should be a ban on growing genetically engineered crops and food in Britain. The other questions were about willingness to eat genetically engineered food (26% yes, 61% no), whether a ban on the growing of genetically engineered crops should be allowed, as had been implemented in France (77% yes, 11% no), and whether transfer of genes to "wild, natural plants" causes concern (73% concerned, 20% not concerned) (Genewatch, 1998). We were somewhat unhappy with this survey, as the questions are slightly loaded, and the smallness of the question set does not allow for detailed analysis of the data.

The main objectives of the research reported here were to attempt to

- identify the knowledge and understanding of biotechnology amongst the public and attempt to identify how much the public knows about modern biotechnology. Do they know what it means or what activities are included in the term?
- analyse the beliefs and perceptions held - Is biotechnology necessary and what risks are there? Are foods produced by biotechnology safe to eat?
- describe attitudes - whether biotechnology is good or bad, acceptable or unacceptable (rather than whether it is safe).
- explain behavioural intentions and behaviour - are people likely to eat the products?

Methodology

The analysis of public acceptance of biotechnology was attempted through Focus Group Research, a survey of a large number of people throughout England, focus groups of teenagers to probe their views, and a telephone survey of scientists and journalists.

The first stage of the research involved a study of public responses about biotechnology and attempted to qualitatively probe awareness, knowledge and opinions. The main reason for this stage was to test and refine a questionnaire for subsequent use in face-to-face interviews in the main survey. There were six focus groups involving eight individuals. Two groups were female only, two were male only and the remaining two mixed. Three each came from the North and South of England.

The main survey was carried out throughout Great Britain. 2,185 individuals were interviewed at home. 47% of these were males, 53% female and the age distribution was as shown in Figure 1:

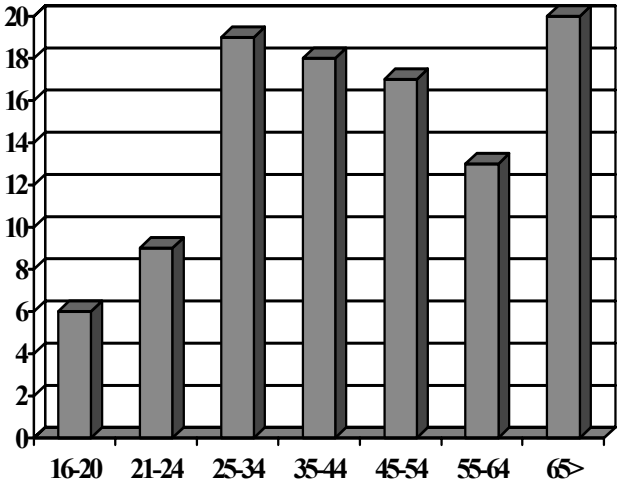

Figure 1 Age distribution for the main survey of 2,185 individuals

96% of the sample were white and from the UK. 84% claimed to be Christians, but a majority (67%) claimed to be not very (32%) or not at all (37%) religious.

Six focus groups involving 16-17 and 18-19 year olds followed the main survey in order to obtain the views of young people in England. All the groups were single sex. Three were recruited in the South and three in the North of England, with eight participants in each group.

It was also felt important to obtain information from specialists; small groups of scientists (30) and journalists (31) were polled using a telephone survey with interviews of about half an hour. Both were asked the same set of questions. The scientists were identified from lists of members of scientific societies; journalists had written articles about biotechnology within the previous 12 months.

Results

Attitudes

It was important to attempt to identify attitudes to technology and awareness amongst those surveyed. They stated that they had changed their eating habits significantly during the previous 10 years (Figure 2). Women were more likely than men to mention some change in their diet or eating habits (75% against 65%). It was the better off who were more likely to mention that their eating habits had changed. More than 30% had reduced their intake of fatty foods, and about 20% were eating less red meat or sugar. 12% mentioned that they had increased their consumption of fresh fruits and vegetables.

The respondents to the main survey were asked about their perception of change in farming practice. Over 70% mentioned that they were aware that some change had occurred. Unprompted responses focused on mentions of greater use of pesticides, fungicides or chemicals in general (24%) as well as the greater use of machines and the industrialisation of agriculture (20%). 14% mentioned changes towards organic farming.

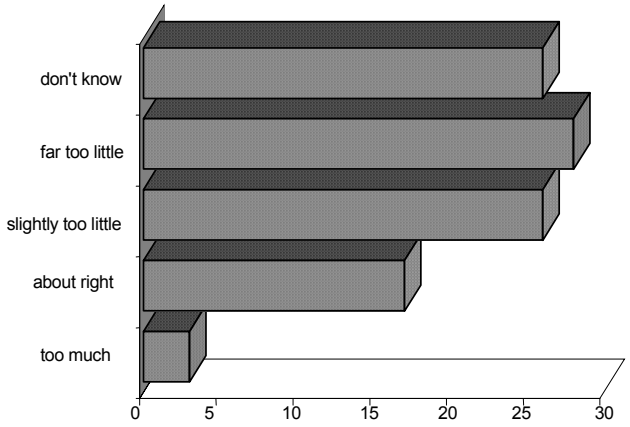

Figure 2 Regulation of biotechnology

Awareness

Most of those interviewed were aware of what constituted healthy food, although it was not often translated into practice. 73% reported at least some concern about food safety although one in four had given it a great deal of thought, and 81% were concerned at environmental pollution. What constituted a healthy food was not absolutely clear, an example being one which had not been tampered with and which was in a natural state. Many of those interviewed were very concerned at food additives, with focus groups mentioning their sensitivity to "E-numbers".

General awareness of biotechnology was low, with only about 55% claiming to have heard of it. Those who read "quality newspapers" were much more likely to have heard of biotechnology than those reading tabloids (85% against 52%). Within the focus groups, initial mention of the term biotechnology aroused suspicion and a general lack of detailed understanding. Many did not distinguish modern biotechnology from modern agricultural practice, and were suspicious of it. In the main survey, respondents were shown a list of fourteen possible applications and processes and asked to indicate those which were most connected with biotechnology. 75% were willing to respond to this list, but 25% claimed to have no idea at all when looking through the list. The lack of knowledge was particularly marked among those aged over 65, those with no science qualifications and those who had left education at 16.

The main associations emerging focused on making pest resistant crops (30%), cloning (26%), altering the genetic structure of bacteria (24%) and applying preservatives to food (23%).

Awareness of regulation

We attempted to discover whether the public were aware that biotechnology is regulated. It was clear from both focus groups and the main survey that the public is uncertain about regulation, but believes that it should be regulated carefully. Within the focus groups there was a general feeling that an unspecified "they" were in some way, at risk of becoming out of control. The results of the main survey, shown in Figure 2, indicate that the majority of those asked felt that there was far too little regulation. Perceptions of there being too little regulation were most marked amongst those respondents with science qualifications to 18 or above (70%), readers of broadsheet newspapers (67%). Feeling that the amount of regulation was about right was most common among the 16-19 age group (25%).

Many of those interviewed were not convinced that the existing systems of control works in the consumers' favour. Within the focus groups some respondents were pessimistic about whether a

regulatory body would have enough "clout" to stop the powerful multi-national chemical companies operating in this area.

Perceived risks and concerns

The latest Eurobarometer research has shown that over half of their interviewees in the United Kingdom thought that there were risks associated with a number of biotechnology applications. In evidence submitted to the House of Lords, Galvin (1998) produced a graph which dramatically illustrated the difference in perception of the technology in different countries (Figure 3). This graph was partially published in *Science* on 31 July 1998 (Ayers, 1998).

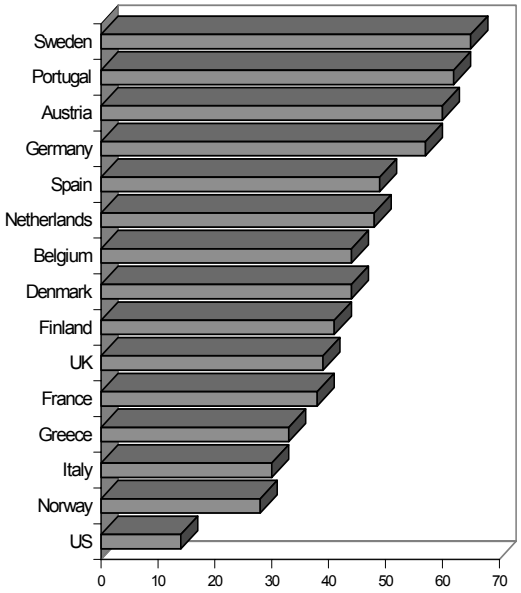

Figure 3 Perception of genetic modification as a serious food risk

Research by Which (1996) found that members of the public in Britain had concerns about the application of modern biotechnology to food products and these concerns were apparently strong enough to make them think very carefully before buying genetically modified foods. Even if such products were cheaper than other brands, few people expressed a willingness to purchase. 77% of those surveyed by Which thought that the food industry stood to benefit more than they did from the use of biotechnology.

On initial questioning about the balance of risks and benefits, our respondents replied that the risks outweighed the benefits in almost every case (Figure 4). A huge proportion, 37%, were not willing to state an opinion. We then asked about specific applications of the technology with the results shown in Figure 5.

After prompting and discussion, the original question relating to the balance of risks associated with the technology was asked again. This time, far fewer were unwilling to state an opinion reduced to 21%, but the proportion rating the technology risky had risen markedly to -15% from the original -4%. The survey attempted to investigate the public image of biotechnology by asking respondents to indicate from a list which descriptors best applied to the genetic modification of plants and animals. The results are tabulated in Table 1.

There was an attempt to identify personal concerns of those interviewed, and the results are plotted in Figure 6.

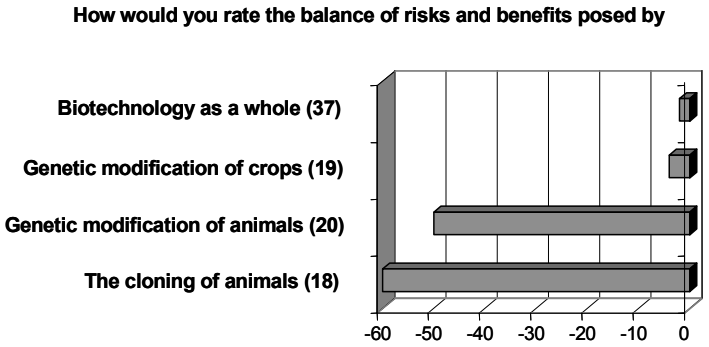

Figure 4 Risks and benefits

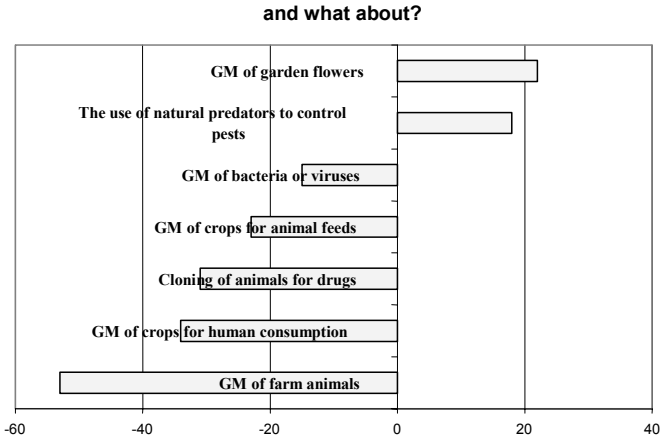

Figure 5 What about specific aspects of biotechnology?

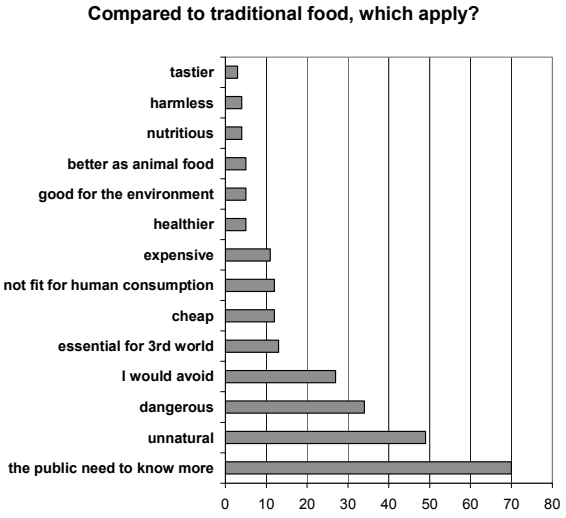

Figure 6 Respondents' reaction to genetically modified foods in comparison to traditional foods

Table 1 Perceptions of genetic modification of crops and animals

	Genetic Modification	
	Crops	**Animal**
safe	10	3
harmless	11	4
beneficial	17	7
willing to buy	17	7
ethically right	14	6
well regulated	8	6
progress	30	20
inevitable	30	25
impossible to regulate	29	42
not willing to buy	29	42
harmful	31	43
dangerous	31	49
should not be allowed	27	46
worrying	40	54
unethical	29	51
would prefer was not done	41	58
tampering with nature	52	66

It is clear from all of these results that the public is extremely concerned about the introduction of this new technology into Britain, and in addition, that any presumption that more information would necessarily improve the perception of the science may be misguided. The results would seem to suggest that a regulatory system that is totally open and known to be concerned with safety and attempting to minimise harm to the environment is more likely to gain public confidence than any "education" approach. It is, however, essential that this process is seen to be acting in the public interest. It is essential that the products appearing on the market are seen by the public to be in the consumers' best interest, rather than just for farmers.

Journalists and Scientists

The survey of journalists and scientists had as its objectives

- to discover if the perception of the risks and benefits are similar amongst these two groups;
- to ascertain the perception of the public's need to know and their understanding of biotechnology;
- to examine the perceptions of the role of the media in enhancing the public understanding and opinion; and
- to explore the perceptions of their own roles and responsibilities in informing the public.

Similar questions were asked of these two groups as had been asked in the main survey, although in this case it was known that they knew about biotechnology. When asked about dietary changes, there was little difference among these two groups from that observed for the general public. Both scientists and journalists had thought about food safety, giving it a great deal more thought than members of the general public do. Scientists (57%) much more likely than journalists (39%) were to say they were very concerned about issues relating to environmental pollution.

Scientists and journalists were asked to estimate the relative balance of risks and benefits associated with biotechnology as a whole, and with specific types of modification or biotechnology processes.

The overwhelming majority of scientists (93%) believed that the benefits of biotechnology in general outweighed the risks. No scientist felt that risks outweighed benefits. Journalists were less confident,

although a clear majority (65%) believed that benefits outweighed risks. Both groups were far more confident about biotechnology as a whole than were the general public. A number of specific applications were put to both groups, summarised in Figure 7. A clear division of opinion can be seen. In looking at other processes, even greater differences were seen between journalists and scientists. For example, irradiation of foods was seen to be an application where benefits outweighed risks by 83% of scientists, but only 51% of journalists. The use of antibiotics in farming was seen to be risky by both journalists (58%) and scientists (63%). The image of biotechnology to these two groups is demonstrated in Figure 8.

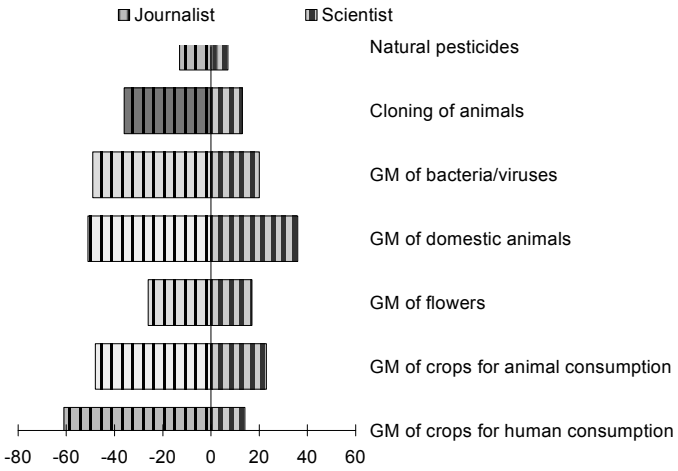

Figure 7 The differing perceptions of scientists and journalists to benefits and risks of technology. The values plotted for journalists have been plotted as "negative numbers" to allow easy comparison with the views of the scientists.

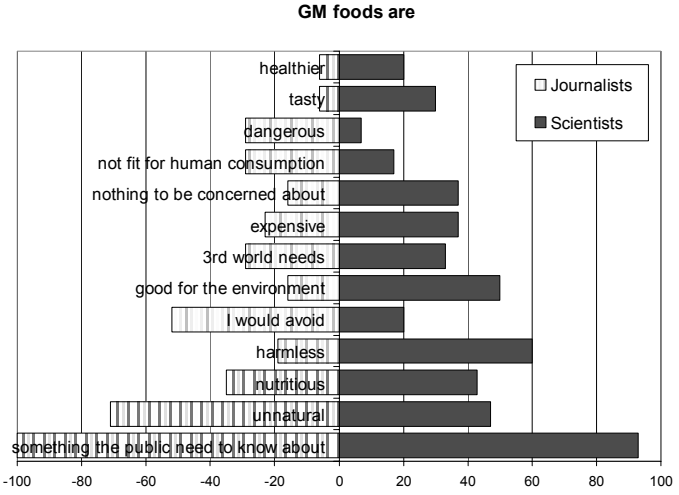

Figure 8 Image of genetically modified food

Perhaps one of the most dramatic results of this survey was the view expressed by scientists and journalists on the public's understanding of the science, illustrated in Figure 9.

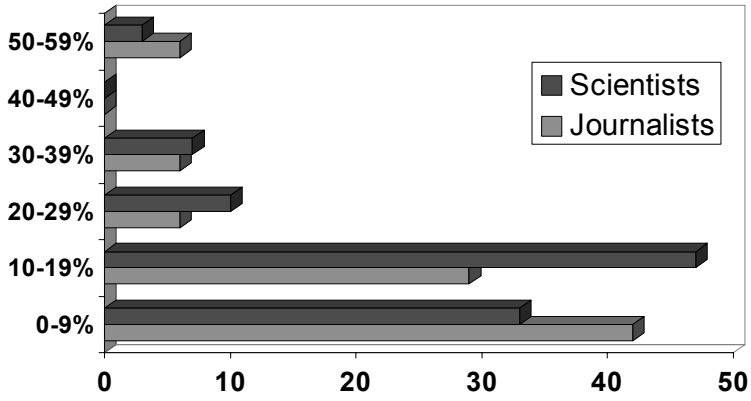

Figure 9 Estimates of the public's knowledge and understanding of biotechnology made by journalists and scientists

Conclusions

This short paper only provides a sample of the welter of results obtained from this extensive series of surveys, and there remains a great deal of information to analyse and understand.

The provision of more detailed information and education to the public may not serve to create a greater acceptance of the technology, nor alleviate the anxieties about the perceived risks. It will be important to provide a mechanism for the public to be more aware of the extensive regulatory structure, and the extensive risk analysis and management procedures that have been applied before any release into the environment or into the food chain has occurred. It may also be necessary to provide much more public involvement in the decision process, and an assurance that their views are considered before technology is accepted by the majority. In Britain at least, segregation of crops to allow individuals choice in the supermarkets is an issue which will not go away.

We thank the Ministry of Agriculture, Fisheries and Food for providing the funds to allow this research to proceed.

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The Swiss endorsement of genetic engineering: What made the difference in the vote

Käppeli, O.

Agency for Biosafety Research and Assessment of Technology Impacts of the Swiss Priority Programme Biotechnology, BATS, Clarastrasse 13, 4058 Basel, Switzerland

Introduction

On 7 June 1998 Swiss citizens decided on the fate of genetic engineering in a national referendum. As one of the world's leading democratic societies, in which a successful popular vote can directly amend the constitution, Switzerland is vulnerable to public initiatives that might otherwise be deactivated by the parliamentary process. In the case of the "Genschutz-Initiative" (gene protection amendment), a diverse coalition of environmental, animal rights, farming, and political groups (green and social democratic) relatively easily gathered more than 100,000 signatures that required the Swiss government to put the proposed amendment to a referendum. If the proposal had been approved by a majority of Swiss voters and a majority of cantons (states) it would have had required enforcement by the government. All research involving transgenic animals, the deliberate release and the patenting of genetically modified organisms would have been banned. In addition, an article on the approval procedure of certain academic research involving genetic engineering was drafted: Prior to approval, scientists would have to submit evidence that their research was of use, was safe, and that there was no alternative available.

There was much concern among industry and the research community that the referendum would attract a majority of voters because of the complexity and the emotional appeal of the issues. In fact, fierce debates between proponents and opponents of genetic engineering took place preceding the vote. Opinion polls predicted a close result even shortly before the voting day.

The result of the voting

At the end of the voting day, the verdict was surprisingly clear. The gene protection amendment had been turned down by 66% of the votes. Some generalizations can be made (Figure 1):

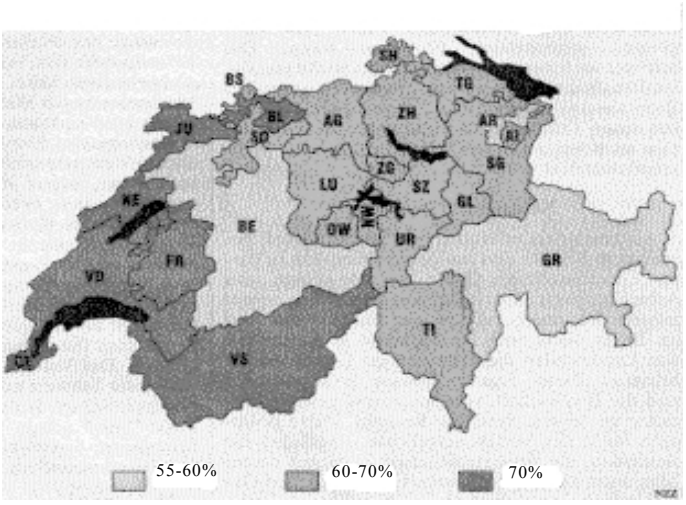

Figure 1 Percentage of "No" votes in the Swiss referendum on the gene protection amendment

1. No rural or urban region was in favour of the initiative.
2. The fraction of "No" votes fell between 55 and over 70%.
3. In the French speaking part of Switzerland the "No" fraction was above 70%.

