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Berlin-Dahlem

# Key Biosafety Aspects of Genetically Modified Organisms

## Workshop 10. – 11. April 1995 in Braunschweig, Germany

edited by

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and
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Biologische Bundesanstalt für Land- und Forstwirtschaft,
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Workshop on

# KEY BIOSAFETY ASPECTS OF GENETICALLY MODIFIED ORGANISMS

**10 - 11 April, 1995**
**Braunschweig, Germany**

The Workshop was organized by:

Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), Braunschweig, Germany
Federal Biological Research Centre for Agriculture and Forestry
Rudolf Casper and Jörg Landsmann

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Editors of the Proceedings:

Jörg Landsmann and Rudolf Casper

On the occasion of the retirement of Professor Dr. Rudolf Casper

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## Zusammenfassung (Workshop-Summary)

## Key Biosafety Aspects of Genetically Modified Organisms

## - Gentechnisch veränderte Organismen: Aspekte der biologischen Sicherheit -

Dieser Workshop stand in der Tradition des 1992 ebenfalls vom Institut für Biochemie und Pflanzenvirologie organisierten Symposiums über "Biosafety Results of Field Tests of Genetically Modified Plants and Microorganisms" in Goslar. In Braunschweig nahmen am Workshop ca. 120 Wissenschaftler teil. In fünf Sektionen wurden die wichtigen risikorelevanten Aspekte der Freisetzung gentechnisch veränderter Pflanzen, Baculoviren und Mikroorganismen behandelt.

Bei den gentechnisch hergestellten virusresistenten Pflanzen wurden von G. A. de Zoeten (East Lansing/USA), R. Hull (Norwich/UK), J. G. Atabekov (Moskau/Rußland), E. Maiß (Hannover) und R. Koenig (BBA Braunschweig) die Möglichkeiten für virale heterologe Enkapsidierung und Rekombination diskutiert. Es zeigte sich, daß beide Ereignisse natürlicherweise vorkommen und bei Pflanzen mit ins Genom eingeführten Virusgenen wahrscheinlich nicht signifikant häufiger auftreten werden. Erweiterungen viraler Wirtskreise sind durch die transgenen Pflanzen hypothetisch möglich, doch gibt es keine Hinweise darauf, daß das pathogene Potential pflanzlicher Viren hierdurch neue Dimensionen erreichen könnte. Ein Forschungsbedarf über die Effekte virusresistenter transgener Pflanzen auf Pflanzenviruspopulationen wurde jedoch aufgezeigt.

Bei den herbizidresistenten Pflanzen wurden von P. Rüdelsheim (Plant Genetic Systems, Gent/Belgien), P. Böger (Konstanz), J. Cremer und K. Trinks (AgrEvo, Frankfurt/Berlin) mögliche Auswirkungen des landwirtschaftlichen Anbaus auf den Naturhaushalt diskutiert. Es wurde aufgezeigt, daß seltene Auskreuzungen des Herbizid-Resistenzgens in die Unkrautflora aufgrund des mangelnden oder diskontinuierlichen Selektionsdrucks kaum zu Problemen führen werden. Eventuelle Nachauflaufprobleme BASTA-resistenter Pflanzen werden mit konventioneller Rotationspraxis umgangen.

P. Ahl Goy (Ciba-Geigy, Basel/Schweiz) diskutierte mögliche Effekte des in Maispflanzen eingeführten *Bacillus thuringiensis*-Endotoxins auf Zielinsekten und Nichtzielorganismen. Die Spezifität des *B.t.*-Endotoxins erwies sich als ein guter Sicherheitsfaktor. Ob der Selektionsdruck durch konstitutiv das Endotoxin ausprägende Pflanzen die Bildung resistenter Insektenpopulationen begünstigt, können nur weitere Forschungen und die Erfahrung zeigen.