From this outcome of the vote an important conclusion may be drawn: In the French speaking part of Switzerland acceptance of genetic engineering is better than in the German speaking part.

The evolution of public opinion

The onset of the main debate can approximately be placed at the beginning of 1996. Since then representative opinion polls were carried out by a renowned Swiss research institute (GfS, Berne) on behalf of the pharmaceutical industry. It is quite clear that the percentage of the public acceptance of genetic engineering was not the same throughout the whole campaigning prior to the referendum (Figure 2). The results were made public in the aftermath of the voting only.

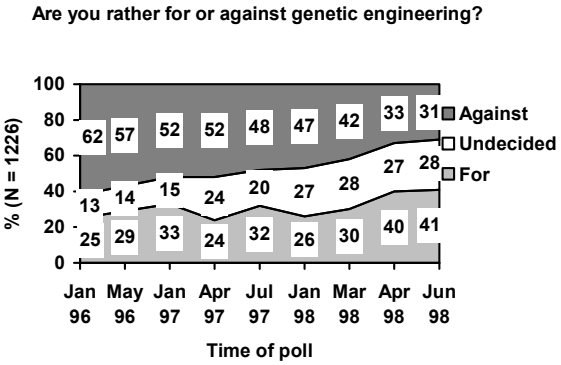

Figure 2 Evolution of public perception on genetic engineering

The fraction of people opposed to genetic engineering dropped steadily during the campaign, from 62% to 31%. At the same time the fraction of people in favour of genetic engineering and the undecided fraction increased in parallel (from 25% to 41% and from 13% to 28%, respectively). It was obviously possible to influence people's minds to a great degree, which is one of the most significant results of the referendum experience. However, the high number of undecided voters made it difficult to predict the final outcome of the voting; forecasts were therefore associated with great uncertainty.

Decisive issues

Initially, the coalition of groups that launched the referendum claimed that the need for bans on genetic engineering is based on risk issues, such as predictability, reversibility, long-term effects, sustainability, biodiversity and the dignity of creature. They argued that safety requirements were not currently being met by known regulations with opposing goals (e.g. revitalizing the economy, competitive edge, decrease of bureaucracy). However, it was not the risk issues that proved decisive for the outcome of the vote. Other factors had a greater influence.

Reluctance towards ban. The initial polls already revealed that a majority of Swiss people are reluctant towards bans (Figure 3). This issue was subsequently emphasized by the opponents of the referendum, i.e. the proponents of genetic engineering. A firm rejection of the extreme proposals of the initiative was the consequence. Simultaneously, a strict regulation was endorsed and even requested by a parliamentary initiative requiring a hastening of the legislative process (gene lex motion) by adapting existing laws (e.g. food act, environmental protection act) to the demands of genetic engineering. A separate gene law was rejected, based on earlier decisions favouring a decentral regulation of gene technology. This strategy led to slogans such as "Yes to regulation - No to bans" and the gene protection amendment was subsequently referred to as the "gene ban amendment".

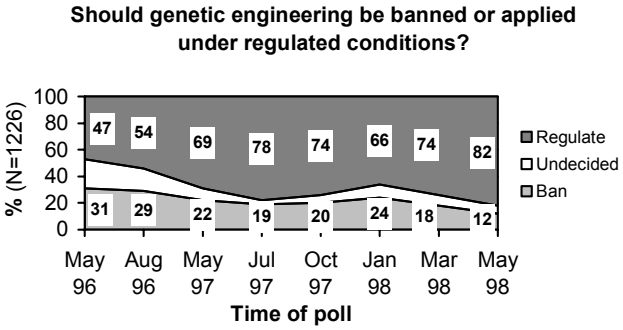

Figure 3 General options on genetic engineering

Responsiveness to public concerns. During the campaign, several milestone events occurred such as the cloning of animals ("Dolly") and the market appearance of genetically modified food crops (herbicide tolerant soybean). The latter case received much public attention, and regulatory issues became dominant. From the polls, it was clear that people were again not in favour of a strict ban on genetically modified food but wished to maintain their freedom of choice between transgenic and non-transgenic food products (Figure 4). The Swiss Food Ordinance requires a marketing authorization of all genetically modified food by the Federal Agency of Public Health, but what was to be the basis for the labelling of transgenic food? The final regulation stated that all food needed to be labelled when recombinant DNA could be detected in the endproduct. This rule was considered as acceptable, thereby decreasing the intensity of the debate. However, a lot of scepticism remains as to whether or not industry complies systematically to the agreement.

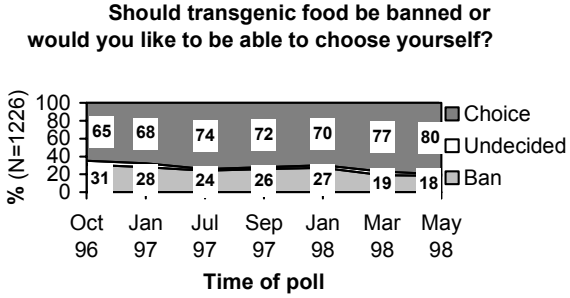

Figure 4 Choice versus ban of genetically modified food

Furthermore, it became clear that gene technology applications on plants were not fundamentally unacceptable (Figure 5). Applications of genetic engineering that reduced crop deficits or the use of agrochemicals were positively viewed by a majority of interviewed people. Other breeding goals such as the improvement of storage quality received a lower approval rate. It can be concluded that beneficial aspects of genetic engineering applications need to be better communicated. It is of paramount importance that people know why certain genetic modifications are performed.

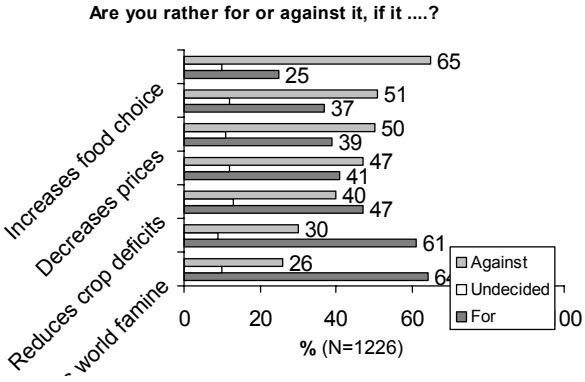

Figure 5 Application of genetic engineering on plants

Prospect of losing opportunities

During the campaign, emphasis was placed on the impacts of the gene protection amendment on medicine, research and economics.

Medicine: As mentioned above, the gene protection amendment would have banned the use of transgenic animals. During the debate it was possible to explain to people that transgenic animals were important tools for investigating basic biological mechanisms and were necessary for understanding the cause of diseases such as cancer, AIDS or Alzheimer. The public realized that a ban would jeopardize the development of new therapies. Restrictive laws governing animal protection and experimentation exist already in Switzerland, as a result of previous referendums.

Research: During the campaign it became clear that the gene protection amendment would threaten a leading research area in Switzerland. The initiative would imperil Switzerland's future as a centre of excellence in biomedical research, molecular biology, neurosciences and other relevant areas.

Jobs: Since the pharmaceutical industry plays an important role in the Swiss economy, it was obvious that the acceptance of the gene protection amendment would have had an important impact on employment. Distinct figures are not available but it was clear to the public that the initiative would affect the employment outlook both in industry and academia.

Consensus among opinion leaders: Since the initiative affected both industry and academia alike a hitherto unprecedented mobilization of their members took place. Scientists held open doors to explain the background of their research and their personal motivation to a larger public. This involvement culminated in a public protest against the ban, staged by more than 5,000 scientists in the streets of Zurich and Geneva. It seems to be this coherence in opinions within the industrial and scientific community on the severity of impacts that convinced the voters to reject the gene protection amendment.

Conclusions

Although the Swiss situation may be unique, a number of lessons may be learned:

- Communication of technology-related issues requires the involvement of all parties, i.e. opinion leaders from industry, authorities and science. Most importantly, a coherent view on basic impacts (e.g. economic and social consequences) and measures (e.g. regulatory approach to ensure safety and prevent misuse) should be elaborated and transmitted.
- Public concerns need to be taken seriously and addressed by taking satisfactory precautions (e.g. labelling of genetically modified food, closing of regulatory gaps).
- Knowledge transfer of science issues to the public should be accepted as a permanent task, involving both communication and educational endeavours.
- Credibility of public opinion leaders is still the most important factor. The final decision on complex issues seems to be made rather on the basis of "I trust" than of "I understand". Hence, the credibility gain of gene technology proponents was probably more decisive than the knowledge gain.

Biosafety communication – today and tomorrow

Sinemus, K.

Genius GmbH, TU Darmstadt, 64287 Darmstadt, Germany

Biotechnology - a word many people today look at with some suspicion but also a word which describes a technology used by mankind since more than 6,000 years for benefits. Already 4000 B.C., Egyptians used yeast to bake leavened bread and to make wine, two quite biblical products of biotechnology. Specific breeding and cultivation of food plants like potatoes and corn (maize), the development of fermentation, brewery, and conservation techniques made it possible to produce healthier and more tasteful foodstuffs.

The results of the experiments made by a very patient Austrian monk named Gregor Mendel were a breakthrough in genetics. In 1865 Mendel founded the field of genetics by the publication of his experimental data in heredity, which most of us still remember from school. When Mendel showed that biological features were passed on from the parental to the filial generation, nobody could imagine the precise details of this process or the nature of the invisible information carrying elements called genes.

At the end of the last century Friedrich Miescher discovered that genes consist of DNA, and in 1910 Thomas Hunt Morgan located them on the so-called chromosomes which are visible in cells before cytokinesis.

Again it is Thomas Morgan to whom we owe the first gene map of Drosophila and who introduced this little fly into genetics, where still today it is the preferred pet of countless scientists all over the world.

The puzzling over the mechanism of genetic heredity ended only in 1944 when Oswald Avery and his colleagues showed that DNA carries a cell's genetic information.

From that time researchers focused on the question of how such a simply constructed chemical molecule like DNA is able to contain such a complex information.

When in 1953 James Watson and Francis Crick described the double helix structure of DNA, using x-ray diffraction patterns of Rosalind Franklin and Maurice Wilkins, it was one of the exceptional results in biological history showing the way to the understanding of gene function on a molecular level.

Modern gene technology was born when in 1973 Stanley Cohen and Herbert Boyer developed ways to cut and splice DNA, and introduced recombinant DNA techniques into molecular biology.

From the very beginning the new technology was accompanied by discussions about the risks and potential biohazards of these experiments. Indeed, the start of gene technology was the birth of the biosafety discussion. In 1971 Paul Berg planned his first experiments to transfer DNA from the SV 40 virus into *E. coli*. This virus causes tumors in monkeys but is also able to transform human cells in tumor cells. So it is not astonishing to learn that already then there were considerable doubts about the insertion of a pathogen virus DNA into a microorganism which is also located in the human gastro-intestinal tract.

With regard to the state of knowledge of DNA transfer at that time you can imagine the importance of a detailed biosafety discussion. Nevertheless, two years later Cohen and Boyer reported on their first successful experiment in constructing recombinant microorganisms. It was Paul Berg, among other scientists, who demanded a pause for experiments dealing with the transfer of tumor genes, antibiotic resistance genes, and genes of SV 40 into *E. coli*. Further, the so-called "Berg letter" asked for the foundation of a committee by the National Institute of Health (NIH) which should formulate guidelines for research in gene technology and, third, for organizing a conference to discuss biosafety aspects and the regulation of recombinant DNA experiments.

This conference took place in February 1975 at the "Asilomar Conference Center" in Pacific Grove, CA, and was the first of a number of milestones in the biosafety discussion until today.

The summary statement of the report on the Asilomar conference by Paul Berg and his colleagues David Baltimore, Sydney Brenner, Richard Roblin, and Maxine Singer was published in "Science" in June 1975. In this paper the authors presented proposals for a classification of different types of containment and experiments regarding recombinant DNA molecules. Further they asked for the development of safer

vectors and especially hosts used in recombinant DNA experiments, and to give high priority to the research in this area.

Following the legendary Asilomar Conference the NIH founded a "Recombinant DNA Advisory Committee" (NIHRAC). This committee formulated guidelines regarding the scientific work with recombinant DNA on the basis of the proposals published in the "Science" article. These NIH guidelines were exemplary for gene technology regulations in other countries such as Great Britain, Germany, and Switzerland.

With the Asilomar Conference and its consequences the biosafety discussion about gene technology and the potential risks was taken into the public for the first time. In the following years the concern of people regarding the new technology was not dispelled, and interest groups like the "Foundation on Economic Trends" or "Friends of Earth" articulated a vociferous protest against recombinant DNA research. It was therefore quite logic that the first attempt to release genetically modified microorganisms into the environment led to enormous problems.

The story started in 1983 when two scientists of the University of California in Berkeley isolated the gene coding for a protein in *Pseudomonas syringae* which function as a sort of scaffolding upon which water molecules can form ice crystals. The deletion mutant, called "Ice Minus", should be tested as a frost protection agent on crops. Although in November 1983 the NIHRAC approved field experiments to be conducted by the researchers, regulators and scientists failed to communicate potential risks to local residents and the local government. So people felt abandoned and exempted from the review and approval process, and as a result, Jeremy Rifkin, president of the "Foundation on Economic Trends", filed suit in US District Court and blocked the experiments. The lack of information, in other words the lack of biosafety communication, was also the reason for blocking field trials with "Ice Minus" on strawberry plants planned by AGS (Advanced Genetic Systems) in 1985. Worst of all, in February 1986 it was revealed that AGS had already performed outdoor experiments on trees located on the roof of the company's research facility without any approval from NIHRAC or EPA. Because of this case, gene technology had to deal with a great loss of credibility in the public also in the years to come. The "Ice Minus" story was widely published in the media so that everybody in the USA and in Europe was aware of the field release of genetically modified microorganisms. In the end "Ice Minus" was field tested in April 1987 after five years of regulatory review.

When in 1989 the Bundesgesundheitsamt in Berlin, Germany's regulatory authority for gene technology (now named Robert Koch-Institut), gave the approval for the first field release of genetically modified organisms in Europe, the discussion about gene technology and especially biosafety regarding field tests with recombinant organisms spread into the public. The fear of incalculable potential risks or hazards in connection with gene technology led to emotionally charged debates, and once again, a real communication between researchers, the media, and the public was missing. The pink blossoms of the genetically modified petunias set a visible sign for the speechlessness of critics and supporters in the biosafety discussion.

Five years later consumers were struck by a message coming in from the USA: America's FDA gave way to market Calgene's "Flavr Savr tomato", a product which is genetically engineered to ripen on the vine for an extended period to become tastier than fresh tomatoes currently available. Media and interest groups built up a horror scenario of genetically manipulated food containing antibiotic resistance genes which therefore may lead to problems in humans also acquiring antibiotic resistance (I think everybody of us remembers the "Greenpeace tomato"). Although Calgene published a biosafety study referring to potential risks of the "Flavr Savr tomato", consumers feel uneasy about the so-called "gene tomato" until today, and media from time to time start up the old-new discussion about "gene food, antibiotic resistance genes, and potential risks to health".

Although in the USA biotech products and also genetically modified foods are more and more regarded as a matter of course, biotech companies in Germany have great difficulties in performing field releases. Since the Robert Koch-Institut, responsible for the approval of field tests in Germany, gave permission for field experiments with crops resistant to herbicides, viruses, and insects, releases should have become a normality in Germany as well. Unfortunately mistakes have been made during the last years regarding incomprehensible, incomplete, and sometimes missing information to the public. This, as well as the

rather fruitless discussion between scientists and nonscientists, often led to opponents blocking and even damaging the field experiments.

However, at a time of globalisation, gene technology and its products would not come to a halt at our borders. In 1998 thousands of tons of soybeans were already imported from the USA. The segregation of soybeans derived from genetically modified, Roundup Ready resistant, soybean plants, and these from plants without modification, is not possible for technical and economic reasons. So, after processing, genetically engineered soybeans will be an ingredient of many dietary products. Although the European Union passed a "Novel Food" guideline considering the labelling of genetically modified food, the exact mode of action is still discussed. As a result consumers, not for the first time, felt themselves and their interests ignored.

In this context it is not surprising that, in difference to the USA, the acceptance of biotech products in Germany is very low, except of the products of the so-called "red" biotechnology; 80-90% of the people accept pharmaceuticals produced with the help of gene technology.

In conclusion, I would like to talk about two actual examples showing us the problems in public acceptance biotechnology still has to cope with. The first of these two cases has been discussed since the approval of the first field trials several years ago, and it is a never-ending story in the printmedia and on TV. The other one, just a month ago, initiated a biosafety discussion and led to a serious set-back of people's trust in gene technology.

After 15 years of biotechnology research the first applications are still under discussion in Europe and particularly in Germany and the North European countries. Herbicide resistant oilseed rape will be among the first transgenic plants ready to be marketed in Europe. The prospect of a commercial release of a genetically modified crop resistant to glufosinate has intensified public concerns about novel food and the potential gene flow from the transgenic plants to their wild relatives, leading to herbicide resistant superweeds.

Oilseed rape is a member of the *Brassica* family, and wild brassica indeed often grow nearby, which would make it possible to transfer resistance genes with the pollen. Actually, when grown together in a common field site, *Brassica napus* (oilseed rape) and *Brassica campestris* (weed) spontaneously produce transgenic hybrids (Mikkelsen et al. 1996). On the other hand it can be shown that under natural conditions, that is: a population of Brassica weeds growing next to a field of commercial oilseed rape, the density of airborne pollen beyond 360 m decreases to less than 10%. Within this radius of 360 m only 0.4-1.5% of the seedlings were transgenic hybrids, and less than 2% of these hybrids survived (Scott and Wilkinson, 1998). So, although the potential for transgene recruitment is real, the process is likely to be very slow and uncertain unless the transgene offers a significant selective advantage particularly during seedling establishment (which is not given with herbicide resistance).

Since the media have to strive for high circulation rates and viewing figures, they are only interested in spectacular headlines. Bearing this in mind it is not astonishing to find that they referred mainly to the results of Mikkelsen and his colleagues, creating huge headlines of a "superweed", although these experiments were not conducted under normal environmental conditions. In this way the media reaction confirmed people's suspicion and concerns about gene technology and transgenic plants. It is therefore the job of scientists and journalists to work together for a credible and balanced information of the public. Development of transgenic plants raises several questions about food and feed safety, environmental and agronomic concerns. The risk assessment of gene flow must take into account the specific trait introduced (herbicide resistance) and the biology of the plant (pollination mechanism, potential wild relatives in the same ecosystem). Experimental tests (and therefore field releases) are the only way of addressing public concerns. They will be conducted thoroughly and the results put at the journalists' disposal.

The other example I would like to refer to is the stir genetically modified potatoes from Scotland caused just one month ago. Scientists at the Rowett Research Institute in Aberdeen transferred genes coding for different types of lectins into potatoes to test a new form of insect protection. Among these genes was the genetic information for the lectin of the snowdrop and of Concanavalin A, a lectin found in a South American bean variety. Both of these lectins are known to be toxic for humans and other mammals. Nevertheless raw pieces of the transgenic potatoes containing these lectins were fed to rats, and as a logic

consequence the animals were intoxicated, lost weight, their immune system weakened, and they appeared otherwise unhealthy. (So, what would you expect after 110 days of this very "special" diet?). The fact that the rats reacted this way to the genetically modified potatoes containing Concanavalin A and the snowdrop lectin is by no means surprising as the genes the researchers introduced into the plant encode substances known to be toxic.

The fact that the Scottish scientists constructed such toxic potatoes caused public outrage, and again countless headlines, news shows, and magazines dealt with the "incalculable risks" of novel food and gene technology. The scientists who obviously believed their action was best scientific manner caused a public hysteria, condemning all genetically modified food products to be dangerous.

Additionally, the researchers of the Scottish Rowett Research Institute did not adhere to the rule set off at the Asilomar conference in 1973. They conducted experiments which foreseeably led to a potentially dangerous result and thereby carelessly destroyed the public's trust in modern biotechnology. Now we have to make every possible effort to restore this loss of faith. And for this reason, not for the first time, scientists, industries, and journalists have to cooperate with the aim of a trustworthy and credible biosafety communication.

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Seeds of Hope – A synopsis for the proceedings of the Biosafety Symposium Braunschweig, 9/98

Tschimmel, U.

Media Projects, Schloss Allner, 53773 Hennef

A synopsis of the production of the TV series for ZDF

"Seeds of Hope" is a six-part documentary of three hours length. It portrays the present, past, and future situation of the world food supplies and crop plants. A special focus is put on biotechnology as an important tool to develop a healthier nutrition, ensure food security, relieve the environment, and boost scientific methods for plant breeding.

The USA and Europe - the latter with its strongly emerging network research and development capacities - will play a pivotal role in that scenario as the advances in molecular plant genetics will ensure high quality and a new functionality in modern nutrition.

Furthermore, new developments from Asia will help secure the food supply in developing countries. There is no doubt that, if we are to forestall the spread of famines, this enormous task has to be performed within a few years. The seeds of biotechnology contain the powers to boost yields on existing farmland without degrading it. Thus, they could support the survival of the last wilderness areas on the planet.

The series will also throw a light on the enormous ecological potential of biotechnology: Where applied, it already has significantly reduced the use of herbicides, fungicides and insecticides. And, on the horizon, biotechnology is promising a closer approach to a sustainable agriculture.

Last not least, the series sets out to explore the role information technology is playing for modern genetics and agriculture. Be it the reduction of agro-chemicals through computer-controlled spraying, the spread of GPS systems or the enormous task of deciphering the genome of the thumb-sized model plant *Arabidopsis* computers are at the very heart of future agricultural revolutions.

The filming for "Seeds of Hope" is now emerging into its third stage. During the first stage, the production has included plant biotechnology research and farm application in the USA, including an in-depth coverage of research at Monsanto's Life Science Center, Calgene, biotech farmers (maize, soybeans, potatoes, cotton, rapeseed). Interviews were made with Rob Horsch, Peter Raven, Jimmy Carter, Roy Fuchs, Terry Medley, Uma Lele, Ismail Serageldin, Norman Borlaug, Roger Beachy, and many, many more.

During the second stage, Peru, India, Nepal, and Africa served as locations for topics like: The Need for the Next Green Revolution, Marginal Agriculture in the Himalayas, The World's Highest Potatoes, Who Will Feed the Masses?, Asia's Rice Bowls, Hunters and Gatherers, Africa's ongoing Nutrition Problems, and more.

Now, in its third stage, the "Seeds" crew is focussing on Europe and its biotechnology research projects. Here, we already have produced films on Integrated Farm Management (R&D and application in plant protection and plant nutrition) including precision farming with GPS etc., organic farming, classical plant breeding, MPIZ projects, BBA evaluation projects, FAL projects, gene bank Braunschweig (wild *Brassica* on Helgoland among the more exotic topics), Vavilov Institute, novel food, field trials.

"Seeds of Hope" is a multimedia package in the true sense of the word. The ZDF and Deutsche Welle will distribute the program in six world languages to more than 90 countries.

Accompanying and already preceding the TV series is our website www.gen-info.de with authors like Professor Jozef Schell, Norman Borlaug, and President Jimmy Carter; the site offers a daily email service with press clippings from more than 50 newspapers. It will be transformed into a science communication research and development project in the near future (BioRegio program).

Finally, radio coverage as a spin off from the film production will start in February 1999 and continue throughout the next year. And, last not least, a book to accompany the TV series is in its final planning stage.

During one and a half year of travelling and producing dozens of hours of broadcast quality video material, the crew has met many key scientists, visionaries, consumers, and - last not least - farmers all over the world. Especially in the developing countries, many of them share the urgent wish to participate in the technological advances which are made in the industrialized world, including biotechnology.

Most scientists in the USA were extremely well versed with media coverage showing a much wider exposure to that field - an advantage which should be taken into account by European firms for their PR work. It seems that in the States the necessity of media work is much more widely accepted than in the Old World.

All in all, the interest, co-operation, and hospitality during our travels was overwhelming. We visited any adventurous places like the Himalayan mountain village of Sikles which we reached by a gruelling two-day trek. If the Sherpas had not carried our equipment, we would not have made it. In Southern India, there were many dangerous situations on the road but thanks to our driver we swerved around a couple of bad accidents. And in America, we were able to relax for a few hours under the wide skies of Mississippi, in the middle of a *Bt* cotton field, listening to the eternal hum of the insects around us.

Summation

Economidis, I.

European Commission, DG XII, Bruxelles, Belgium

This report should not be considered as a summary of the Symposium but a report based on a rather personal and general appreciation of the Symposium. This review is made against the following pertinent questions:

- Are there gaps in our knowledge preventing risk measurement of the fate of released GMOs? Can we derive priorities for future research?
- Has the accumulated knowledge contributed to the development and practice of the existing regulatory framework?
- How can the results from the biosafety research improve the public understanding about biotechnology and its environmental impact?

The presentations made clear that several companies already have products at the commercialisation stage. Scientific managers from these companies presented data showing mainly the high yield of these products, with unfortunately very little data on safety.

The discussion of productivity of the US products was very striking in comparison to the EU where the "debate" on the safety of these products continues. It was made clear by the different speakers that the public in Europe shows a reluctance to accept these products. Hence, the field releases are limited in number.

Other European (non-EU) countries do not yet have field releases. They make an effort to harmonise with the EU regulatory framework.

There is a limited activity of field tests in Japan and a strong interest (little activity) in developing countries. Exception to this observation are the large releases in China. For the current releases, China applied a recently adopted regulatory framework.

The US speakers and participants came from academia, industry, and regulatory agencies. The impression given was that they make every effort to link regulations (and decisions) to R&D activity (USDA/EPA), a very important issue if we want to base regulatory regimes and decisions on scientific grounds. It was rather disappointing that European participants represented mainly the academic and the industrial establishments. The absence of regulators and competent authorities' representatives (despite being invited) was negatively perceived by the organisers and the participants.

The presentations dealt with the following issues:

Gene flow: Experimental or large-scale releases have not indicated up to now that gene flow is significant. The lack of obvious consequences may lay on the fact that the first generation of transgenics is based on single traits. Observations must be repeated with transgenic organisms with multiple recombinant traits and gene stalks.

Centre of origin: This is an issue which deserves monitoring for transgenic crops which are released in areas close to the centre of origin (diversity). The possibility of weediness is higher in areas close to the centre of diversity and close to areas where wild relatives of a given crop are found.

Large-scale releases: We have already experiences from large-scale releases of transgenic crops in the USA, in Canada, China, and other countries. Observations made in the field do not show any alarming phenomenon which might indicate risk.

Monitoring: Several speakers advocated for a post-release monitoring to assess any environmental impact due to long-time applications and large-scale releases. There is, therefore, a need for a reliable monitoring, however, it is debatable who should undertake these tasks. Factors such as reliable methodologies, the financial burden, and the public trust should be taken into serious consideration.

Public concern: Several speakers, mainly Europeans, focused on the public concern and the need for communication. A workshop was devoted to this issue. Presentations analysed the Swiss referendum and

the recent "turn" in the UK and in other countries having experience in transgenic crop releases. A well-prepared video presentation, aimed at TV programmes, raised the fundamental questions of the right methodology to approach public concern.

The other three workshops dealt with topics of microbial ecology (e.g., rhizosphere dynamics); *Bt* transgenic crops; and virus resistant transgenic plants. According to the rapporteurs all three scientific issues deserve further research efforts.

The discussions during the sessions raised several issues. One of the main concerns was the poor definition of environmental risk. In some cases, the participants advocated the broadening of the definition of risk beyond the environmental issue. In some extreme cases they proposed that risk should be defined by including not only issues associated to health and environment but also issues including socio-economical criteria.

In the general discussion it was pointed out correctly that discussion on risk without taking into account base-line information and the notion of substantial equivalence was fruitless.

Finally, it was mentioned that the high yield which can be achieved by transgenic crops should give the opportunity to avoid the over-intensification of agriculture, leaving some space for non-agricultural ecosystems.

It was suggested that the 6th International Biosafety Symposium in Canada in July 2000 should take into consideration the following issues:

- The presentations by the industry must be more analytical and present more safety-related data. Promotion-like presentations should be avoided.
- It is important to encourage presentations from consumers and users (farmers).
- It is necessary to continue discussions on emerging issues.
- The collaboration between R&D projects with regulatory practices is of paramount importance.
- The progress in the field of biosafety is based on the accumulation of experiences. Therefore, the presentation of experiences from different releases (different species, different ecosystems) is needed.

The 5th International Biosafety Symposium was successful, keeping the momentum of the debate of biosafety in its right track, i.e. the accumulation of experiences from the field releases, the accumulation and correlation of scientific information based on biosafety research, and the effort to link regulatory regimes to the scientific experiences.

The 6th Biosafety Symposium hosted by Canada in the year 2000 should continue in the same track enlarging its scope to issues such as food/feed biosafety issues without necessarily downplaying the environmental aspects. Finally, we expect that in the future we will have a more active participation of the regulatory authorities for a dynamic and fruitful discussion. Last, but not least, the organisers should make every effort to encourage attendance by participants from developing countries. Biosafety is an international endeavour and it is the duty of these Symposia to keep it as such.

Assessment of the invasiveness of transgenic sugar beet in populations of wild beet *Beta vulgaris* subspec. *maritima* (L.) Arcang

Pohl-Orf, M., Hesse, P., Brand, U., Schuphan, I., Bartsch, D.

Chair of Biology V, Ecology, Ecochemistry and Ecotoxicology, Aachen University of Technology - RWTH, Worringerweg 1, 52074 Aachen, Germany

Abstract

With the introduction of new genes in cultivated plants that do not normally occur in its species or in its crossable relatives, the evolutionary principle of mutation and selection is accelerated in a way that cannot be expected under natural conditions. The distribution of beet, *Beta vulgaris*, is ecologically limited due to their low competitiveness and frost tolerance. Therefore, an escape of transgenic attributes through gene flow into populations of beet relatives is more reliable than the escape of the transgenic sugar beet plant itself. It is important to emphasise that all cultivated and wild varieties of the beet (sugar beet, swiss chard, red beet or fodder beet) belong to the same species. That implies that there should be no barrier for outcrossing of genetically fixed qualities within this species.

The aim of this work was to evaluate if this outcrossing is probable, in which cases it is relevant, and which parameters are decisive to determine invasive effects of a transgenic plant.

In our studies, the potential ecological advantage of a coat protein-mediated virus resistance against infection with the *Beet necrotic yellow vein virus* (BNYVV) was assessed under conditions similar to those in wild beet habitats (salt irrigation). This should give answers to the following questions:

Is the rhizomania virus present at the wild beet habitats?

Are wild beets susceptible to infection with the rhizomania virus and if so, would the resistance of transgenic plants cause an ecological advantage?

Does this advantage help the plant to become invasive in natural plant communities?

Investigations were made to localize wild beet populations in Italy near the center of seed production in the Po valley. In these habitats, the occurrence of BNYVV was checked. To assess the influence of salt irrigation, tests on salt tolerance and rate of infection with the rhizomania virus under mesohaline soil conditions were performed. Related to results from earlier experiments about the competitiveness of transgenic and non-transgenic sugar beets, a clearer picture of the effects and the relevance of outcrossing and establishment of the transgenic virus resistance could be drawn.

The first result was that with immunological tests (ELISA) the occurrence of BNYVV could not be detected in the investigated Italian wild beet habitats.

In greenhouse tests on virus infection under different soil conditions there was a significant decrease of the infection rate with increasing salinity of the soil. The different wild variants showed a different range of extinction values. One of the populations even proved to be completely tolerant against virus infection. Especially in the case of the cultivar “Edda”, morphological changes due to salt irrigation were noticed. The plants developed succulent characteristics like thick leaves with a strong cuticle and more compact growth thus causing morphological similarity to their wild relatives.

Varying levels of infection between wild beet populations have been noted previously but not the strong dependence on soil salinity. One possible reason for this dependence may be the absence of the vector *Polymyxa betae* in the naturally mesohaline habitats. Based on our results, we cannot determine why this virus is unable to infect the plants but we can state that selective pressure for rhizomania resistance does not appear to be the decisive factor for the potential invasiveness of the transgenic beet into the natural Italian populations at the Adriatic coast.

In previous experiments on competitiveness of transgenic and non-transgenic sugar beet a higher performance of transgenic hybrids was expected under disease pressure. We found that the transgenic hybrids were superior to controls only at high BNYVV infestation levels (approximately 20%).

To answer the questions stated at the beginning the results of this investigation confirm that in this special case the risk for the modified sugar beet to become invasive is expected to be low because:

- The virus could not be detected in the wild beet habitats, and the susceptibility of wild beet to rhizomania is rather low especially under the salty conditions in coastal areas.
- Ecological implications due to transgenic BNYVV-resistant hybrids might only be observable in natural beet habitats with high levels of BNYVV infestation and where susceptible *Beta vulgaris* genotypes grow.

The possibility of outcrossing has been proved by investigations on outcrossing but in our case the ecological relevance of the new traits is less important. As confirmed by the experiments on competitiveness, there is no ecological advantage for the transgenic plants when the virus is absent, which is the case under natural mesohaline soil conditions. On the other hand, the transgenic plants appear to be more competitive under high virus pressure. If the virus would be able to establish itself in these habitats, an increased fitness in wild habitats could be the consequence, regarding that this case has not been found yet and an occurrence of the virus is only common on agricultural areas.

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Gene flow between cultivated and wild forms of beet (genus *Beta*) in California

Bartsch, D., Clegg, J., Cudney, D.W., Norris, R.F., Ellstrand, N.C.

Department of Botany and Plant Sciences, University of California, Riverside, CA 92521-0124, USA

Introduction

The main target of genetic engineering in the genus *Beta* is sugar beet, *Beta vulgaris* ssp. *vulgaris* var. *altissima* DÖLL, which is ecologically limited due to its low competitiveness and susceptibility to several plant pathogens and phytophagous animals. Nearly all beets are wind-pollinated. Thus, gene flow between sympatric cultivated and compatible wild forms is unavoidable (Boudry et al. 1993; Raybould and Gray, 1993; Bartsch and Schmidt, 1997). To address biosafety concerns for beet in California three questions should be answered: 1. Where are potential areas of sympatry of wild and cultivated beet in California? 2. Can we use isozyme marker to assign wild beets of California species and subspecies? 3. Is there genetic evidence of gene flow between wild and cultivated beets in California?

Materials and Methods

Fresh leaf material of wild and cultivated beet was sampled at several locations in California (Figure 1) and transported at 4ºC for immediate examination in the laboratory. Greenhouse-cultivated plant material generated from seed donation of USDA-ARS WRPIS (Pullman, USA), FAL (Braunschweig, Germany), Speckels Sugar (USA), Betaseed (USA), and KWS (Einbeck, Germany) was used as standards. 100 mg of fresh young leaf tissue was ground up in 0.5 ml extraction buffer (0.1 M Tris-HCl pH 7, 4% polyvinylpyrrolidone (PVP), 0.1% Dithioerythritol (DTT), 0.1% Ascorbic acid). Gel buffers, and techniques are as described by Devlin and Ellstrand (1989). Data analysis was carried out with POPGENE[1].

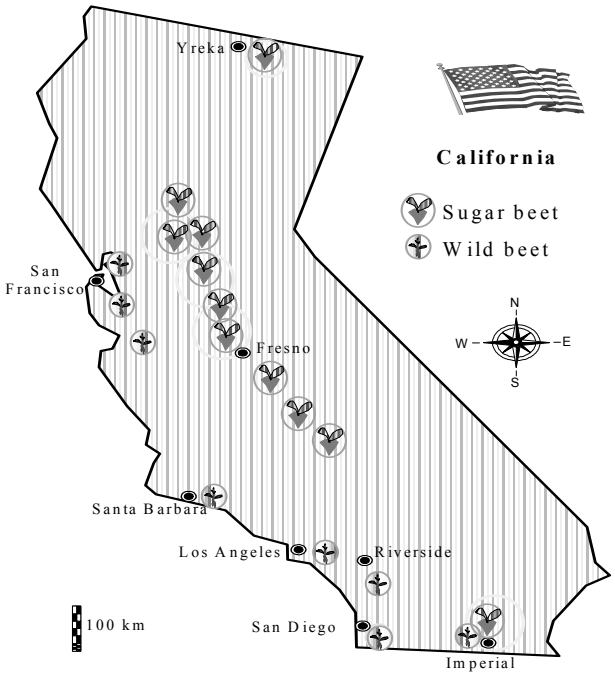

Figure 1 Areas of sugar beet and wild beet populations in California

[1] http://www.ualberta.ca/~fyeh/index.html

Results and Discussion

Wild beet populations in California were found in several locations[1]. Our isozyme study suggests some small populations (less than 200 individuals each) of vegetable beet have escaped cultivation and became naturalized in Riverside, Los Angeles, and San Diego Counties. Populations closely related to sea beet (*Beta vulgaris* subspec. *maritima*) are common in salt marshes near Santa Barbara and the San Francisco Bay Area, as well as waste land in northern San Benito County. The weed beet *B. macrocarpa* was found in sugar beet fields in the Imperial Valley.

In the Imperial Valley, McFarlane (1975) reported phenotypic observations of hybridization between wild and cultivated beets. In this area sugar beet is grown in winter culture, and vernalization of the biennial plants is a common phenomenon due to moderate cold winter temperature. An examination based on 15 sugar beet fields (representing an area of approximately 2 million m²) showed a sugar beet bolting rate of 0.6 plants/m². This rate seems to be higher than typical in this area, probably due to an extraordinary cool winter 1997/98 with periods of low freeze in some parts of the area. The density of the annual weed *B. macrocarpa* is in the range of 2.7 plants/m² (representing an area of approximately one million m² sugar beet plantation examined). Although the annual *B. macrocarpa* usually flowers earlier than sugar beet bolters, a flowering time overlap could be detected in May 1998. Based on 10 specific isozymes (Table 1), introgression in this area could be detected at a rate of 2% wild beet individuals (13 of 594 examined plants), which were morphologically similar to *B. macrocarpa*, but had isozyme alleles specific to *B. vulgaris*.

Table 1 Mean allele frequences for 13 isozymes of genus *Beta*. Isozyme alleles specific to *B. vulgaris* which were found in 2% of Californian *B. macrocarpa* individuals, are underlined. I = Sugar beet (16 varieties from Europe and California), II = Swiss Chard (4 varieties from California), III = Red beet (3 varieties from California), IV = Sea Beet (13 populations from Europe), V = Sea Beet (1 population from Egypt); VI = Imperial Valley weed beet (9 populations from Imperial Valley, California), VII = *Beta macrocarpa* (3 populations from Europe), VIII = *Beta macrocarpa* (1 population from Israel)

		I	II	III	IV	V	VI	VII	VIII
Mdh1	-3	0.765	0.525	0.827	0.885	0.809	0.692	0.726	0.961
	-4	0.157	0.443	0.005	0.023	0.000	0.001	0.000	0.000
	-5	0.078	0.032	0.168	0.092	0.191	0.307	0.274	0.039
Mdh2	-1	0.536	0.000	0.004	0.019	0.000	0.006	0.000	0.000
	-2	0.464	0.993	0.965	0.924	1.000	0.496	0.500	0.500
	-3	0.000	0.000	0.000	0.000	0.000	0.498	0.500	0.500
	-4	0.000	0.007	0.031	0.057	0.000	0.000	0.000	0.000
Aco	-2	0.914	0.176	0.254	0.728	1.000	1.000	1.000	1.000
	-3	0.086	0.824	0.746	0.272	0.000	0.000	0.000	0.000
Skd	-1	0.036	0.000	0.061	0.055	0.000	0.045	0.000	0.000
	-2	0.005	0.000	0.010	0.082	0.000	0.946	0.988	1.000
	-3	0.826	0.973	0.822	0.781	0.524	0.009	0.012	0.000
	-4	0.133	0.027	0.107	0.082	0.476	0.000	0.000	0.000
Lap	-3	0.390	0.725	0.551	0.821	0.937	0.001	0.000	0.000
	-4	0.610	0.275	0.449	0.179	0.063	0.008	0.000	0.000
	-5	0.000	0.000	0.000	0.000	0.000	0.991	1.000	1.000

[1] California County Flora Database. Http://plants.usda.gov/plants; California flora occurrence database. http://galaxy.cs.berkeley.edu/calflora/botanical.html

		I	II	III	IV	V	VI	VII	VIII
Tpi1	-1	0.499	0.500	0.500	0.474	0.500	0.500	0.545	0.500
	-2	0.501	0.500	0.500	0.526	0.500	0.500	0.455	0.500
Tpi2	-1	0.000	0.000	0.000	0.064	0.000	0.000	0.000	0.000
	-2	0.971	1.000	0.911	0.878	1.000	1.000	0.000	0.000
	-3	0.029	0.000	0.089	0.057	0.000	0.000	0.000	0.000
Aat1	-1	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	-3	0.533	0.500	0.496	0.570	0.048	0.005	0.000	0.096
	-4	0.000	0.000	0.004	0.210	0.476	0.991	1.000	0.904
	-5	0.466	0.500	0.500	0.220	0.476	0.004	0.000	0.000
Aat2	-1	0.000	0.000	0.000	0.000	1.000	1.000	0.985	1.000
	-2	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000
Udp	-1	0.556	0.482	0.831	0.180	0.469	0.000	0.000	0.000
	-2	0.444	0.518	0.169	0.820	0.531	1.000	1.000	1.000
Pgm1	-3	0.000	0.000	0.000	0.052	0.000	0.984	0.988	1.000
	-4	0.998	1.000	1.000	0.930	1.000	0.016	0.012	0.000
	-5	0.002	0.000	0.000	0.019	0.000	0.000	0.000	0.000
	-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Pgm2	-1	0.000	0.000	0.000	0.013	0.000	0.992	0.933	0.519
	-2	0.266	0.028	0.009	0.109	0.000	0.003	0.067	0.000
	-3	0.686	0.964	0.991	0.828	0.690	0.005	0.000	0.481
	-4	0.048	0.008	0.000	0.050	0.310	0.001	0.000	0.000
Gdh	-1	0.824	0.933	1.000	0.742	0.048	0.002	0.000	0.000
	-2	0.176	0.067	0.000	0.215	0.238	0.008	0.000	0.000
	-3	0.000	0.000	0.000	0.042	0.714	0.989	1.000	1.000

Conclusions

Engineered cultivars grown in the Imperial Valley would have an increased probability of gene escape to wild relatives if their bolting properties were similar or higher than tendencies of the current cultivars planted in this area. Because of this increased tendency for beets to bolt in this area, the impact of an engineered trait escaping into wild populations would have to be assessed.