J. M. Vlak (Wageningen/Niederlande) demonstrierte Möglichkeiten des Einsatzes gentechnisch veränderter Baculoviren zur Schädlingsbekämpfung. Hier sollen eingeführte Insektentoxingene die Wirkung beschleunigen.

J. D. van Elsas (Wageningen/Niederlande) - in Zusammenarbeit mit K. Smalla (BBA Braunschweig) - demonstrierte molekulare Methoden zum Nachweis mikrobieller Antibiotikaresistenzen in Umweltproben.

Es wurde deutlich, daß die in der Gentechnik als Markierung eingesetzten Resistenzen natürlicherweise weit verbreitet sind und somit keine neuen ökologischen Belastungen darstellen.

K.-D. Jany (BFE Karlsruhe) erklärte, daß die Frage der Bezeichnung von Inhaltsstoffen bei Lebensmitteln ein Diskussionsthema ohne Ende sein kann. Welche Stoffe gekennzeichnet werden, ist schon heute nicht logisch organisiert. Selbstverständlich soll dem Verbraucher die genaueste Information und Auswahl ermöglicht werden - auch bei gentechnisch hergestellten Produkten. Doch wird eine Grenzziehung zwischen zu

bezeichnenden und nicht mehr zu bezeichnenden Folgeprodukten erschwert, wenn man von dem Prinzip abrückt, nur direkt veränderte lebende Organismen zu kennzeichnen.
D. Bartsch (Aachen) sprach über Möglichkeiten ökologischer Sicherheitsforschung bei Zuckerrüben. Hier stellte sich heraus, daß sog. verwilderte Rüben besonders in der Nähe entsprechender Züchtungsbetriebe anzutreffen sind. Die Finanzierung ökologischer Langzeitforschung über Einflüsse genetisch veränderter Organismen auf den Naturhaushalt hat in der Vergangenheit ein Schattendasein geführt.
J. Schiemann (BBA Braunschweig) stellte die Forschungen der Kollegen im Institut für Biochemie und Pflanzenvirologie zur biologischen Sicherheit vor. Die Notwendigkeit zur Sammlung von Daten in Begleitung von Freisetzungen gentechnisch veränderter Organismen - auch im Hinblick auf die öffentlichen Diskussionen zur Gentechnik - wurde deutlich. Spezifische Risiken bei Freisetzungen sind allerdings nicht zu Tage getreten.
W. van den Daele (WZB Berlin) berichtete über die Ergebnisse einer sozialwissenschaftlich geleiteten Technikfolgenabschätzung. Hierbei kam er zu dem Fazit, daß man die Gentechnik insgesamt nicht in Abwägung geringer Risiken gegen mäßigen Nutzen befürworten oder ablehnen kann, sondern daß es einer gesellschaftlichen Willensentscheidung bedarf. Es gilt, die Alternativen in jedem Einzelfall gegeneinander abzuwägen.
Dieser Workshop stellte einen weiteren wichtigen Beitrag zur Diskussion um Chancen und Risiken der Gentechnik in Deutschland dar. Öffentliche Darlegungen des Wissensstandes auf dem Gebiet der Freisetzung und Sicherheitsbewertung gentechnisch veränderter Organismen sind notwendige Bausteine zur Absicherung eines vorbildhaften Umgangs mit der Gentechnologie und ihrer Akzeptanz in der Bevölkerung. Wissenschaftliche Treffen dieser Art müssen im Schritt mit der technologischen Entwicklungsgeschwindigkeit auch in Zukunft stattfinden.

**Jörg Landsmann**

## Farewell notes

During more than 30 years of service in the Federal Biological Research Centre I have experienced such great support and help from so many colleagues that I have to thank you all for this good company through all the years. In science only teamwork leads to success, and I realized that the good atmosphere in our virology group was the key to good results in research. Changing cooperations, changing research topics, and even changing financial support caused some times closer and then looser contacts during the years. Therefore, I was very happy to see so many old and very old friends at this symposium. It was the suggestion by Gus de Zoeten to have a colloquium on the occasion of my retirement which gave us the last kick to organize this meeting. Nearly all invited speakers accepted our call, and the large number of participants today shows that we have a topical subject. Plant virology has changed from a more or less passive control of virus diseases to an active elimination of viruses in plants. This was possible by the application of genetechnology but it implied biosafety measures. This topic will be of interest for more years to come and we hope that this symposium will become a regular event from now on.
I thank all colleagues who made this symposium a success, and last not least, we thank the Federal Ministry for Food, Agriculture and Forestry for financial support.