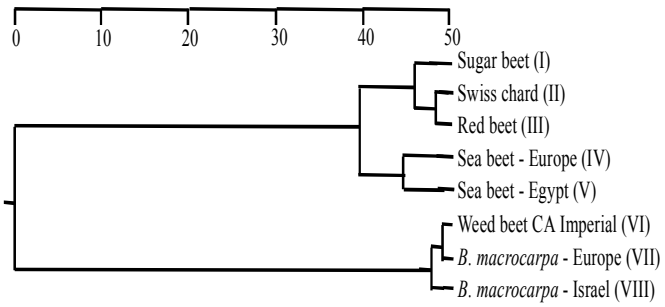

Figure 2 UPGMA dendrogram based Nei's (1978) genetic distance method of allele frequences for 13 isozymes of *Beta vulgaris* (see Table 1).

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Strategy to control heteroencapsidation-associated risks in transgenic plants

Jacquet, C., Ravelonandro, M., Dunez, J.

Station de Pathologie Végétale, INRA, Centre de Recherche de Bordeaux, BP 81, 33883 Villenave d'Ornon Cedex, FRANCE

Introduction

Engineering of the virus coat protein (CP) genes in plants is a strategy frequently used to fight virus infection. Successful results have been related against numerous virus groups (Beachy et al. 1990), and the resistance mechanisms have been commenced to be understood (Wilson, 1993). Since these last five years tremendous progress has been made and permitted to claim that the transgene products RNA or protein could interfere with the virus replication and lead to the protection against virus infection (Baulcombe, 1996).

Referring to CP as the transgene product, the exchange of CP sub-units with the virus incoming, a phenomenon also called heteroencapsidation, could happen in transgenic plants synthesizing viral CP and modify the epidemiological characteristics of the virus (Farinelli et al. 1992; Lecoq et al. 1993). Thus, complementation of deficient or absent biological properties of an incoming virus could be achieved through heteroencapsidation which is recognized as a potential biological risk. Lecoq et al. (1993) demonstrated that a strain of zucchini yellow mosaic potyvirus non aphid-transmitted (ZYMV-NAT) could become transmissible by aphids when encapsidated by the plum pox potyvirus (PPV) CP produced in transgenic *Nicotiana benthamiana*.

This article deals with the strategy used to control biological risks associated with heteroencapsidation. Taking a potyvirus CP gene as a model, some specific modifications were introduced in the CP gene construct according to two strategies. The first approach has been based on the deletion of the DAG amino acid triplet, involved in potyvirus aphid transmission (Atreya et al. 1990), and the second approach was to alter the CP transgene in order to obtain an encoded CP which would fail to assemble (Jagadish et al. 1991), thereby preventing possible heteroencapsidation in transgenic plants.

Results and discussion

In order to study the impacts of such modifications, the use of a procaryotic system like *E. coli* has been chosen for checking the feasibility of the tasks. The wild type PPV-FLCP gene has been manipulated (Ravelonandro et al. 1992). The modifications achieved either by PCR or by site-directed mutagenesis targeted to remove respectively the nucleotides coding for the DAG amino acid triplet or those for R_{220}, Q_{221} or D_{264}. These three latter amino-acids were targeted because they are crucial for the assembly of other potyviral CP (Jagadish et al. 1991; Dolja et al. 1994). Different forms of modified PPV CP (e.g. ΔDAGCP,ΔRQCP, ΔDCP or the 3ΔCP characterized by a CP lacking the three respective sites coding for DAG, RQ and D amino-acids) have been produced and evaluated in *E. coli.* Electron microscopy studies (IESM) showed that VLPs could only be observed in the extracts prepared from bacteria expressing FLCP and ΔDAGCP. These VLPs were highly heterogeneous in length and were fully decorated with PPV CP antiserum (Jacquet et al. 1998a). VLPs were never observed in the extracts from induced cells containing ΔRQCP and ΔDCP constructs.

To check for the potentiality of these gene constructs *in planta*, *N. benthamiana* has been transformed to assess their effectiveness. Transgenic lines transformed with FLCP, ΔDAGCP and 3 ΔCP were selected following their high accumulation of capsid protein (Ravelonandro et al. 1992; Jacquet et al. 1998b). Experiments made by Lecoq et al. (1993) have been reproduced to demonstrate heteroencapsidation-associated risks. All the transgenic lines were inoculated with ZYMV-NAT and infection controlled by ELISA. In most of the ZYMV-NAT infected FLCP and ΔDAGCP lines the presence of the virus was correlated with a significant increase in accumulation of the engineered CP whereas transcript-derived transgene levels were unchanged. This phenomenon was never observed in infected three ΔCP plants. The observations by ISEM revealed a phenotypic mixing in leaf extracts from infected FLCP and ΔDAGCP plants, and ZYMV particles in three ΔCP leaf extracts were never decorated by PPV antiserum.

In aphid transmission experiments, we confirmed that PPV FLCP could complement the deficient ZYMV-NAT through heteroencapsidation. Although a large number of heteroencapsidated ZYMV-NAT particles were observed, no aphid transmission occurred from the ΔDAGCP plants. This result confirms the demonstration that the DAG triplet is directly involved in potyvirus aphid transmission through interactions with the helper component.

Following this report that heteroencapsidation of a non aphid-transmitted potyvirus by a native CP could lead to the complementation of the viral defective function, we described here the different approaches to avoid such a risk and to develop new gene constructs for a rational design of environmental protection.

Acknowledgements

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Field resistance tests to plum pox virus infection of transgenic *Prunus domestica* plants under nursery conditions

Minoiu, N.[1], Ravelonandro, M.[2], Scorza, R.[3]

[1] Statiunea de Cercetera & Pomicola, Bistrita, Romania;
[2] Station de Pathologie Végétale - INRA Bordeaux, BP 81, 33883 Villenave d'Ornon, France;
[3] USDA-ARS Appalachian Fruit Research Station, Kearnsville, WV 25443, USA

Introduction

Plum pox virus (PPV) is one of the plant viruses which cause considerable yield losses for stone fruit production in Europe. This virus was first described as the agent responsible for Sharka disease in Bulgaria (Atanassov, 1932). The aphid vectors are responsible for the spread throughout European borders. Search for natural gene resistance to transfer into economically important Prunus cultivars by conventional breeding is the main target of every scientist involved in the control of the disease but this task is extremely difficult. Only a few examples of apricot or plum cultivars have been clearly identified as tolerant to PPV infection (Dosba et al. 1994; Kegler and Hartmann, 1998; Minoiu et al. 1997). In situations like these, fruit production can be exploited but the trees remain to be infected. The difficulty met in the control of PPV led the EEC to classify this virus as a quarantine pathogen (1993). Tremendous progress has been made with the studies of PPV genomic expression and notably with the technologies of plant transformation in the last twelve years.

More recently, transgenic plums expressing PPV capsid (CP) gene have been produced (Scorza et al. 1994) and shown under confined greenhouse conditions to be resistant to PPV infection (Ravelonandro et al. 1997). The same clones have been transferred in Bistrita (Romania) and tested under natural field conditions. We conducted field experiments with transgenic scions in 1996, 1997 and 1998 to assess the resistance phenotype of transgenic plums containing PPV CP gene under open field and nursery conditions. The number of control plants naturally infected was disappointingly low, the present report describes the results obtained from the experiments under nursery conditions.

Results and Discussion

To validate the observations we had in confined greenhouse conditions, different plum clones (C-2,-3,-4,-5 and -6) carrying the mini-T-DNA which included the NPTII, PPV CP and GUS genes (Scorza et al. 1994) and the non-transformed cultivar B70146 have been transferred to the field. As only a small amount of plums (15-25%) have been naturally inoculated in the open field for two years, the routine protocol developed for nursery conditions has been applied. In August 1997 buds from non-infected clones (transgenic and non-transformed) were co-grafted with infected buds from six local cultivars designated BN6, BN7, BN9, Tuleu, 5/125 and 6/283 onto Myrobolan rootstocks. Foliar symptoms like mosaic, ring, chlorotic blotches, deformation were evident in the majority of both control (infected Romanian cultivar and B70146) and transgenic plums (C-2,-3,-4 and -6) in spring 1998. Greenhouse assays already showed that these clones were susceptible, and that C-5 clone was highly resistant (Ravelonandro et al. 1997). Since the double-grafting made in August 1997, surprisingly the transgenic C-5 clone showed very discrete spots or lines along some veins of the expanded leaves located at the bottom part of the shoots. Leaves have been collected and DAS-ELISA analysis permitted to show that PPV is present in these areas of the scions. One month later, most of the susceptible plants including the local varieties were severely infected, while the transgenic C-5 scions grew vigorously like the non-inoculated control and appeared healthy. Older leaves (bottom part) were the only section infected by PPV. Similar observations have been made in transgenic *N. benthamiana* plants expressing PPV CP gene as previously described by Ravelonandro et al. (1993). Irrespective of the PPV isolates tested PPV-BN6, -BN7, -BN9, -Tuleu, -5/125 and-6/283, the transgenic clone C-5 displayed a recovery phenotype. Only partial data are available about these PPV isolates, they have been serologically and molecularly characterized as D or M strains (Ravelonandro and Minoiu, 1997).

Interpretation of these assays permitted to show that the double-grafting assays tested here are very efficient for infecting plants. Following the dormancy and irrespective of the clones tested, PPV was able to move through the vascular tissue. Such a movement can only occur when the C-5 shoots are still

small. However, when the scions were developing fully expanded leaves, the transgenic C-5 clone recovered and confined PPV to the older leaves adjacent to the grafting point.

Part of our investigations is to characterize the behaviour of transgenic plums in field natural conditions. While it was not surprising to observe one to three plants of the clones C-2, -3, -4, -6 exhibiting infection, the C-5 plum remains healthy. Such observations are corroborating our findings in confined greenhouse conditions when plum C-5 has been aphid-inoculated with PPV (Ravelonandro et al. 1997). The observations are continuing, the understanding of the resistance mechanisms displayed by the C-5 clone is underway. These findings would be determinant in the qualification of plum C-5 as an elite that can be used for a breeding programme.

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Infection by a pathogen can cause transgene instability!

Al-Kaff, N., Kreike, M., Covey, S., Dale, P.

John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Summary

Transgenic plants are being approved for widespread use in agriculture and for food. Before transgenic crops are accepted for this, a risk assessment must examine various factors, including expression and stability of the transgene(s). We have investigated the instability of transgene expression after pathogen infection. We used the cauliflower mosaic virus (CaMV) in different plant host species. Different responses were found depending on the plant host and transgene content. In *Brassica napus* (oilseed rape) infected with CaMV, three types of instability of transgenes were found, transcriptional silencing when the transgene contained promoter homology (35S promoter), post-transcriptional silencing when there was RNA homology to the virus (35S terminator), and enhanced expression of the transgene when there was no homology to CaMV but it contained the bacteria promoter NOS. In this poster we discuss the up and down regulation of transgene expression during infection in different host species.

Introduction

The commonly used regulatory elements in transgenic crops are the 35S promoter or its terminator from the cauliflower mosaic virus (CaMV). Since viral or other pathogenic elements are useful components of transgene constructs, it is important to investigate the consequences and effects for transgene expression after infection by these viruses or pathogens. Here we are presenting some of our results which show different responses to CaMV infection depending on the interaction between plant host species and the CaMV virus.

Materials and Methods

Several lines of transgenic oilseed rape (*Brassica napus)* were used, some of which contained the 35S promoter of the cauliflower mosaic virus (CaMV). Transgenic oilseed rape was infected with two isolates of CaMV Cabb B-JI and Aust, severe and very severe, respectively. RNA was extracted for the transgenes at different days post inoculation and then detected by Northern blot analysis. In all experiments, transgene expression was compared between infected and non-infected plants.

Conclusions

1. Infection of transgenic oilseed rape *(Brassica napus)* with CaMV causes transgene instability.
   - Expression level of transgenes containing sequence homology to CaMV (35S promoter) were silenced at the transcriptional level.
   - Expression level of transgenes containing RNA homology to CaMV (35S terminator) were silenced at the post-transcriptional level.
   - Expression levels of transgenes which did not contain sequence homology (Nos promoter) were enhanced.
2. Transgene instability in CaMV-infected transgenic plants depends upon virus and plant host species interaction.

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Environmental risk assessment of transgenic cucumber introduced rice chitinase

Koga-Ban, Y.[1)], Tabei, Y.[1),2)], Ishimoto, M.[3)], Nishizawa, Y.[1)], Tsuchiya, K.[1)], Kayano, T.[1)], Tanaka, H.[1)]

[1)] National Institute of Agrobiological Resources, MAFF, Tsukuba, Japan,
[2)] MAFF Research Council Secretariat, Tokyo, Japan,
[3)] National Agricultural Research Centre, Japan

Abstract

We reported that transgenic cucumber plants introduced rice chitinase cDNA acquired gray mold (*Botrytis cinerea*) resistance (Y. Tabei et al. 1998). These plants are introduced by *Agrobacterium* mediation and rice chitinase cDNA was driven by the CaMV 35S promoter. Following the above results we have assessed environmental risk of transgenic cucumber plants.

According to the guideline of Science and Technology Agency (STA), we have performed environmental risk assessment of transgenic cucumber plants in a closed and in a semi-closed greenhouse. The following items were compared between transgenic plants and non-transgenic cucumber plants; 1) morphological characteristics of pustules and fruits during their maturation periods, 2) reproductive characters, e.g. pollen form and fertility, longevity of the pollen, pollen dispersal by wind, seed fertilities and cross compatibility of melon with wild relatives, 3) possibility of harmful influences on environment due to the presence of detrimental substances, i.e. volatile compounds, allelochemical substances, 4) presence of remaining *Agrobacterium tumefaciens* strain LBA 4404, which was used as a vector in the production of the transgenic cucumber. Transgenic cucumber indicates no characteristic differences except expression of rice chitinase gene and resistance to gray mold.

Results and Discussion

Evaluation of resistance against gray mold in transgenic cucumber plants and its heredity to the next progeny

We had examined 20 transgenic cucumbers and found three types of sensitivity to gray mold, i.e. strongly resistant (non-extensible lesion), resistant (weak extensible lesion), sensitive (extensible lesion) (Table 1).

Table 1 Susceptibility of transgenic cucumber plants bearing the rice chitinase cDNA (RCC2) to *Botrytis cinerea* infection

Resistance	Lesion diameter (mm)[1)]	Transgenic cucumber strain
Highest resistance	<5	CR-29, 32, 33
Intermediate resistance	5~10	CR-1, 3, 4, 6, 8, 31, 34
Sensitive	10<	CR-15, 18, 20, 37, 42,
		Shimoshirazu[2)]
		Shimoshirazu[2)]

[1)] Diameter of lesion was measured after four days of infection; [2)] non-transgenic cucumber

Detection of the RCC2 protein by ELISA

The amounts of RCC2 products from three CR strains (CR- 33, CR-32 and CR-20) were compared with the control by ELISA analysis (Table 2). The level of gene expression of two resistant strains, CR-32 and CR-33, were 2.3 and 3.1 times higher than that of the control, respectively. On the other hand, the susceptible strain CR-20 was only 1.6 times higher.

Table 2 Detection of RCC2 protein product in transgenic cucumber plants by ELISA analysis

	CR 33	CR 32	CR 20	Control
MV[1)]	1.12	0.83	0.58	0.36
RV[2)]	(3.1)	(2.3)	(1.6)	(1.0)

[1)] Measured value of absorbance at 405 nm; [2)] relative value of RCC2 protein compared to the control

Comparison of pollen characteristics between transgenic and non-transgenic cucumber plants

The pollen size of transgenic and non-transgenic plants were found to be the same, i.e. 50~60 mm. Pollen fertility, when it was stained with acetocarmine, also showed no difference (Table 3). Table 4 shows longevity of pollen. Thus it can be predicted that there is no difference between transgenic and non-transgenic cucumber.

Table 3 Comparison of pollen fertility between transgenic cucumber and non-transgenic cucumber plants

	Transgenic cucumber	Non-transgenic cucumber
Fertility (%)	78.1 ± 12.0%	74.2 ± 7.7%

Table 4 Comparison of pollen longevity on media plate between transgenic and non-transgenic cucumber plants

	Germination Percentage	
Time of pollen collection	**Transgenic cucumber**	**Non-transgenic cucumber**
7:30	29	36
9:30	30	37
11:30	38	41
13:30	45	43
15:30	37	34
17:30	12	19
13:30, next day	16	5
9:30, two days later	0	0

Pollen dispersal and natural seed setting

Ten pots of transgenic and eight pots of non-transgenic cucumber plants were placed in 50 cm, and the seeds of naturally set fruits formed without artificial cross and insect mediation were investigated. Fruits after 35 days of flowering were examined, and no seeds were found in both transgenic and non-transgenic cucumber plants.

Comparison of detrimental substances between transgenic cucumber and non-transgenic cucumber plants employing biological assay

i. Influence of volatile compounds: Germination rate of transgenic and non-transgenic cucumber plants in the plant box was found to be 98.0 % and 98.4 %, respectively. Employing t-distribution testing indicates no significant difference between transgenic cucumber and non-transgenic cucumber plants (Table 5).

ii. Influence of allelochemical substances secreted from root: Germination rate of broccoli immersed in water, containing root secreta of transgenic or non-transgenic cucumber plants, was 91.2% and 93.2%, respectively. Employing t-distribution testing indicates no significant difference between transgenic and non-transgenic cucumber plants (Table 5).

iii. Influence of allelochemical substances produced in plants. Germination rate of broccoli immersed in crude extract of transgenic cucumber or non-transgenic plants was 99.2% and 99.6%, respectively. Employing t-distribution testing indicates no significant difference between transgenic and non-transgenic cucumber plants (Table 5).

Table 5 Influence of biological products on broccoli germination in transgenic or non-transgenic cucumber plants

	Germination Percentage	
Growth condition	**Transgenic cucumber**	**Non-transgenic cucumber**
Co-culture in box	98.0 ± 2.0	98.4 ± 2.4
Root secreta	91.2 ± 6.1	93.2 ± 5.4
Plant extracts	99.2 ± 1.1	99.6 ± 0.9

Chromatographical comparison of transgenic cucumber and non-transgenic cucumber plants

i. Chromatographical comparison of volatile compounds
Elution profile of volatile compounds from transgenic and non-transgenic cucumber plants was compared by gas chromatography. Gas chromatography data of transgenic and non-transgenic cucumber plants showed no significant difference in peak position and peak form.

ii. Chromatographical comparison of leaf products
High-pressure liquid chromatography (HPLC) of transgenic and non-transgenic leaf extracts showed some difference of peak height, however, it is considered as individual variation and not the significant difference, i.e. peak position and peak form.

iii. Chromatographical comparison of root secreta
HPLC of transgenic and non-transgenic cucumber root secreta also showed some difference of peak height, however, it is considered as individual variation and not the significant difference.

Effect of transgenic cucumber cultivated soil to succeeding crop

No significant difference in germination rate and growth of broccoli was observed when soil cultivated by transgenic or non-transgenic cucumber was used (Table 6). Thus the influence of transgenic cucumber cultivated soil was the same as of non-transgenic cucumber.

Table 6 Influence of soil grown previously with transgenic or non-transgenic cucumber plants on germination and growth of broccoli seed

Broccoli grown soil	Germination (%)	Root length (cm)	Fresh weight of seedling (g)
Transgenic cucumber	93.0 ± 3.3	9.81 ± 0.20	24.7 ± 1.57
Non-transgenic	94.0 ± 2.0	9.6 ± 1.0	25.5 ± 1.8

Effect of transgenic cucumber cultivation on soil microorganisms

Number of microorganisms in the soil is indicated in Table 7. Number of bacteria and actinomycetes in non-transgenic cucumber cultivated soil was little larger than that in transgenic soil. Number of fungi was found to be vice versa. However, the value was quite similar and we considered that there should be no effect on soil microbiota by transgenic cucumber cultivation.

Table 7 Number of microorganisms in the soil in which transgenic or non-transgenic cucumber plants were grown, respectively

	Number of microorganisms (CFU/g)		
Soil	**Bacteria**	**Actinomycetes**	**Fungi**
Grown with transgenic cucumber	8.6×10^6	1.3×10^5	1.4×10^4
Non-transgenic cucumber	9.6×10^6	1.8×10^5	1.2×10^4

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T4-Lysozyme as a tool for resistance engineering in potato

Mahn, A.[1)], Porsch, P.[1,3)], Brinkmann, O.[2)], Gieffers, W.[2)], Düring, K.[1,3)]

[1)] Institute for Breeding Methods in Vegetables, Federal Centre for Breeding Research on Cultivated Plants, Quedlinburg, Germany;
[2)] Max-Planck-Institute for Plant Breeding, Köln, Germany;
[3)] MPB Cologne GmbH, Köln, Germany

Transgenic potato plants were engineered for resistance to microbial pathogens using the lysozyme gene of bacteriophage T4 fused to a plant signal peptide encoding DNA sequence for import of the newly synthesized protein into the endoplasmic reticulum and subsequent secretion to the intercellular space (transgenic lines DL4 and DL5). In addition, MAR sequences flanking the lysozyme expression cassette have been introduced into the T-DNA for normalization of position effects in one construct (transgenic lines DL10-15). The level of T4 lysozyme expression in transgenic plants was determined by Western blotting.