**Rudolf Casper**

# Opening of the Workshop

Ladies and Gentlemen,
I have the honour of opening the workshop on „Key biosafety aspects of genetically modified organisms".
But before I do this I want to welcome all of you to Braunschweig and especially to the Federal Biological Research Centre for Agriculture and Forestry (whose acronym BBA is derived from the German name Biologische Bundesanstalt für Land- und Forstwirtschaft).
The occasion for organizing this workshop is, as you know, the release into the retirement of Professor Casper, who will be going to leave the BBA for good in May.
The BBA has taken this event to call together all those experts who, in their specific work, have, in one way or another, accompanied Professor Casper in his work as virologist or as director of the Institute for Biochemistry and Plant Virology, the institute which also is a cooperative authority for permitting the release of genetically modified organisms into the environment.
Before I open the workshop and before we enter into the specific subjects of the workshop let me give you a brief introduction into the work and activities of the Federal Biological Research Centre in order that you might know where and in what kind of institution you have gathered.

Let me start from the year 1898, the year the Biological Department at the Imperial Health Office in Berlin was founded. Already this Biological Department, our predecessor, had to do research in the entire field of plant health in agriculture and forestry.
Plant protection and the realization of legal aspects in plant protection procedures belonged to the duties of the Biological Research Centre ever since.
Today the BBA is a research centre and an autonomous higher federal authority attached to the Federal Ministry of Food, Agriculture and Forestry.
The BBA derives its duties and responsibilities from the Plant Protection Act, the Genetechnology Act, from parts of the Federal Epidemics Act and the Chemicals Act.
Within the legal functions of BBA the examination and the licensing of plant protection chemicals and equipment used in the protection of plants and stored products are comprising the biggest part. However, in its 15 institutes, research is done on plant diseases and pests or, in other words, on phytopathology in its widest sense and on plant protection systems, e. g., on IPM (Integrated Pest Management or Integrated Plant Protection).
The variety of research work within the BBA provides a basis for decision-making in respect of food, agricultural and forestry policies as well as with regard to consumer policies.
Apart from the headquarter there exist 15 research institutes, the Department of Plant Protection Products and Application Techniques, the libraries and the Office for Economic and Legal Affairs in Plant Protection.
For a number of reasons, the BBA is not a centralized institution but, instead, has facilities in seven different locations, scattered all over the country.
Coming back to the work which is done by the Federal Biological Research Centre I would like to list, besides the already mentioned, a few more:

- Studies on plant pests (nematodes, insects, mites, rodents, birds etc.), pathogens (fungi, bacteria, viruses) and weeds, and the development of suitable methods of control
- Integrated pest management, including risk assessment in plant protection and ecotoxicology, warning service and plant quarantine measures, modelling and electronic data processing

- Research on crop losses caused by non-parasitic diseases
- Studies on resistance, transfer of resistance to plants by employing classical and biotechnical methods
- Development of methods for the diagnosis of plant diseases, including genetechnological methods
- Investigations on the integration of chemical, biological and agrotechnical measures in order to minimize the use of pesticides
- Development of suitable methods of biological control
- Studies on problems in the protection of stored products
- Research on equipment and methods of application
- Investigation on the mode of action and use of pesticides and their environmental side effects
- Work on residue problems arising from pesticide application with a view to safeguarding the health of humans and animals
- Collection, evaluation and information on national and international scientific literature on phytomedicine and plant protection, and of relevant laws and regulations of the Federal Republic of Germany and of foreign countries in the field of plant protection and plant quarantine
- Participation in crop protection projects in developing countries.