Greenhouse-grown transgenic potatoes have been analysed for resistance to *Erwinia carotovora* ssp. *atroseptica* (Eca) using a tuber disc assay. Two out of eight lines were also used for field experiments. Expression of T4 lysozyme in the field-grown plants was stable throughout the growth periods within three years of field tests. For evaluation of resistance to soft rot and blackleg tubers were inoculated before planting with Eca using vacuum infiltration or inoculation of bacteria into size-defined wounds, respectively. Plants were screened for incidence of blackleg. In addition, harvested tubers were used for a storage assay.

To compare the agricultural value of transgenic and control lines in terms of yield of healthy daughter tubers after field cultivation and storage an index for the practical value of the lines was designed. After definition and ranking of assessment criteria, their relative value was multiplied by the respective ranking factor. According to that calculation the index for the practical value of both the transgenic DC1 and the non-transgenic Désirée control lines was about 0.8 whereas the transgenic line DL5 obtained an index of 0.95. Line DL4 showed a remarkable growth depression and delayed development most probably due to somaclonal variation. As a consequence its performance in the field was less good and an index of only 0.65 was calculated. Although a statistical analysis of these indices is not possible, they demonstrate that the practical value of the phenotypically normal line DL5 is promising.

For evaluation of soft rot resistance of field tubers a half tuber assay was employed showing significant differences between transgenic and control tubers. The results available from the first field resistance testing of 1997 provide the following tendencies:

1. DL5 shows better performance in respect of blackleg
2. DL4 is disadvantaged in blackleg
3. DL4 and DL5 both perform better in the storage assay for determination of soft rot
4. DL4 and DL5 both perform better in the half tuber assay.

All results are from one-year field experiments so far. These experiments are repeated in 1998 and results are under evaluation which is made always relative to the non-transgenic and transgenic marker gene - only control lines.

Significantly increased resistance of transgenic potato plants to *Phytophthora infestans* was shown in leaf disc inoculation assays. Reduction of the diseased leaf areas after inoculation with a mixture of races 1-11 of *P. infestans* by 60-70% was shown with lines DL10, DL12, DL13 and DL14 to be statistically significant.

This effect of T4 lysozyme cannot be explained by its muramidase activity. Additional experiments to elucidate another mode of action of T4 lysozyme revealed that enzymatically inactive T4 lysozyme retains its full bactericidal activity. The region conferring this bactericidal activity was localized to a 13 amino acid C-terminal peptide of T4 lysozyme. The corresponding chemically synthesized peptide possesses as expected no enzymatic but bactericidal activity. Further lines of evidence for this new mode of action could be elaborated by demonstrating fungistatic activity of enzymatically inactive T4

lysozyme and isolated peptide on growth of fungal germination tubes of *Phytophthora nicotianae* and *Fusarium oxysporum*. In addition, a second peptide of T4 lysozyme could be identified conferring fungistatic activity. Both peptides contain amphipathic helices which are well known to mediate cell membrane and cytoplasmic disrupture, most likely by pore formation leading to leakiness.

A possible effect of T4 lysozyme on integrity of plant cell membranes was analysed in survival assays with potato protoplasts. Survival of protoplasts was determined using Sytox, a green fluorenscent dye that stains DNA and is unable to penetrate intact cell membranes. Thus, protoplasts with permeable membranes fluoresce green whereas protoplasts with an intact cell membrane show red background fluorescence of chloroplasts. The number of living protoplasts was clearly reduced after incubation with T4 lysozyme and hen egg white lysozyme in contrast to control experiments with bovine serum albumin.

Using this knowledge new resistance strategies against plant pathogens e.g., expression of peptides of T4 lysozyme in transgenic plants, are conceivable giving a new basis for safety evaluation. Biosafety aspects of T4 lysozyme potatoes are assessed in a co-operative project funded by the German Federal Ministry of Education, Science, Research and Technology.

Assessing the impact of transgene encoded T4-lysozyme on *Rhizobium leguminosarum* bv. *viciae*

Broer, I.[2)], Kriete, G.[1)], Neumann, K.[1)]

[1)] Universität Rostock, Fachbereich Biologie, Doberanerstr. 143, 18051 Rostock, Germany;
[2)] Georg-August-Universität Göttingen, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Untere Karspüle 2, 37073 Göttingen, Germany

Transgenic potato plants expressing T4-lysozyme display enhanced resistance against the plant pathogen *Erwinia carotovora* (Düring et al. 1993). T4-lysozyme, as other lysozymes, is a bacteriolytic enzyme destroying bacterial cell walls. Hence, the transgene encoded T4-lysozyme may not exclusively harm bacterial pathogens but also affect other soil bacteria necessary for plant growth and health. We therefore analysed the impact of T4-lysozyme in comparison to hen egg white lysozyme on symbiotic bacteria, using *Rhizobium leguminosarum* as a model organism. We showed that when growing in liquid medium *R. leguminosarum* was extremely sensitive to both kinds of lysozymes. Additionally, the impact of crude extracts from transgenic potatoes on *R. leguminosarum* could be demonstrated. The sensitivity of *R. leguminosarum* was dependent on the number of cells present in the culture, and it was highest using a starting titer of 10^3 bacteria.

From this we conclude that *R. leguminosarum* is a suitable model organism to study the putative impact of transgene encoded T4-lysozyme on soil microorganisms (de Vries et al. 1998).

Although, in the laboratory, the nodulation of the untransformed macrosymbiont *Vicia hirsuta* was diminished by a pre-treatment of the bacteria with T4-lysozyme, no influence could be detected after the application on the root itself. Nevertheless, when T4-lysozyme was produced directly by transgenic *V. hirsuta* roots on culture medium, the number of nodules was reduced to less than 50% (Quandt et al. 1993; Broer et al. in prep.)

We therefore analyzed the nodulation of wild-type V. *hirsuta* roots grown in the direct neighbourhood of transgenic potatoes in the greenhouse and during a three-year field trial. Although the nodulation rate of the *V. hirsuta* plants was influenced by the location, season, weather, and even by the potato cultivar, no correlation between the nodulation rate or the nitrogen fixation rate and the T4-lysozyme excreted by the transgenic root could be detected, neither in the field nor in the greenhouse.

Hence, under non sterile conditions, there seems to be no bacteriolytic lysozyme activity affecting the extremely lysozyme-sensitive Rhizobia in the close vicinity of the transgenic potato roots used in the experiments. Nevertheless, since the lysozyme production of the two transgenic lines used is not very high, this statement cannot be generalized for all lysozyme producing lines that might be used in agriculture.

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Monitoring the bacterial diversity of rhizosphere soil around transgenic Barnase/Barstar and non-transgenic potato plants with molecular techniques

Lukow, T., Liesack, W.

Max-Planck-Institut für terrestrische Mikrobiologie, Marburg, Germany

Introduction

During the last two decades the use of the 16S rRNA gene (rDNA) as a culture-independent marker has provided insights into the naturally occurring diversity of the microbial world. Studies using the PCR-based retrieval of 16S rDNA, followed by cloning and comparative sequence analysis, have resulted in the detection of many previously unknown microbial lineages. But this technique is not suitable for screening many different samples because of the labour and time intensiveness involved. Several 16S rDNA-based methods have therefore been developed for molecular fingerprinting of microbial communities, e.g., ARDRA (amplified ribosomal DNA restriction analysis), SSCP (single-strand conformation polymorphism), TGGE (temperature gradient gel electrophoresis), and DGGE (denaturing gradient gel electrophoresis). However, one major drawback of all these techniques is that they often result in very diffuse patterns when applied to complex microbial communities, making their interpretation rather difficult (Jaspers and Overmann, 1997).

Here we demonstrate the usefulness of the T-RFLP (terminal restriction fragment length polymorphism) method (Liu et al. 1997) for molecular analysis of complex microbial communities.

The transgenic potato plants under study carry two modified gene constructs (Strittmatter et al. 1995). One gene construct consists of a bacterial ribonuclease from *Bacillus amyloliquefaciens*, termed Barnase, coupled with the *prp 1-1*-promoter from potato. The promoter should be selectively induced by pathogen attack. To minimize the detrimental effects of a potential background Barnase-activity in non-infected tissue, the second gene construct is inserted into the genome of the transgenic plants: the *barstar*-gene coupled with the constitutively expressed viral *CaMV 35S*-promoter. The barstar-gene which inhibits the Barnase synthesis is derived from *B. amyloliquefaciens* as well.

Field trials are being conducted at the Max Planck Institute for Plant Breeding in Cologne to investigate possible risks of the release of such transgenic plants (the coordinator of the project is Professor W. Rohde). In this context our aim is to elucidate if transgenic potato plants promote shifts in the bacterial community structure of the rhizosphere soil compared to non-transgenic plants. The seven different transgenic Barnase/Barstar plants and the one non-transgenic plant (*Solanum tuberosum* cv. "Bintje") covered an area of about 100 m^2. Samples have been taken near the plants at a depth of 20 cm.

Methods

The T-RFLP method is a culture- and cloning-independent technique which allows the identification of fragment length polymorphisms among individual sequence types of mixed 16S rRNA gene PCR products. In parallel, the bacterial diversity present in the rhizosphere soil was characterized by comparative analysis of 66 randomly selected environmental 16S rRNA gene sequences. We were able to correlate defined OTUs (operational taxonomic units) of the T-RFLP patterns with defined phylotypes.

Results and Discussion

Rhizosphere soil samples from seven different transgenic Barnase/Barstar potato plants and one non-transgenic plant were investigated by T-RFLP to comparatively analyse the bacterial diversity. The use of T-RFLP resulted in very similar patterns for all eight samples. The analysis of variance of the relative signal intensities of the abundant OTUs did not reveal any significant difference among the rhizosphere soil samples taken from the eight investigated potato plants.

Additionally, the bacterial diversity present in the rhizosphere soil was characterized by comparative analysis of 66 randomly selected environmental 16S rRNA gene sequences. Members of the following taxa could be identified as abundant populations in the field site examined: *Proteobacteria*, *Holophaga/Acidobacterium* phylum, CFB-phylum, *Planctomycetales*, and *Verrucomicrobiales*.

Sequence information of the 66 rRNA gene clones allowed the correlation of defined phylotypes to individual OTUs of the community patterns. However, a linkage between individual OTUs and phylogenetic information is not one word because different phylotypes may contribute to the same OTU.

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Methods to assess horizontal gene transfer in soil bacteria

Nielsen, K.M.[1)], Bones, A.M.[1)], Smalla, K.[2)], Elsas, J.D. van[3)]

[1)] UNIGEN - Center for Molecular Biology, and Department of Botany, Norwegian University of Science and Technology, 7005 Trondheim, Norway;
[2)] Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, 38104 Braunschweig, Germany;
[3)] Research Institute for Plant Protection (IPO)-DLO, 6700 GW Wageningen, The Netherlands

The possibilities of horizontal gene transfer (HGT; no parental-to-offspring transfer of genes) from genetically modified plants (GMP) to microorganisms are often assessed during evaluation of the biosafety of GMP before release into the field. The relevance of this type of assessment is controversial due to that experimental approaches in both field and laboratory have not been able to confirm the occurrence of such HGT from transgenic plants to naturally occurring bacteria. However, recently, two studies have shown transfer of marker genes from transgenic plants to bacteria based on homologous recombination (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998). Also, indications of natural HGT from plants to bacteria have been given after comparison of their DNA sequences. Here we briefly describe the methods used to investigate such HGT, discuss their limitations, and summarize the results obtained (Nielsen et al. 1998).

HGT of transgenic plant DNA to bacteria exposed to the plant material in soil has been investigated by at least two different approaches:

1. Field studies with the aim to detect bacteria transformed with marker genes released from transgenic plants during field trials (Smalla et al. 1994; Smalla, 1995).
2. Laboratory studies with the aim to detect HGT under optimized conditions for gene transfer into transformable bacteria (Broer et al. 1996; de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Nielsen et al. 1997; Schlüter et al. 1995).

In addition, HGT of native plant genes to bacteria has been suggested after comparison of DNA sequences obtained from plant and bacterial genes (Nielsen et al. 1998). The HGTs suggested after the comparisons may indicate that plant genes transferred to bacteria do not overcome an expression barrier (e.g. introns), that the fragments transferred are too short to be detected, or that native plant DNA does not confer any selective advantage in the bacteria. Thus, these observations might therefore not be relevant for the assessment of HGT of novel genes from transgenic plants.

Investigations with the aim to detect bacteria transformed with marker genes released from transgenic plants during field trials have been performed (Becker et al. 1994; Smalla et al. 1994; Smalla, 1995). These studies were based on detection of transfer of plant-harbored antibiotic resistance genes to bacteria. A successful detection of HGT events using this method requires that the relevant species or sub-populations of bacteria would:

1. Be naturally exposed to transgenic plant DNA at a quality and quantity required for uptake and integration. At the same time, the bacteria must experience conditions that enable them to express genes needed for stabilization of the foreign DNA.
2. Be able to regulate their barriers to recombination with diverged transgenic plant DNA (unless sequence homology is present or the antibiotic resistance genes from the plants are functional gene cassettes).
3. Can express the incorporated antibiotic resistance genes at sufficient levels for the applied selection to be successful, without experiencing a significant reduction in fitness. They should also be able to respond to the selective medium used, with a growth rate which enables their detection.

From the above criteria, it is clear that many potentially transformable bacteria in soil cannot be detected by this method. Only a minor fraction of soil bacteria, approximately 10%, will grow when plated on commonly used growth media. Screening of the bacterial fraction unavailable to cultivation techniques can be done by PCR analyses of total DNA extracted from soil samples. However, such analyses seldom prove incorporation of the plant marker genes in the genomes of the bacteria. Advantages using this

method are the detection of naturally occurring gene transfers (of the selected marker gene), and the screening of potential HGT in numerous species of soil bacteria.

Applying this method, screening of HGT in field trial sites from transgenic sugar beet (*Beta vulgaris*) to naturally occurring soil bacteria has been performed. Analyses of bacterial fractions (both culturable and non-culturable fractions) of soil samples were done without obtaining any positive results of HGT of the selectable kanamycin resistance gene (*nptII*), as confirmed by colony hybridization and PCR (Smalla et al. 1994; Smalla, 1995). In another study, a soil microcosm was used to investigate possible HGT of the *nptII* and hygromycin resistance (*hph)* genes from homogenates of transgenic tobacco (*Nicotiana tabacum*) into bacteria present in the microcosm. No transformants were detected in these studies after selective plating for antibiotic resistance followed by DNA hybridization (Becker et al. 1994).

Several studies on HGT from genetically modified plants to bacteria have been performed under optimized laboratory conditions with the hypothesis that such gene transfer takes place by natural transformation. The reported studies have all been done with readily culturable, Gram-negative, soil or plant-associated bacteria. An advantage of this method is the high sensitivity obtained. Some limitations emerging from the use of this method include the following:

a) Although optimized conditions are commonly used (high concentrations of DNA, optimal cell growth and competence development) the actual gene transfer systems are often far from characterized and developed with respect to optimal conditions for gene transfer. For instance, the level of natural competence of the bacterium used may be low, or even unknown. In some investigations, electroporation or artificial transformation has been used to infer knowledge of natural gene transfer events. The relevance of data produced by such methods, at large developed for plasmid transfer, to the investigation of natural transfer of linear chromosomal genes is unclear. For instance, conditions or environmental signals needed for integration of chromosomal genes might not be present when applying techniques developed for the transfer of DNA across bacterial membranes.
b) The presence and stability of the transgenic plant DNA used, and most importantly, the transforming activity of the plant material often remains to be demonstrated. Also, due to the large size of plant genomes, the inhibitory effect of the excess plant DNA present might reduce the transformation efficiency below the level of detection.
c) The expression level of the selectable antibiotic resistance marker may be low in the transformed bacterium.
d) Conditions or sub-populations of bacteria needed for recombination with diverged DNA might be overlooked in the system used.

Using the bacterium *Agrobacterium tumefaciens* as a recipient for transgenic tobacco (*Nicotiana tabacum*) DNA with a selectable gentamycin resistance (*accI*) gene, Broer et al. (1996) were not able to detect HGT from the tobacco. The transformation frequency was found to be the below detection limit ($<6x10^{-12}$). Similarly, Schlüter and coworkers (1995) failed to detect HGT when investigating transformation of the bacterium *Erwinia chrysanthemi* with DNA from transgenic potato (*Solanum tuberosum*) containing a selectable ampicillin resistance gene. In another study, the soil bacterium *Acinetobacter calcoaceticus* was used as a recipient of DNA from transgenic sugar beet and potato DNA; both containing the *nptII* gene. Transformants were also not detected in this study (Nielsen et al. 1997), and it was concluded that sequence homology or a stabilizing sequence like an origin of replication was required for stable maintenance of introduced genetic material in the transformants. Introducing homology between transgenic plant and bacterial DNA, two recent studies (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998) have shown the uptake of transgenic plant DNA by natural transformation in *A. calcoaceticus.* Both studies used homology based recombination to ensure restoration of a bacterial *nptII* gene, which harbored an internal deletion, with transgenic plant DNA containing a complete *nptII* gene. Transgenic plant DNA from *Solanum tuberosum, Nicotiana tabacum, Beta vulgaris, Brassica napus* and *Lycopersicon esculentum* was able to restore resistance to kanamycin after natural transformation of the bacterium (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998).

With the exception of the above mentioned studies which used artificially introduced homology between the donor plant and recipient bacterium, to our knowledge experimental evidence demonstrating HGT of antibiotic resistance genes from GMP to bacteria is presently not available. Given the limitations of the

methods as indicated above, the data obtained indicate that HGT either did not occur, that the transfer frequencies and expression were too low to be detected, or that the environment used or techniques applied were not appropriate for the detection of this type of HGT. It should be emphasized that all the early attempts to monitor such HGT events have focused on transfer of functional and expressed genes, instead of shorter DNA fragments, such as used in the later studies (de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998) which also utilized homology between the donor and the recipient DNA. Genes homologous to prokaryotic genes are frequently introduced in GMP (Nielsen et al. 1998). Much of the discussion of putative HGT from GMP to bacteria has been based on transfer frequency estimates. However, the frequency of HGT is, in an evolutionary perspective, probably only marginally important compared with selection acting on the bacterial transformants. We therefore draw attention to the need to enhance the understanding of selection processes acting in natural environments. Also, information of the natural background levels of the resistance genes (and not phenotypes) in question would be helpful when assessing the impact of the genes in the environment. Only an accurate understanding of the ecology and selection of antibiotic resistance genes will allow the prediction of their fate and possible consequences following a large-scale introduction into the environment.

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Environmental effects of GMOs: The variation of structure and function of microbial communities studied as an endpoint of ecotoxicological testing

Backhaus, H.[1)], Engelen, B.[1)], Malkomes, H.-P.[2)], Tebbe, C.[3)], Miethling, R.[3)], Wieland, G.[1)]

[1)] Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Messeweg 11-12, 38104 Braunschweig;
[2)] BBA, Institut für Unkrautforschung, Messeweg 11-12, 38104 Braunschweig;
[3)] Bundesforschungsanstalt für Landwirtschaft (FAL), Institut für Bodenbiologie, Bundesallee 50, 38116 Braunschweig, Germany

Ecotoxicological testing procedures evaluate the impact of potentially harmful agents on the environment by monitoring non-target effects on single species or communities. Being aware of the role of microorganisms in sustaining global cycling of matter or of their varied functions in supporting the growth and welfare of higher organisms, any manmade stressors on microbial communities should be investigated in cases of potential negative interference. For example, impacts of herbicides on soil microorganisms are monitored by analysing changes of microbial biomass and general metabolic parameters in laboratory testing protocols. Data of this type of testing are used for herbicide registration, however, to a widely divergent degree internationally.

The assessment of chemical impacts on microbial soil communities by conventional testing protocols served as a defined reference in a first approach to analyse the potential of novel methods in microbial ecology (Engelen et al. 1998). Standard agricultural soil was inoculated with two herbicides, Herbogil® (1x) and Goltix® (10x), and Oleo (10x), a mineral oil used as an additive, at normal application dosage or at 10 times that concentration, as indicated. Inhibitions of biomass related activities (short-term induced respiration, dehydrogenase activity) and a stimulation of nitrogen mineralization were the most significant effects caused by the application of Herbogil. The application of Goltix resulted in much smaller effects, and the additive Oleo was the least active compound with minor stimulation of test parameters (including long-term respiration) at later observation times. Changes in catabolic activities induced by treatments were also analysed using the 95 carbon sources as provided by the Biolog system. Variations in the complex „metabolic fingerprints“ demonstrated an inhibition of many catabolic pathways with Herbogil. Again, the effects of the other compounds were much less expressed and comprised stimulation as well as inhibitions. The testing for significance by a multivariate t-test of 8 experimental replicates comparison to the results of the conventional testing procedures (Table).

Table Statistical significance of observations (t-test, a = 0,05) of effects from inoculations in the conventional tests and in the multivariate test of Biolog results

	Short-term respiration				**Dehydrogenase activity**				**Long-term respiration**				**nitrogen mineralisation**				**BIOLOG multivariate test**			
	Week				**Week**				**Week**				**Week**				**Week**			
	1	**2**	**5**	**8**	**1**	**2**	**5**	**8**	**1**	**2**	**5**	**8**	**1**	**2**	**5**	**8**	**1**	**2**	**5**	**8**
Herbogil (1x)	*	*	*	*	*	*	*	*			*	*	*	*			*	*	*	*
Oleo (10x)							*	*			*	*								
Goltix (10x)			*		*			*			*		*						*	

The identification of sensitive carbon sources by a detailed analysis of F-values (factor weights) was used to characterize the shift induced by the Herbogil* treatment in more detail. The variation of factor weights during the time course of observations indicated the dynamics of induced changes in the metabolic potential of the microbial community (Figure 1).

The Biolog system affords a detailed and sensitive view on induced changes of the functional spectrum of the community. The limited observation window is also biased, depending on the physiological states of inoculated cells as well as on selective growth. Data interpretation in terms of detailed cause-effect

mechanisms is not possible in general. For evaluation, any reduction in the spectrum of catabolic functions indicated by the test system might primarily be considered as an unfavourable effect. Depending on the knowledge about functions in a particular habitat, the approach of the system may be modified to include more specific endpoints by the use of alternative/additional carbon sources. Also, the sensitivity of data analysis might be improved (Glimm et al. 1997).

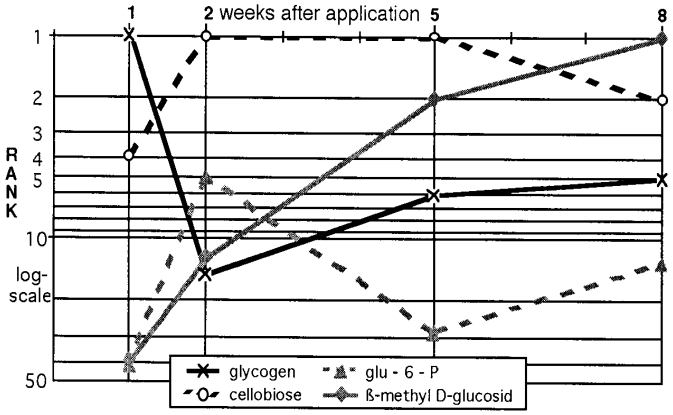

Figure 1 Rank of factor weights of some variables (substrates) of the Biolog system contributing to group differentiation at different times after inoculation (Herbogil® application versus control).

To reveal any effects of the chemicals application on the structural composition of the soil bacterial community, we analysed the abundance of sequence variants of an amplified segment of rDNA, isolated from the soil bacteria fraction. The sequence dependent separation of the amplified segments in a Temperature Gradient Gel Electrophoresis (TGGE) resulted in highly reproducible banding patterns. This "fingerprint" of the distribution of variants of phylogenetically meaningful sequences is a representation of the species composition of the bacterial community. Impacts on these banding patterns resulting from the applications were analysed and quantified. The significant shift in community composition in particular resulting from the Herbogil® treatment is shown in Figure 2. The variation in band appearance or intensity (sampling after eight weeks) is demonstrated by the comparison of the average density profiles (three replicates) of the Herbogil® pattern versus the control.

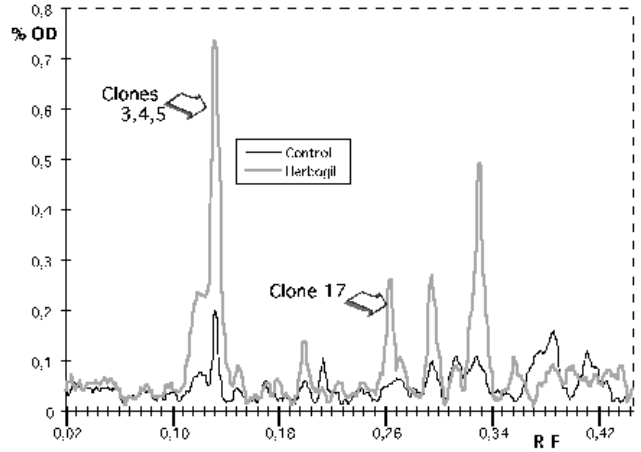

Figure 2 TGGE patterns of rDNA amplicon separations represented as the average of three density profiles

A quantitative measure of the impact was obtained by integrating the differences between control and application patterns in absolute terms. The Herbogil® treatment (61%) again ranks first in such a measure of pattern modification. More than 20% of the pattern difference of the Oleo® treatment (55% differences) results from the abundance of one band which increased during the time course of the experiment. The Goltix® treatment ranks lowest in this ordination (38%), at a value which is close to the variability of individual replicates (about 30%), indicating the limit of sensitivity. Some pattern differences were also characterized by extraction and sequencing of bands with increased intensity. The most similar sequence to (homologous) clones 3-5 in the database was that of *Nitrospina gracilis* (similarity 85-87%). Clone 17 of the Herbogil® pattern (Figure 2) had a similarity of 97% to *Xanthomanas campestris*, a plant pathogen. Clone 24, isolated from the band characterizing the shift in the Oleo® pattern, had a similarity of 92% with *Shewanella alga* (Engelen et al. 1998).

In a different experimental approach, the natural variability of different soil and plant habitats served as a reference for ordination and evaluation of an induced impact on microorganism communities. A design with plant microcosms was set up to monitor the fate of a Sinorhizobium strain, genetically modified with a luc-marker (Selbitschka et al. 1992), and to analyse any effects of the inoculation on resident communities. Microcosms containing two different agricultural soils were sowed with seeds of alfalfa (*Medicago sativa*, host for nodulation) and rye (*Secale cereale*). In a randomized design with four replicates of each variant, 50% of the microcosms were inoculated with 10^6 CFU of *S. meliloti* strain L33/ g soil.

After 10 weeks (alfalfa) and 11 weeks (rye) the microcosms were sampled for analysis. DNA and rRNA were extracted from soil, rhizosphere, and rhizoplane habitats (defined by the kind of washing procedure) to analyse amplification products of the 16S sequence. Due to the higher ribosome content of active microbial cells, rRNA extracted by a ribosome isolation protocol was used as an additional target molecule (Felske et al. 1996). Products of amplification with conserved bacterial primers (between pos. 968-1346, *E. coli* numbering) were separated in the TGGE to obtain “fingerprints” of the community in the respective habitat of the model system variant. For comparison of these TGGE patterns, scans of the gels were analysed for similarity with the DIVERSITY DATABASE software. Dice coefficient and UPGMA were used for representation of similarity by clustering.

Figure 3 represents the clustering of the patterns obtained with rRNA products from the rhizosphere habitats. Apparently, plant species had the greatest effect on pattern differences (and community composition). The influence of soil type ranks next. In comparison, effects from the GMO inoculation were minor and variation was only significant (patterns of inoculated systems form different clusters than non-inoculated ones) in experimental variants with the host plant alfalfa. The respective analysis from the other habitats and with DNA as target molecules gave essentially similar results.

Our experiments demonstrate the suitability of novel endpoints for ecotoxicological testing of effects on microbial community composition and function. They could be used in a tiered testing for potential impacts of varied stress factors, including the application of GMOs, if risk analysis does not suggest different specified endpoints in particular cases. Depending on objectives, the modification of experimental parameters can be used to adapt observation windows and to extend the analysis for more defined effects endpoints (e.g. by modifying primer selectivity).

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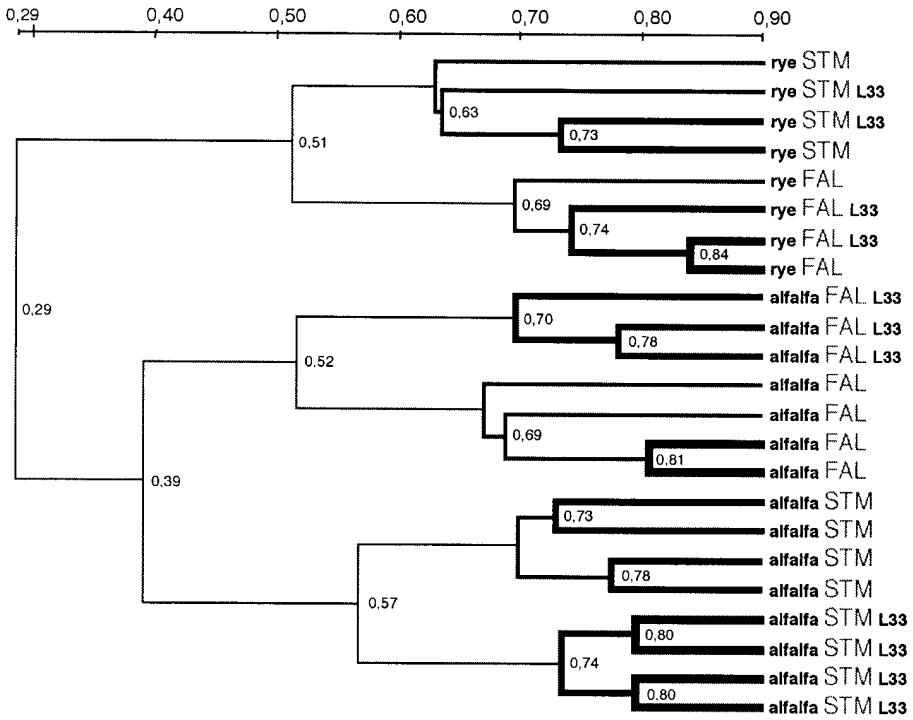

Figure 3 Clustering of patterns of rRNA (RT) amplification products representing the similarity (scale: 29-90%) of rhizosphere communities. Plant, soil type, and inoculation with the GMO strain L33 of the model system variant are indicated.

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ICGEB and the "Safe use of biotechnology"

Ferraiolo, G.

ICGEB Biosafety Unit, Padriciano 99, 34012 Trieste, Italy

The International Centre for Genetic Engineering and Biotechnology (ICGEB) has been established in 1987 as a Centre of Excellence for Research and Training in Biotechnology in a project of the United Nation Industrial Development Organization (UNIDO). The Centre is today an autonomous, international, intergovernmental, organization with the special mandate "to promote the safe use of biotechnology worldwide and with special regard to the needs of the developing world" and with a total of 60 countries signatories to the Statutes, 42 of which are now full member states.

In its ten years of existence ICGEB has developed much experience in scientific training and capacity building. It hosts a network of 32 Affiliated Centres, which are national laboratories in member states, whose research activities are coordinated with, and partially funded by the Centre. More than 250 people from 28 different countries are involved in the scientific activities in the two main laboratories in Trieste (Italy) and New Delhi (India) and more than 400 scientific articles have been published in international journals since 1989. During the period 1989-1996 over 300 post-doctoral fellowships were awarded to member country scientists and 140 grants were issued to ICGEB Affiliated Centres in the developing world in the same period. Over 600 scientists participate annually in the courses and workshops organized by the Centre (approximately 14 per year) with 15 meetings/courses and 2 conferences being scheduled for 1998.

ICGEB has been involved, since its inception, in the UNIDO/WHO/UNEP/FAO Informal Working Group on Biosafety and in the preparation of the "Voluntary Code of Conduct" for the release of genetically modified organisms (GMOs) into the environment (1991).

Since September 1992 ICGEB organized annual workshops on "Biosafety and Risk Assessment" for the release of genetically modified organisms with participation of over 400 scientists involved in bio-safety issues in more than 40 different countries.

In 1995, ICGEB has collaborated with UNIDO in the publication of BINAS News and participated in the creation of BINAS, a Biosafety Information Network and Advisory Service with the objective to monitor global developments in regulatory issues in biotechnology.

In early 1998, the Centre added the ICGEB Biosafety WebPages (Internet address: http://www.icgeb.trieste.it/biosafety/) as a new section to ICGEBnet, its bio-informatic network. These web pages are totally dedicated to "Biosafety and Risk Assessment for the Environmental Release of Genetically Modified Organisms (GMOs)" with the aim of offering to member states all the available information on biosafety concerns and proceedings worldwide and on the ongoing process for the adoption of a biosafety protocol by the signatory countries to the Convention on Biological Diversity (CBD) expected for February 1999.

In May 1998, during the Fourth Meeting of the Conference of the Parties (COP), ICGEB offered to the CBD to share its experience and the work performed in its network of Affiliated Centres. Moreover, following the indication by ICGEB of its interest in being involved in these activities the Centre has been invited to participate in the Steering Committee of the UNEP/GEF Pilot Biosafety Enabling Project in order to "offer scientific and technical advice and other professional guidance/service for the effective implementation of this important biosafety activity".

In the next few months the Centre will make available on Internet, with free access, an updated scientific bibliographic database on biosafety and risk assessment in biotechnology. The database contains, to date, more than 1,700 scientific articles (full references and abstract), published in international scientific journals since 1990, selected and classified from ICGEB scientists by the main topics of concern for the environmental release of GMOs as reported in Table 1. ICGEB is following with great attention the negotiations for the adoption of a Biosafety Protocol within the Open End Ad Hoc Working Group on Biosafety to the CBD and, in general, the development of the convention on the issues relating to biotechnology. The Centre has already received, from its member states, formal requests for assistance in formulation and harmonization of biosafety procedures and regulations in the framework of the United

Nations System. Therefore, the foreseen biosafety activity for the next years will be focused on the creation of an ICGEB Biosafety Network in its member states.

Table 1 Main topics of concern for the environmental release of GMOs

Risks for human health:
Toxicity & food quality/safety
Allergies
Pathogen drug resistance (antibiotic resistance)
Risks for the environment:
Persistency of gene or transgene (volunteers, increased fitness, invasiveness)
Transgene products (accumulative effects)
Resistance/tolerance of target organisms
Increased use of chemicals in agriculture
Unpredictable gene expression or transgene instability
Risks for agriculture:
Weeds or super-weeds
Alteration of nutritional value (attractiveness of the organisms to pest)
Reduction of cultivars (increase of susceptibility) and loss of biodiversity
General concerns:
Loss of familiarity
Higher cost of agriculture
Field trials not planned for risk assessment
Ethical issues (labelling)
Risks of interaction with non-target organisms:
Genetic pollution through pollen or seed dispersal
Horizontal gene transfer (transgene or promoter dispersion)
Transfer of foreign gene to microorganisms (DNA uptake)
Generation of new live viruses by recombination (transcapsidation, complementation etc.)

The Centre aims to share its experience and the work performed in its network of 32 Affiliated Centres with the CBD and its Clearing House Mechanism (CHM). This cooperation would be fully within the mandate and the activities of the ICGEB, while at the same time, the Convention and its Signatory Parties, without necessarily duplicating existing organization, would take full advantage of the technical competence, the experience in developing training programmes and the international recognition obtained by ICGEB in its first ten years of activity.

Developing resistance management strategies for herbicide tolerant rice in temperate production systems

Mitten, D.H.[1)], Smith, L.[1)], Johnson, K.[1)], Linscombe, S.[2)], Abreu, A.[3)]

[1)] AgrEvo USA;
[2)] Louisiana State University;
[3)] AgrEvo dò Brasil

For the direct-seeded, mechanized rice culture typical of temperate rice production, both cultural practices (e.g., crop rotation and irrigation management) and herbicides are needed to control weeds. A weed problem of significance to temperate rice production systems is red rice, *Oryza sativa.* It is considered to be a companion weed for the crop, white rice, *O. sativa*. Red rice was introduced with the initial cultivation of rice as an impurity in the planting seed (Hill et al. 1994). Cultivated rice and red rice are sexually compatible, thus the advent of herbicide tolerant rice varieties could lead to transfer of a herbicide tolerant gene into red rice (Clegg et al. 1993).

Herbicide tolerant rice varieties (IMI, Roundup Ready™ and Liberty Link™ rice varieties, which are tolerant to sulfonylureas and imidazilinones, both are ALS inhibitors, glyphosate and glufosinate, respectively) representing three separate herbicidal modes of action (Schmidt 1997) are currently in advanced breeding programs targeted for temperate rice production. Commercial release of herbicide tolerant rice cultivars is imminent. Rice varieties tolerant to herbicides offer unique management tools providing flexibility in the timing of herbicide application, and in some regions which lack economically viable crop rotation systems, maybe the only management tools to control problem weeds, like red rice. Commercial scale development of herbicide tolerant rice varieties requires concomitant biosafety assessment and management strategies for the potential development of herbicide resistant weeds. Herbicide resistance may be achieved by either gene flow to sexually compatible species or through intensive use of the herbicide, which can select for resistant individuals in weed populations. For example, propanil resistant barnyardgrass and bensulfuron resistant sedges were found where continuous cropping to rice and no rotation to alternative herbicides was practiced (Hill et al. 1994, Heap 1998). Both these herbicides have residual activity and thus, offer a prolonged selection pressure for weed populations.

In 1992, the World Bank brought together members of the rice research and regulatory agencies representing the rice producing countries of the world. This group recommended two inquiries needed to access the likely consequences of a herbicide tolerance gene moving into red rice or a wild rice species; 1) fitness evaluation of the potential hybrids, and 2) an evaluation of the likely effects on current agricultural systems. A key question identified by the symposium for herbicide tolerance genes is: Will the transfer of herbicide tolerance to wild and weedy rice relatives exacerbate problems of weed control and thus lead to decreased rice production yields? (Clegg et al. 1993). For temperate rice production regions, the potential for gene transfer is limited to red rice.

A review of studies that address the questions surrounding gene flow to red rice and its consequences indicate that the large-scale release of herbicide tolerant rice varieties will not exacerbate red rice weed control problems because

1. In the temperate rice production regions, both crop and red rice grow only in agricultural settings, neither has escaped cultivation and neither has naturalized (Morishima 1984).
2. Studies to measure the fitness of herbicide (glufosinate) tolerant rice/red rice hybrid populations (two cultivars, transgenic and non-transgenic, handcrossed into two red rice types) have not recorded any change in the fitness of populations (in the absence of the selection pressure of the herbicide) containing a gene for herbicide tolerance (Oard et al. 1998; Gealy and Gravois, 1998). Therefore, hybrid populations of herbicide resistant red rice will behave like the parents and remain confined to agro-ecosystems.
3. Current management practices for red rice control are effective on herbicide tolerant (glufosinate) crop or weed rice (Sanders et al. 1998; Sankula et al. 1998).
4. Studies using a common garden design interplanted red and crop rice of varying maturities. Outcrossing was the greatest when flowering periods of the red rice and crop rice overlapped

(Langevin et al. 1990). Several management techniques are in practice to prevent red rice flowering to overlap the flowering period of the crop rice. The ability to apply herbicides that can selectively control red rice can be used to prevent the red rice from flowering, and thus, greatly reduce the opportunity for gene flow.

5. Herbicides selective for crop white rice have similar activity on red rice. Thus, red rice which is resistant to some herbicides already exists. Herbicide resistant red rice is not new to the rice growing region. Knowledge of red rice biology and currently developed weed management practices can be applied to minimize the potential occurrence of herbicide resistant red rice populations and to control those populations that may develop.
6. Case-by-case biosafety evaluations must consider the current agriculture management and weed control practices in the granting of commercial clearance. In most cases, if the red rice populations do become resistant as a result of gene flow, agriculture will revert to the current situation. An exception may be in regions with limited herbicide choices, for example, regions in which no-till practices are based upon herbicides with a common mode of action. It would be unwise to release a rice variety with genetic tolerance to the same herbicide class in such a situation.
7. Herbicide resistance management recommendation, monitoring and response plans have the same elements as those already in use by the agricultural community for managing weeds resistant to conventional herbicide technology (Anonymous, 1995).

Prior to commercial release, resistance management strategies should be in place for agronomic management recommendations, communication, monitoring and response. An example outline plan for herbicide tolerant rice follows:

- Management recommendations
  - Rotation of crops and herbicide mode of action
  - Techniques to prevent red rice from flowering
  - Techniques for depletion of red rice seed in the soil
  - Identification of the best practices for the region.
- Communication
  - Develop grower education program combining best agronomic practices for local region and monitoring for volunteers
  - Training for field representatives, local pest control advisors and local extension agents
  - Point of sale product education brochures
  - Alternation of herbicides with different modes of action.
- Monitoring
  - Independent surveys by private and/or public sector weed specialists
  - Grower responsibility.
- Response
  - Management plan for unexpected results
  - 800 phone access and follow up
  - Apply the “Weed Resistance Management Action Tree” recommendations
  - Sampling procedures to confirm presence of herbicide tolerant gene.

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Sankula, S., Braverman, M.P., Oard, J.H. (1998): Genetic analysis of glufosinate resistance I crosses between transformed rice (*Oryza sativa*) and Red Rice (*Oryza sativa*). Weed Technology 12, 209-214.

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Program

Sunday, 6 September

Registration, Reception

Monday, 7 September

Frank Laplace (Federal Ministry of Education, Science, Research and Technology, Germany)
Opening remarks

Karl-Wilhelm Schulze-Weslarn (Federal Ministry for Food, Agriculture and Forestry, Germany)
Welcome address

Panel I: New developments in science for biosafety evaluation

Moderator: John Beringer (University of Bristol, School of Biological Sciences, UK)

Clive James (The International Service for the Acquisition of Agri-biotech Applications, Cornell University, Ithaca, USA)
Current global status of commercialized transgenic crops

Allison Snow (Ohio State University, Dept. of Plant Biology, Columbus, USA)
The assessment of gene flow and of potential effects of genetically engineered sunflowers on wild relatives

Dave Ellis (Forest Biotechnology Centre, Vancouver, Canada)
Deep rooted strategies for the deployment of transgenic trees

Glynis Giddings (University of Wales, Institute of Biol. Sciences, Aberystwyth, UK)
Modelling the establishment and spread of transgenes in plant populations

William Schneider (EPA, Washington, USA)
US regulatory experiences with *Bacillus thuringiensis* toxins in transgenic plants and genetically engineered baculoviruses

Alfred Pühler, Mathias Keller and Werner Selbitschka (University of Bielefeld, Germany)
Field release of a RecA-deficient *Sinorhizobium meliloti* strain - Test of a novel safety concept

Panel II: Novel uses and products of agricultural biotechnology

Moderator: Klaus Ammann (University of Bern, Botanical Garden, Switzerland)

Desh Pal Verma (Plant Biotechnology Center, The Ohio State University, Columbus, USA)
Development of new drought tolerant/salt resistant plant varieties

Fructans as prebiotic food additives
Arnd Heyer (Max-Planck-Institute for Molecular Plant Physiology, Golm, Germany)

Engineering plant viruses to produce peptides or proteins of medical, veterinary or industrial importance (talk presented by Peter Palukaitis)
Michael A. Wilson (Scottish Crop Research Institute, Dundee, UK)

Panel III: Products for the market – Field releases and commercialization of transgenic plants worldwide

Moderator: Ken-ichi Hayashi (STAFF, Tokyo, Japan)

Thomas Nickson (Monsanto Environment Technology Center, St. Louis, USA)
Status of the commercialization of Monsanto transgenic crops

Henk Joos (AgrEvo/PGS, Astene, Belgium)
AgrEvo/PGS in the global oilseed rape seed business

Roger Hull (John Innes Centre, Norwich, UK)
Pragmatic approach to farmer release of transgenic rice in Africa

Yutaka Tabei (Innovative Technology Division, MAFF, Tokyo, Japan)
Field releases for commercialization of transgenic plants in Japan

Poster session I

Tuesday, 8 September

Panel III: Products for the market – Field releases and commercialization of transgenic plants worldwide (cont.)