Not mentioned in the list of activities and objectives, but to which I would like to draw your attention, is the involvement in risk assessment of genetically modified organisms deliberately released into the environment. This is the special duty of Professor Casper's institute.

Since the mid-eighties questions of biotechnology and genetechnology, respectively, have become of paramount interest to the German Government.

New research activities were initiated and, at the same time, first legal regulations were set up.

Although BBA was engaged for already quite a while in biotechnological research a new incentive for working on biosafety came in May 1986 from the „Directive of protection against dangers brought about by *in vitro* recombinant nucleic acid".

In one of its paragraphs the directive was specifying the actions which the Federal Biological Research Centre had to take in the course of its responsibility for deliberate releases of genetically modified organisms.

To take over the new responsibility of giving its consent to the deliberate releases, a working group of nine people was set up, which since 1987 has been busy answering the many enquiries of our government, going through hundreds of files handed in for field releases, and in public hearings, held prior to the open field releases.

Eventually, the responsibilities of BBA in gentechnology were fixed in the Gentechnology Act of 20 of June 1990. In paragraph 16 the BBA is commissioned to give its consent for the release of genetically modified organisms.

In the past there has been an enormous interest in the public hearings which were to be held preliminary to the field releases.

In this connection I must mention the role Professor Casper had to play in these hearings as well as in all the discussions of the PRO and CONS of genetechnology. His diplomatic skill enabled him to calm down the often heated discussions, and to find new points of agreement on a scientifically sound base.

Since the first experimental releases of genetically modified plants in 1986 the number of releases have increased exponentially.

As I learned from Professor Casper, thus far there have been no unexpected alterations in the behaviour of transgenic plants in the environment, especially not, when the characteristics of the receiver plants and the nature of the transmitted genes are taken into consideration.

This sounds quite appeasing, yet, in certain cases one is not completely sure that there is no risk at all.
Thus, there is need to assess the perceived levels of risk and to determine if they represent an increase over acceptable 'natural' levels. If any significant increases are discovered the task is then to balance them against the benefits that might occur from the use of transgenic organisms in question.
I suppose this kind of research work will keep us busy for quite a while.

Finally, I may be allowed to direct a few words to Professor Casper, to whom this workshop is dedicated:
Dr. Casper was born on 5th of May 1930 in Riesa, situated at the river Elbe in Saxonia.
From 1954 until 1959 he studied biology at the University of Heidelberg.
As holder of a Fulbright-scholarship he studied for one year at the University of Kentucky/USA, from where he got his Master of Science degree.
In 1963 he received his PhD from the University of Göttingen.
In his theses he worked on the effects of blue and red light upon the composition of leaves of *Calanchoe rotundifolia*.
Right after his promotion he began his career at the Federal Biological Research Centre in Braunschweig.
As member of the scientific staff of the Institute of Virus Serology he was intensively engaged in the development of diagnostic methods for viruses.
He combined a serological test with an enzymatic dependent colour reaction, the ELISA.
This test was of an immense value, especially for potato breeders. Today it is a cheap and reliable routine test in phytopathology.
Quite early, in the mid-eighties, he also was engaged in the development of genetechnological methods for virus diagnostics.
In 1986 he published an article with the title „Gentechnologische Methoden zur Diagnose und Bekämpfung des Scharkavirus" (Gentechnological methods for diagnosis and control of the plum pox virus.
Thus, investigation of genetically engineered cross protection with the plum pox virus was during the last years, as I believe, one of his favourite subjects.
Dr. Casper also has been working at the University of Göttingen. In 1973 he started as a lecturer and from 1983, after his habilitation, as Professor, reading plant virology.
In 1989 Professor Casper became head of the BBA-Institute of Biochemistry, which in 1991 merged with the Institute of Virus Diseases of Plants.
As director of the Institute of Biochemistry and Plant Virology he, up to this date, was responsible for carrying out research on risk assessment of genetically modified organisms deliberated into the environment.
In this capacity as well as in earlier performances Professor Casper has contributed nationally and internationally to the high recognition of the work of the Federal Biological Research Centre.
As president of the Federal Biological Research Centre I do thank you, Professor Casper, for your long membership in the BBA and for the work you have done to defend the high scientific standard of our institution.
We all are wishing you a good start into the new period of life, free from professional obligations.
And now, I declare the floor open for the workshop on „Key biosafety aspects of genetically modified organisms".