Zhangliang Chen (Peking University, College of Life Sciences, Beijing, China)
Commercialization of GMOs in China

Ariel Alvarez-Morales (CINVESTAV, IPN Unidad Irapuato, Dept. of Plant Genetic Engineering, Mexico)
Agricultural biotechnology in Mexico: Release, research and commercialization of GMOs

Tomasz Twardowski (Institute of Bioorganic Chemistry PAS, Poznan, Poland)
Conditions for commercialization of GMOs in Central and Eastern Europe

Gwendolyn McClung (EPA, Washington, USA)
Commercialization of a genetically modified symbiotic nitrogen-fixer, *Sinorhizobium meliloti*

Panel IV: Experience gained during commercialization

Moderator: Terry L. Medley (DuPont Agricultural Products, Wilmington, USA)

Alan McHughen (University of Saskatchewan, Saskatoon, Canada)
Commercialization of transgenic linseed: International regulations and economic considerations

Dennis Gonsalves (Cornell University, Dept. of Plant Pathology, Geneva, USA)
PRSV-resistant papaya in Hawaii

Randy Deaton (Monsanto, St. Louis, USA)
Global commercial experience with transgenic cotton

Naoto Nitta (Plant Breeding and Genetics Research Laboratory, Japan Tobacco Inc., Shizuoka, Japan)
Experience in the development of low protein japonica rice

Workshops: Four workshops, running in parallel

Workshop 1: Evaluation of commercial scale usage of virus-resistant transgenic plants

Moderator: Mark Tepfer (INRA, Laboratoire de Biologie Cellulaire, Versailles, France)

James White (USDA-APHIS, Riverdale, USA)

Overview on cultivation of virus-resistant crops in the US

Marc Fuchs (INRA, Laboratoire de Pathologie Végétale, Colmar, France)
Is gene flow a serious environmental safety issue for virus-resistant transgenic squash?

Richard F. Allison (Michigan State University, East Lansing, USA)
Significance of RNA recombination in virus-resistant transgenic plants

Rachid Aaziz, Katalin Salanki, Ervin Balázs, Mireille Jacquemond and Mark Tepfer (INRA, Laboratoire de Biologie Cellulaire, Versailles, France)
Strategies for detection of recombination in virus-infected plants expressing a viral transgene

Peter Palukaitis (Scottish Crop Research Institute, Virology Dept., Dundee, UK)
Synergy of virus accumulation and pathology in transgenic plants expressing viral sequences

Allen Miller (Iowa State University, Ames, USA)
Issues surrounding transgenic resistance to luteoviruses

Philip J. Dale, Simon N. Covey, Maria M. Kreike, Anthony M. Page, Rachel Pinder and Nadia S. Al-Kaff (John Innes Centre, Norwich, UK)
Pathogen induced transgene instability in *Brassica napus*

Workshop 2: Resistance management strategies for Bt toxin transgenic plants

Moderator: Blair Siegfried (University of Nebraska, USA)

Richard L. Hellmich (USDA, ARS Corn Insects and Crop Genetics Research Unit, Iowa State University, Ames, USA)
Establishing research priorities for managing insect resistance to transgenic corn

Sue MacIntosh (AgrEvo/PGS America, Des Moines, USA)
A science based development of a resistance management strategy

Graham Head (Monsanto, St. Louis, USA)
How differences among *Bt* crops and agronomic practices affect resistance management: reconciling science, economics and practicality

William D. Hutchison and David A. Andow (University of Minnesota, St. Paul, USA)
Resistance management for *B.t.*-corn: Progress and challenges to consensus in US policy

Jia Shi-Rong (Biotechnology Research Center, Chinese Academy of Agricultural Sciences, Beijing, China)
Development of insect resistance management strategies for the commercial cultivation of *B.t.*-cotton in China

David Ferro (University of Massachusetts, Dept. of Entomology, Amherst, USA)
Transgenic potatoes: Resistance management of the Colorado potato beetle

Angelika Hilbeck (Swiss Federal Research Station for Agroecology and Agriculture, Zurich, Switzerland)
Implications of transgenic plants expressing *Bacillus thuringiensis* protein on insect natural enemies

Workshop 3: Monitoring of microbial communities

Moderator: Kornelia Smalla (BBA, Braunschweig, Germany)

Janet K. Jansson (University of Stockholm, Dept. of Biochemistry, Sweden)
Marker and reporter genes in microbial ecology

Larry Forney (University of Groningen, Dept. of Microbiology, The Netherlands)
Potentials and limitations of T-RFLP of 16S rDNA for microbial community analysis

Andy Lilley and M. J. Baily (Institute of Virology and Environmental Microbiology, Oxford, UK)
Field release, gene flux and monitoring the impact of GMMs in the phytosphere

Jan Dirk van Elsas (IPO-DLO, Wageningen, The Netherlands)
Fate and effects of genetically modified plant growth promoting rhizobacteria in rice in China

Ray Seidler (EPA, Corvallis, USA)
Polyphasic approach to field risk assessments: recombinant alfalfa inoculated with recombinant Sinorhizobium with enhanced N_2 fixing abilities

Christoph Tebbe (Federal Research Centre for Agriculture, Braunschweig, Germany)
Monitoring effects of released transgenic *Sinorhizobium meliloti* strains on rhizosphere and bulk soil microbial communities

Kornelia Smalla (BBA, Braunschweig, Germany)
Monitoring effects of transgenic T4-lysozyme-expressing potatoes on the bacterial rhizosphere population

Workshop 4: Communicating biosafety issues and results to the wider public

Moderator: Willy de Greef (Novartis Seeds, Basel, Switzer-land)

Martina McGloughlin (University of California, Davis, USA)
Communication of biosafety results

Willy de Greef (Novartis Seeds, Basel, Switzerland)
Getting the results of biosafety research into the policy debate

Julian Kinderlerer (University of Sheffield, Sheffield Institute of Biotechnological Law and Ethics, UK)
Public opinion about biotechnology in Britain

Othmar Käppeli (Fachstelle BATS, Basel, Switzerland)
The Swiss endorsement of genetic engineering: What made the difference in the vote

Siglinde Fischer (BioAlliance Deutschland GmbH, Frankfurt, Germany)
A European information network for safety relevant issues of biotechnology

The future of scientific communication:

Kristina Sinemus (Genius GmbH, University of Darmstadt, Germany)
Biosafety communication today and tomorrow

Udo Tschimmel (MediaProjects, University of Darmstadt, Germany)
Communicating biotechnology: The journalist's view

Reception at Dornse, Altstadtrathaus, Altstadtmarkt

opened by Fred Klingauf (BBA, Braunschweig, Germany)

Wednesday, 9 September

Panel IV: Experience gained during commercialization (cont.)

Karl Hurle (University of Hohenheim, Institute for Phytomedicine, Germany)
Cultivation of herbicide resistant crops: weed management and environmental aspects

Robert MacDonald (AgrEvo/PGS Canada, Regina, Canada)
Bringing monitoring concepts into practice

Ian Grant (Pioneer Hi-Bred, Aussonne, France)
Experience in the breeding process using novel gm traits

Steve Wentworth (Oreana, Illinois, USA)
An Illinois corn and soybean farmer's three years of experience with genetically modified crops

Panel V: Lines of intersection in different regulations / International harmonization of regulations

Moderator: Joachim Schiemann (BBA, Braunschweig, Germany)

Michael Schechtman (USDA-APHIS, Washington, USA)
Strategic regulation of agricultural biotechnology products in the United States

Grant Watson (Canadian Food Inspection Agency, Plant Products Division, Nepean, Canada)
The process in Canada for regulating plants with novel traits (PNTs) from field testing to commercialization

Karsten Hohgardt, Joachim Schiemann and Peter Zwerger (BBA, Braunschweig, Germany)
Authorization of herbicides complementary to herbicide resistant crops

George Tzotzos (UNIDO, Vienna, Austria)
Decision support systems for safety assessments of agri-biotech applications

Excursions

E1: KWS, a Breeding Company in Einbeck; visiting the historical town Einbeck - famous for its long brewery tradition

E2: BAZ, the Federal Centre for Breeding of Cultivated Plants in Quedlin-burg; visiting Quedlinburg - famous for its historical town centre

E3. BBA, the Federal Biological Research Centre for Agriculture and Forestry Braunschweig; visiting the Riddagshausen Monastery

E4: Braunschweig and its historical „Islands of Tradition"

Thursday, 10 September

Poster session II

Presentation and Discussion of the document:

„Prenormative Biosafety Research Related to the Release of Genetically Modified Organisms" prepared by the EU Commission

Panel V: Lines of intersection in different regulations / International harmonization of regulations (cont.)

Simon Barber (OECD, Paris, France)
Regulatory harmonization: Where are we today?

Hans-Jörg Buhk (Zentrum Gentechnologie, Robert Koch-Institut, Berlin, Germany)
An overview about the regulatory process on the basis of Directive 90/220/EEC

Ervin Balázs (Agricultural Biotechnology Center, Institute for Plant Sciences, Gödöllö, Hungary)
Janus face of biotechnology and biosafety in Central and Eastern European Countries

Les Levidow (Centre for Technology Strategy, Open University, Milton Keynes, UK)
Managing international disharmonies through market-stage precautions

Rapporteurs' reports
Moderators of Panels I-V, Workshops 1-4; 10 minutes each

Summation
Ioannis Economidis (EU, DG XII, Brussels, Belgium)

Closing remarks
Rudolf Casper (Braunschweig, Germany)

Poster Presentations

Matthias Pohl-Orf, Peter Hesse, Ulrike Brand, Ingolf Schuphan, Detlef Bartsch (RWTH Aachen, Aachen, Germany)
Assessment of the invasiveness of transgenic sugar beet in wild beet populations of *Beta vulgaris* subspec. *maritima* (L.) Arcang.

Detlef Bartsch, Janet Clegg, Norm Ellstrand (Dept. of Botany and Plant Sciences, University of California, Riverside, USA)
Gene flow between cultivated and wild forms of beet (Genus *Beta*) in California

C. Jacquet, M. Ravelonandro, J. Dunez (Station de Pathologie Végétale, INRA-Bordeaux, France)
Strategy to control heteroencapsidation-associated risks in transgenic plants

Nicolae Minoiu, Michel Ravelonandro, Ralph Scorza (Statiunea de Cercetera & Pomicola, Bistrita, Romania; Station de Pathologie Végétale, INRA-Bordeaux, France; Appalachian Fruit Research Station, USDA, Kearneysville, USA)
Field resistance tests to plum pox virus infection of transgenic *Prunus domestica* plants under nursery conditions

Nadia Al-Kaff, Maria Kreike, Simon Covey, Philip Dale (John Innes Centre, Norwich, UK)
Infection by a pathogen can cause transgene instability

Yasunori Koga-Ban, Yutaka Tabei, Masao Ishimoto, Yoko Nishizawa, Kenichi Tsuchiya, Tosiaki Kayano, Hiroshi Tanaka (National Institute of Agrobiological Resources; MAFF Research Council Secretariat; National Agricultural Research Centre, Japan)
Environmental risk assessment of transgenic cucumber introduced rice chitinase

Andreas Mahn, Petra Porsch, Olaf Brinkmann, Werner Gieffers, Klaus Düring (Federal Centre for Breeding of Cultivated Plants, Quedlinburg, Germany; Max-Planck-Institute for Plant Breeding, Cologne, Germany)
T4 lysozyme as a tool for resistance engineering in potato

Jana Lottmann and Gabriele Berg (University of Rostock, Dept. of Microbiology, Rostock, Germany)
The influence of transgenic potatoes producing T4 lysozyme on plant-associated beneficial bacteria

I. Broer, G. Kriete, S. Wedell (University of Rostock, Dept. of Biology, Rostock, Germany)
Field tests to assess the impact of transgene encoded T4 lysozyme on *Rhizobium leguminosarum* bv. *Viciae*

Thomas Lukow and Werner Liesack (Max-Planck-Institute for Terrestrial Microbiology, Marburg, Germany)
Monitoring the bacterial diversity of rhizosphere soil around transgenic Barnase/Barstar and non transgenic potato plants with molecular techniques

M. Lin, W. Lu, H. Heuer, K. Smalla, J.D. van Elsas (Institute for Plant Protection, Wageningen, The Netherlands; Institute for Application of Atomic Energy, CAAS, Beijing, P.R. China; Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany)

Effect of inoculation with *Alcaligenes faecalis* on the bacterial community in flooded soils planted with rice seedlings

Kaare M. Nielsen, Atle M. Bones, Kornelia Smalla, Jan D. van Elsas (Norwegian University of Science and Technology, Trondheim, Norway; Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany; Institute for Plant Protection, Wageningen, The Netherlands)

Methods to assess horizontal gene transfer in soil bacteria

Horst Backhaus, Bert Engelen, Hans-Peter Malkomes, Gabriele Wieland (Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany)

Environmental effects of GMOs: The variation of structure and function of microbial communities studied as an endpoint of ecotoxicological testing

Gesine Schütte, Bettina Heidenreich, Volker Beusmann (FSP BIOGUM, Technology Assessment on Biotechnology, University of Hamburg, Hamburg, Germany)

A review on green biotechnology and its ecological risks

Giovanni Ferraiolo (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy)

The ICGEB and the „safe use of biotechnology“

Mark Varrelmann and Edgar Maiss (Institute for Plant Disease and Plant Protection, University of Hannover, Hannover, Germany)

N-terminal deletion and mutations in the plum pox virus (PPV) coat protein gene abolish particle assembly, heterologous encapsidation and complementation in transgenic *N. benthamiana* plants

Donna H. Mitten, Lane Smith, Kirk Johnson, Steve Linscombe, André Abreu (AgrEvo USA; Louisiana State University; AgrEvo dò Brasil)

Developing resistance management strategies of herbicide tolerant rice in temperate production systems

List of Participants' Names and Addresses

Aaziz, Rachid	INRA	Route St. Cyr	78026 Versailles Cédex France
Alawady, Ali E.	Institut für Pflanzengenetik und Kulturpflanzenforschung	Correnssstr. 3	06466 Gatersleben Germany
Al-Kaff, Nadia	John Innes Centre	Colney Lane	Norwich NR4 7UH UK
Allison, Richard F.	Dept. of Botany and Plant Pathology, Michigan State University		East Lansing, MI 48824, USA
Alvarez Morales, Ariel	CINVESTAV IPN Unidad Irapuato Dept. of Plant Genetic Engineering	Apdo. Post. 629	Irapuato, Gto. C.P. 36500 Mexico
Ammann, Klaus	Universität Bern, Botanischer Garten	Altenbergrain 21	3013 Bern Switzerland
Anderson, Bill	Ag-West Biotech Inc. Saskatchewan Regulatory Affairs Service	101-111 Research Drive	Saskatoon, SK S7N 3R2 Canada
Apel, Petra	Umweltbundesamt	Seecktstr. 6-10	13581 Berlin Germany
Arndt, Nicola	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Artelt, Petra	Bezirksregierung Braunschweig	Obergstr. 1	38100 Braunschweig Germany
Arvanitis, Nikos	Microbiology Lab., Section of Botany, Dept. of Biology	University of Athens	15781 Athens Greece
Attathom, Supat	Plant Genetic Engineering Unit Kasetsart University	Kamphaengsaen	Nakorn Pathom 73140, Thailand
Auberson, Lillian	Fachstelle BATS	Clarastr. 13	4058 Basel Switzerland
Bachmann, Susanne	Bundesforschungsanstalt für Land-wirtschaft, Institut für Agrarökologie	Bundesallee 50	38116 Braunschweig Germany
Backhaus, Horst	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Balázs, Ervin	Agricultural Biology Center, Institute for Plant Sciences	P.O. Box 411	2101 Gödöllö Hungary
Ban, Yasunori	National Institute of Agrobiological Resources, MAFF	2-1-2, Kannondai	Tsukuba, Ibaraki 305-0856 Japan
Barber, Simon	OECD Environmental Directorate	2, rue André Pascal	75775 Paris Cédex 16 France
Bartsch, Detlef	RWTH Aachen Biologie V	Worringer Weg 1	52074 Aachen Germany
Becker, Regina	ZALF Müncheberg; LfL Güterfelde	Berliner Str.	14532 Güterfelde Germany
Beißner, Lutz	Institut für Zuckerrübenforschung	Holtenser Landstr. 77	37079 Göttingen Germany

Berg, Gabriele	Universität Rostock Abt. Mikrobiologie	Gertrudenstr. 11a	18051 Rostock Germany
Beringer, John	University of Bristol School of Biological Sciences	Woodland Road	Bristol BS8 1UG UK
Bora, Alfons	Wissenschaftszentrum Berlin	Reichpietschufer 50	10785 Berlin Germany
Brandt, Peter	Robert Koch-Institut Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Broer, Inge	Universität Rostock, Fachbereich Biologie	Doberanerstr. 143	18051 Rostock Germany
Buchholz, Christiane	Forschungszentrum Jülich GmbH, Projektträger BEO		52425 Jülich Germany
Buhk, Hans-Jörg	Robert Koch-Institut Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Burrows, Paul	Department of the Environment, Transport and The Regions	3/HII Ashdown House, 123 Victoria Street	London SW1E 6DE UK
Busch, Ulrich	Landesuntersuchungsamt für das Gesundheitswesen Südbayern	Veterinärstr. 2	85764 Oberschleiß- heim, Germany
Buschhorn, Heike	Niedersächs. Umweltministerium	Archivstr. 2	30169 Hannover Germany
Casper, Rudolf		Am Hasselteich 42	38104 Braunschweig Germany
Chandler, Steve	Florigene Europe	Waardlaan 4A	2231 NA Rijnsburg Netherlands
Chen Zhangliang	Peking University, College of Life Sciences		Beijing 100873 Republic of China
Corell, Birgit	Bezirksregierung Braunschweig	Obergstr. 1	38102 Braunschweig Germany
Dale, Philip J.	John Innes Centre	Norwich Research Park, Colney	Norwich NR4 7UH UK
Dancsházy, Zsuzsanna	Plant Health and Soil Conservation Station of the Ministry of Agriculture	Budaörsi út 141-145	1118 Budapest Hungary
de Greef, Willy	Novartis Seeds R-1008-7.15		4002 Basel Switzerland
de Kathen, André	btc-BioTechConsult [1998: Molekulargenetik, Universität Hannover]	Tannengrund 7 [Herrenhäuser Str. 2]	31303 Burgdorf Germany [30419 Hannover]
Deaton, Randy	Monsanto Company BB1E	700 Chesterfield Parkway North	St. Louis, MO 63198 USA
Deml, Günther	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Denys, Pascale	SES Europe	Industriepark, Soldatenplein 22, n° 15	3300 Tienen Belgium
Ding, Yong	China National Center for Biotechnology Development	7B, Zao Jun Miao, Haidian Qu POB 8118	Beijing 100081 PR China
Dorsch-Häsler, Karoline	Swiss Expert Commission for Biosafety	c/o BUWAL	3003 Bern Switzerland
Dreyer, Marion	Institut für Soziologie, Universität München	Konradstr. 6	80801 München Germany

Duque, Estrella	CSIC, Estacion Experimental del Zaidin	Profesor Albareda, 1	18008 Granada Spain
Economidis, Ioannis	European Commission, DG XII	Rue de la Loi 200	1049 Bruxelles Belgium
Ehlers, Ulrich	Robert Koch-Institut Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Ellis, Dave	Forest Biotechnology Centre, BC Research, Inc.	3650 Wesbrook Mall	Vancouver, B.C. Canada
Elsas, Jan Dirk van	Plant Research International [1998: IPO-DLO]	POB 9060	6700 GW Wageningen Netherlands
Engelke, Thomas	Ministerium für Umwelt, Natur und Forsten, Referat Biotechnologie/ Gentechnik	Grenzstr. 1-5	24149 Kiel Germany
Feldmann, Sigrun	Niedersächsisches Landesamt für Ökologie	An der Scharlake 39	31139 Hildesheim Germany
Ferraiolo, Giovanni	International Centre for Genetic Engineering and Biotechnology (ICGEB)	Padriciano 99	34012 Trieste Italy
Ferro, David	Department of Entomology, University of Massachusetts		Amherst, MA 01003 USA
Fiebig, Claudia	Ministerium für Umwelt, Raumordnung und Landwirtschaft	Schwannstr. 3	40476 Düsseldorf Germany
Fischer, Siglinde	BioAlliance Deutschland GmbH	Steinlestr. 6	60596 Frankfurt Germany
Forney, Larry	University of Idaho Department of Biological Sciences [1998: Dept. of Microbiology University of Groningen]	[POB 14]	Moscow, ID 83844 USA [9750 AA Haren Netherlands]
Franke, Frank	Institut für Acker- und Pflanzenbau, Martin-Luther-Universität	Ludwig-Wucherer-Str. 2	06108 Halle Germany
Friemann, Andreas	Staatliches Umweltamt Köln	Blumenthalstr. 33	50670 Köln Germany
Fuchs, Marc	INRA, Laboratoire de Pathologie Végétale	28 rue de Herrlisheim	68021 Colmar France
Gallitelli, Donato	Dipartimento di Protezione delle Piante dalle Malattie University of Bari	Via Amendola 165/A	70126 Bari Italy
Giddings, Glynis	The University of Wales, Institute of Biological Sciences	Sir George Stapledon Bldg.,	Aberystwyth SY23 3DD, UK
Gland-Zwerger, Astrid	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Gonsalves, Carol	Cornell University Dept. of Plant Pathology, NYSAES		Geneva, NY 14456 USA
Gonsalves, Dennis	Cornell University, Dept. of Plant Pathology, NYSAES		Geneva, NY 14456 USA
Götz, Reinhard	Institut für Pflanzenkrankheiten und Pflanzenschutz, Universität Hannover	Herrenhäuser Str. 2	30419 Hannover Germany
Grant, Ian	Pioneer Hi-Bred S.A.R.L.	Chemin de l'Enseigure	31840 Aussonne France

Gronenborn, B.	Centre National de la Recherche Scientifique, Institut des Sciences Végétales	Avenue de la Terrasse	91198 Gif-sur-Yvette France
Gupta, Subhash	USDA/APHIS/Biotechnology	4700 River Road, Unit 147	Riverdale, MD 20737 USA
Hagler-Mendonça, Leda	Universidad Federal do Rio de Janeiro, Conselho Nacional de Desenvolvimento Cientifico e Tecnologico	Cidade Universitaria, CCS, Bloco I	21949-590 Rio de Janeiro Brazil
Hardy, Tony	Central Science Laboratory	Sand Hutton	York YO41 ILZ, UK
Hassa, Anke	Universität Hannover Lehrgebiet Molekulargenetik	Herrenhäuser Str. 2	30419 Hannover Germany
Hayashi, Ken-ichi	Society for Techno-Innovation of Agriculture, Forestry and Fisheries (STAFF)	Sankaido Bldg. 2F, Akasaka 1-9-13	Minato-ku, Tokyo Japan
Head, Graham	Monsanto Company, GG3E	700 Chesterfield Parkway North	St. Louis, MO 63198 USA
Heidenreich, Bettina	Universität Hamburg FSP BIOGUM	Ohnhorststr. 18	22609 Hamburg Germany
Heissenberger, Andreas	Umweltbundesamt – Austria	Spittelauer Lände 5	1090 Wien Austria
Hesse, Peter René	RWTH Aachen, Biologie V	Worringerweg 1	52074 Aachen Germany
Heyer, Arnd	MPI für Molekulare Pflanzenphysiologie	Karl-Liebknecht-Str. 25	14476 Golm Germany
Hilbeck, Angelika	Eidgenössische Forschungsanstalt für Agrarökologie und Landbau	Postfach	8046 Zürich Switzerland
Hoffmann, Marcus	Justus-Liebig-Universität	Ludwigstr. 27	35390 Gießen Germany
Hoffmann, Torsten	Landesumweltamt Brandenburg, Abt. Gentechnologie	Postfach 601061	14410 Potsdam Germany
Hofmann	Niedersächs. Umweltministerium	Archivstr. 2	30169 Hannover Germany
Hohgardt, Karsten	BBA, Fachgruppe Chemische Mittelprüfung	Messeweg 11-12	38104 Braunschweig Germany
Hommel, Bernd	BBA, Institut für integrierten Pflanzenschutz	Stahnsdorfer Damm 81	14532 Kleinmachnow Germany
Hull, Roger	John Innes Centre	Norwich Research Park, Colney	Norwich NR4 7UH UK
Hutchison, William	University of Minnesota	219 Hodson Hall	St. Paul, MN 55108 USA
James, Clive	ISAAA	P.O. Box 427	Savannah Grand Cayman Cayman Islands
Jansson, Janet K.	Section for Natural Sciences, Södertörns högskola [1998: Stockholm University, Arrhenius Laboratories of Natural Sciences]	Box 4101	141 04 Huddinge Sweden [106 91 Stockholm]

Jia Shi-Rong	Biotechnology Research Center, Chinese Academy of Agricultural Sciences	30 Baishiqiao Road	Beijing 100081 China
Jonsson, Bodil	Svalöf Weibull AB		26881 Svalöv Sweden
Joos, Henk	Aventis CropScience N.V. [1998: Plant Genetic Systems N.V.] Research Station	Nazarethsesteenweg 77	9800 Astene Belgium
Kameke, Conrad von	Monsanto	Unter den Linden 21 [1998: 272, Avenue de Tervuren]	10117 Berlin Germany [1150 Bruxelles Belgium]
Kamradt, Barbara	Greenpeace e.V. Gentechnik	Große Elbstr. 38	22767 Hamburg Germany
Kandawa-Schulz, Martha	University of Namibia	P/Bag 13301	Windhoek Namibia
Käppeli, Othmar	Fachstelle BATS	Clarastr. 13	4058 Basel Switzerland
Katul, Lina	DSMZ c/o BBA [1998: BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit]	Messeweg 11-12	38104 Braunschweig Germany
Katzek, Jens	KWS	Grimsehlstr. 31	37574 Einbeck Germany
Kinderlerer, Julian	The University of Sheffield, Dept. of Molecular Biology & Biotechnology	P.O. Box 594, Western Bank	Sheffield S10 2UH UK
Klonus, Dirk	Aventis CropScience Deutschland GmbH [1998: Hoechst Schering AgrEvo GmbH]	Industriepark Höchst K607	65926 Frankfurt Germany
König, Ariane	Belfer Center for Science and International Affairs, Kennedy School of Government, Harvard University [1998: Monsanto Services International]	79, John F Kennedy Street [270-272, Avenue de Tervuren]	Cambridge, MA 02138, USA [1150 Bruxelles Belgium]
Kraus, Josef	Planta Angewandte Pflanzengenetik und Biotechnologie GmbH	Grimsehlstr. 31	37574 Einbeck Germany
Kreike, Maria	John Innes Centre	Colney Lane	Norwich NR4 7UH UK
Krug, Manfred	Landesamt für Umweltschutz Sachsen-Anhalt	Reideburger Str. 47	06116 Halle Germany
Kühne, Thomas	BAZ Aschersleben	Postfach 1505	06435 Aschersleben Germany
Laimer da Câmara Machado, Margit	Institut für Angewandte Mikrobiologie Universität für Bodenkultur	Nußdorfer Lände 11	1190 Wien Austria
Landsmann, Jörg	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Langelüddeke, Peter		Nelkenweg 5	65719 Hofheim Germany
Langenbruch	BBA, Institut für biol. Pflanzenschutz	Heinrichstr. 243	64287 Darmstadt Germany

Laplace, Frank	BMBF	Heinemannstr. 2	53170 Bonn Germany
Larssen, Hilde Christin	Directorate for Nature Management	Tungasletta 2	7005 Trondheim Norway
Laucke, Guido	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11/12	38104 Braunschweig Germany
Levidow, Les	Centre for Technology Strategy	Open University	Milton Keynes MK7 6AA, UK
Lilley, Andy	Institute of Virology & Environmental Microbiology	Mansfield Road	Oxford OX1 3SR UK
Lobas, Detlef	Gesellschaft für Biotechnologische Forschung mbH	Mascheroder Weg 1	38124 Braunschweig Germany
Lottmann, Jana	Universität Rostock Abt. Mikrobiologie	Gertrudenstr. 11a	18051 Rostock Germany
Lu, Wei	Institute for Application of Atomic Energy, CAAS	Yuan Ming-yuan West-road 2, Hai Dian District	Beijing P.R. China
Lukow, Thomas	Fraunhofer-Institut für Umweltchemie und Ökotoxikologie, Abt. Mol. Biotechnologie [1998: MPI für terrestrische Mikrobiologie]	Auf dem Aberg 1 [Karl-von-Frisch-Straße]	57392 Schmallenberg Germany [35043 Marburg]
Lyngved, Ragnhild	Norwegian University of Science and Technology, Dept. of Botany		7034 Trondheim Norway
MacDonald, Robert	AgrEvo Canada Inc.	295 Henderson Drive	Regina Saskatchewan S4N 6C2, Canada
MacIntosh, Sue	AgrEvo USA	7200 Hickman Road	Des Moines, IA 50322, USA
Madkour, Magdy A.	Ministry of Agriculture and Land Reclamation, Agricultural Genetic Engineering Research Institute	9 Gamaa St.	Giza 12619 Egypt
Mahn, Andreas	MPB Cologne GmbH [1998: Bundesanstalt für Züchtungs-forschung]	Neurather Ring 1 [Neuer Weg 22/23]	51063 Köln Germany [06484 Quedlinburg]
Maiss, Edgar	Institut für Pflanzenkrankheiten und Pflanzenschutz, Universität Hannover	Herrenhäuser Str. 2	30419 Hannover Germany
Maruyama, Takao	Hoechst Schering AgrEvo K.K.	10-33, Akasaka 4-chome Minato-ku	107 Tokyo Japan
Matzk, Anja	Planta Angewandte Pflanzengenetik und Biotechnologie GmbH	Grimsehlstr. 31	37574 Einbeck Germany
Mauro, Marie-Claude	Moët & Chandon	6, rue croix de Bussy	51200 Epernay Cédex, France
McCammon, Sally	USDA-APHIS Room 312E	J.L. Whitten Federal Bldg.	Washington, DC 20016, USA
McClung, Gwendolyn	US EPA (7403), Office of Prevention, Pesticides and Toxic Substances	401 M Street, SW	Washington, DC 20460, USA
McGloughlin, Martina	University of California, Biotechnology Program	355 Briggs Hall	Davis, CA 95616 USA
McHughen, Alan	University of Saskatchewan, Crop Development Center		Saskatoon SK S7N 5A8, Canada

Medley, Terry	DuPont Agricultural Products	P.O. Box 80083	Wilmington, DE 29880, USA
Menßen, Adriane	Max-Planck-Institut für Züchtungsforschung	Carl-von-Linné-Weg 10	50829 Köln Germany
Metz, Birgit	BMBF		53170 Bonn Germany
Miller, Allen W.	Iowa State University, Developmental Biology	351 Bessey Hall	Ames, IA 50011 USA
Mitten, Donna H.	AgrEvo USA	414 Fourth Street Suite A	Woodland, CA 95695, USA
Momose, Masaki	Kirin Agribio EC B.V.	Postbus 26	2678 ZG De Lier The Netherlands
Narula, Neeru	Dept. of Microbiology	CCS HAU. Hisar	125004 Haryana India
Nasr El-Din, Taymour	Agricultural Genetic Research Institute AGERI	9 Gamaa St.	Giza Egypt
Nehls, Reinhard	Planta Angewandte Pflanzengenetik und Biotechnologie GmbH	Grimsehlstr. 31	37574 Einbeck Germany
Nickson, Thomas E.	Monsanto Company	700 Chesterfield Parkway, N.	St. Louis, MO 63198 USA
Nielsen, Kåre Magne	UNIGEN, MTFS	Norwegian University of Science and Technology	7005 Trondheim Norway
Nitta, Naoto	Japan Tobacco Inc., Plant Breeding and Genetics Laboratory	700 Higashibara, Toyoda, Iwata	Shizuoka 438 Japan
Njuguna, Michael Macharia	International Service for the Acquisition of Agri-biotech Applications	P.O. Box 25171	Nairobi Kenya
O'Gara, Fergal	Biomerit Laboratory, Microbiology Dept.	University College Cork	Cork Ireland
Palkovics, Lásló	Agricultural Biotechnology Center	P.O. Box 411	2101 Gödöllö Hungary
Palukaitis, Peter	Scottish Crop Research Institute, Virology Dept.		Invergowrie, Dundee DD2 5AD, UK
Pedersen, Holger	Danish Environmental Protection Agency	Strandgade 29	1401 Copenhagen K Denmark
Pinck, Lothaire	I.B.M.P. - C.N.R.S.	12, rue du Général Zimmer	67084 Strasbourg France
Pitkäjärvi, Jyrki	Finnish Environment Institute	P.O. Box 140	00251 Helsinki Finland
Pohl, Matthias	TÜV Hannover/ Sachsen-Anhalt e.V. Bereich Energie- und Systemtechnik, Servicestelle Biotechnologie/ Gentechnik	Am TÜV 1	30519 Hannover Germany
Pühler, Alfred	Universität Bielefeld	Universitätsstr. 25	33615 Bielefeld Germany
Pythoud, François	Federal Office for Environment, Forestry, Landscape		3003 Bern Switzerland
Ramos, Juan L.	CSIC, Estacion Experimental del Zaidin	Profesor Albareda, 1	18008 Granada Spain

Ravelonandro, Michel	INRA - Centre de Recherches de Bordeaux Station de Pathologie Végétale	B.P. 81	33883 Villenave d'Ornon Cédex France
Remler, Peter	Technische Universität Graz, Dept of Biochemistry and Food Chemistry	Petersgasse 12	8010 Graz Austria
Rossi, Luca	Fabriques de Tabac Réunies SA/Philip Morris; R&D	Quai Jeanrenaud 56	2003 Neuchâtel Switzerland
Röver, Manuela	[1998: SLFA Neustadt, FB Ökologie]	Kuckhoffstr. 110d [Breitenweg 71]	13156 Berlin Germany [67435 Neustadt]
Röver, Markus	Robert Koch-Institut, Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Rudan, Ferenc	National Committee for Technological Development	Szervita tér 8	1053 Budapest Hungary
Rüdelsheim, Patrick	Aventis CropScience N.V. [1998: Plant Genetic Systems N.V.]	Jozef-Plateaustraat 22	9000 Gent Belgium
Sakai, Michihiko	Zeneca KK	Akasaka Ohji Bldg., 8-1-22 Akasaka, Minato-ku	Tokyo 107-0052 Japan
Salama, Mohamed	Agricultural Genetic Engineering Research Institute, AGERI	9 Gamaa st.	Giza 12619 Egypt
Saleh, Mohamed T.	Agricultural Genetic Engineering Research Institute, AGERI	9 Gamaa st.	Giza 12619 Egypt
Scharnhorst, Christina	Bezirksregierung Braunschweig	Obergstr. 1	38102 Braunschweig Germany
Schauzu, Marianna	Bundesinstitut für gesundheitl. Verbraucherschutz und Veterinär-medizin (BgVV)	Thielallee 88-92	14195 Berlin Germany
Schechtman, Michael	USDA, APHIS, PPQ	12th and Independence Aves., SW	Washington, DC 20250, USA
Schiemann, Joachim	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Schmalenberger, Achim	Bundesforschungsanstalt für Land-wirtschaft, Institut für Agrarökologie	Bundesallee 50	38116 Braunschweig Germany
Schneider, William	EPA, BioPesticides & Pollution Prevention Division, OPP	401 M Street, S.E.	Washington, DC 20460, USA
Schneiker, Susanne	Universität Bielefeld Lehrstuhl für Genetik Fakultät für Biologie	Postfach 100131	33501 Bielefeld Germany
Schofield, Geraldine	Unilever Research, Colworth Laboratory	Colworth House, Sharnbrook	Bedford MK44 1LQ UK
Schreiber, Birgit	Bezirksregierung Hannover Dezernat 501	Am Waterlooplatz 11	30169 Hannover Germany
Schrey, Christiane	Phytowelt GmbH	Kölsumer Weg 33	41334 Nettetal Germany
Schulze, Manuela	Staatliches Lebensmitteluntersuchungsamt	Dresdenstr. 2+6	38124 Braunschweig Germany
Schulze-Weslarn, Karl-Wilhelm	Bundesministerium für Ernährung, Landwirtschaft und Forsten	Postfach 140270	53107 Bonn Germany
Schütte, Gesine	Universität Hamburg FSP BIOGUM	Ohnhorststr. 18	22609 Hamburg Germany

Seidler, Ray	US EPA, Western Ecology Div., National Health and Environmental Effects Research Lab.		Corvallis, OR 97333 USA
Sessitsch, Angela	Österr. Forschungszentrum Seibersdorf, Division of Life Sciences		2444 Seibersdorf Austria
Siegfried, Blair	Dept. of Entomology University of Nebraska		
Sinemus, Kristina	Genius GmbH	Robert-Bosch-St.r. 7	64293 Darmstadt Germany
Smalla, Kornelia	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Snow, Allison	The Ohio State University, Dept. of Plant Pathology	1735 Neil Ave.	Columbus, OH 43210, USA
Sonnenberg, Bernd	BASF AG Agrarzentrum Limburgerhof	Postfach 120	67114 Limburgerhof Germany
Stäber, Horst	Robert Koch-Institut Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Stegemann, Hermann		Am Sandkamp 15	38104 Braunschweig Germany
Steinheuer, Werner	Aventis CropScience Deutschland GmbH, Registrierung D [1998: Hoechst Schering AgrEvo]	Industriepark Höchst - K 607 -	65926 Frankfurt Germany
Stelling, Dieter	Aventis CropScience Deutschland GmbH [1998: Hoechst Schering AgrEvo]	Werftstr. 37	40549 Düsseldorf Germany
Storeng, Anne Britt	Directorate for Nature Management	Tungasletta 2	7005 Trondheim Norway
Tabei, Yutaka	Ministry of Agriculture, Forestry and Fisheries, Innovative Technology Division	1-2-1 Kasumigaseki, Chiyoda	Tokyo 100-8590 Japan
Tebbe, Christoph	Bundesforschungsanstalt für Landwirtschaft, Institut für Agrarökologie	Bundesallee 50	38116 Braunschweig Germany
Tepfer, Mark	INRA, Laboratoire de Biologie Cellulaire		78026 Versailles Cédex France
Tewari, Neera	Molekulargenetik, Universität Hannover	Herrenhäuser Str. 2	30419 Hannover Germany
Thierfelder, Andreas	Monsanto	Obere Zeil 2	61440 Oberursel Germany
Tooming, Ave	Norwegian Biotechnology Advisory Board	P.O. Box 8027 DEP.	0030 Oslo Norway
Trinks, Klaus	Aventis CropScience Deutschland GmbH, Registrierung D [1998: Hoechst Schering AgrEvo]	Industriepark Höchst K607	65926 Frankfurt Germany
Tschimmel, Udo	MEDIA Projects	Schloß Allner	53773 Hennef Germany
Twardowski, Tomasz	Institute of Bioorganic Chemistry	ul. Noskowskiego 12/14	61-704 Poznan Poland
Tzotzos, George	United Nations Industrial Development Organization	Vienna International Centre, P.O. Box 300	1100 Wien Austria
Uijtewaal, Bert	Aventis CropScience N.V. [1998: Plant Genetic Systems N.V.]	Jozef Plateaustraat 22	9000 Gent Belgium

Uwer, Ursula	Planttec Biotechnologie GmbH	Hermannswerder 14	14473 Potsdam Germany
Valenzuela, Sofia A.	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11/12	38104 Braunschweig Germany
van den Eede, Guy	JRC Ispra, EC	TP 361	21020 Ispra (VA) Italy
Varrelmann, Mark	Institut für Pflanzenkrankheiten und Pflanzenschutz, Universität Hannover	Herrenhäuser Str. 2	30419 Hannover Germany
Verma, Desh Pal S.	Molecular Genetics and Biotechnology Center, The Ohio State University	1060 Carmack Rd.	Columbus, OH 43210 USA
Vollmer, Günter	Fraunhofer-Institut für Umweltchemie und Ökotoxikologie	Postfach 1260	57377 Schmallenberg Germany
Waters, Stephen	Monsanto Services International	Avenue de Tervuren 270-272	1150 Bruxelles Belgium
Watson, Grant	Canadian Food Inspection Agency, Plant Health and Production Division	59 Camelot Court	Nepean, Ontario K1A 0Y9 Canada
Weber, Andrea	DSMZ Abt. Pflanzliche Zellkulturen	Mascheroder Weg 1b	38124 Braunschweig Germany
Weber, Barbara	Öko-Institut e.V.	Postfach 6226	79038 Freiburg Germany
Wegelin, Tanja	Molekulargenetik Universität Hannover	Herrenhäuser Str. 2	30419 Hannover Germany
Welling, Michael	Senate of the Federal Research Centres	Messeweg 11-12	38104 Braunschweig Germany
Welters, Peter	Phytowelt GmbH	Kölsumer Weg 33	41334 Nettetal Germany
Wentworth, Steve		RR#1 Box 262	Oreana, IL 62554 USA
White, James	USDA, APHIS, PPQ, Biotechnology Evaluations	4700 River Road, Unit 147	Riverdale, MD 20737 USA
Wolpers, Karl H.	GTZ	Postfach 5180	65726 Eschborn Germany
Wurmbach, Peter	Robert Koch-Institut Zentrum Gentechnologie	Postfach 870161	13161 Berlin Germany
Wyke, Lene	GX Biosystems A/S	Symbion Fruebjergvej 3	2100 Copenhagen Denmark
Zock, Angela	BBA, Institut für Pflanzenvirologie, Mikrobiologie und biol. Sicherheit	Messeweg 11-12	38104 Braunschweig Germany
Zwerger, Peter	BBA, Institut für Unkrautforschung	Messeweg 11-12	38104 Braunschweig Germany

Author Index

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- Alan McHughen (University of Saskatchewan, Canada)
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