**Fred A. J. Klingauf**, President of the BBA

# VIRUS RESISTANT PLANTS: HETEROLOGOUS ENCAPSIDATION AND RECOMBINATION

## Virus resistance: Biosafety research needs

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"Agricultural biotechnology can and likely soon will, focus on all crops of the world; on most, if not all, of the cultural practices; and be used in virtual myriads of environments. In all of this it is more complex and pervasive, and thus likely more vexatious, than biomedical biotechnology which focuses only on humankind, a single species with but little variation among its parts. This is a large part of our confounding context" (Hullar, 1993).
The risk assessment that needs to be performed to meet the challenge that a confounding context provides is perceived to be of dissimilar magnitude to scientific, industrial and public interests. This provides tensions between these groups that have come repeatedly to the surface. The tendency to think in extremes or to conjure up the extremes is inherent in that conflict.
Some see risk assessment as the nemesis of the application of biotechnology, while others feel strongly that risk assessment will only become a nemesis to biotechnology if we neglect to understand that it is part of the science applied in biotechnology. I hope I will convince you that when it pertains to engineered virus resistance risk assessment is a must.
Let me give you here for clarity a working definition of Biotechnology: Biotechnology refers to any technique that uses living organisms or substances from those organisms to make or modify a product, to improve plants or animals, or to develop microorganisms for specific use.
Let me also set the context in which I would like to think about risk 'Safety assessment of a recombinant-DNA-modified organism should be based on the nature of the organism and the environment into which it will be introduced, not the method by which it was modified" (NAS 1987). Implicit in this statement is the expectation that environmental release will be handled on a case by case basis.
The conclusion that both the NRC (1989) and the OECD (1991) reached was: The characteristics of modern biotechnology products that have already been produced, or are now under development, or are anticipated are generally similar to those produced with traditional techniques, and are consequently familiar to regulatory authorities.
Since such familiarity is totally lacking in the case of virus gene transgenic plants, I suggest that special risk assessment approaches should be taken when plants are engineered with virus derived sequences. It is generally acknowledged that recombination between complete and/or partial RNA sequences in plants occurs. The problem is that RNA recombination has a direct baring on the risks of the environmental release of plants expressing viral gene sequences.

### What is the risk and what are its components?

Risk is the product of the frequency of occurrence of an unfavourable event X, the exposure X, the qualitative value of the effect (good, bad or indifferent). Risk analysis pertains to the overall process, risk assessment assigns a value to the possibilities, and risk management addresses mainly the management of the frequency of occurrence.

Recalling escaped genes from the environment may not be a trivial option in the reduction of risk and the inability to do so may make the equation go to infinity.

If we now return to the special case of virus sequence transgenic plants and our suggestion that their release should be looked at on a case by case basis as suggested by the NAS panel, we need to have an understanding of both virus evolution as well as of virus replication. Figure 1 gives a schematic representation of virus replication. It is generally accepted that viral RNA upon infection of the cell communicates in some way its need for a membrane system that can function as the backbone for virus replication. It is assumed that part of this early communication involves the manufacture (translation of part of the viral message) of (an) integral membrane protein(s) that catalyze(s) the replication of the viral genetic material via a presumed single- or double stranded RNA intermediate into a negative sense complimentary RNA that itself will become the template for progeny RNA. The progeny RNA in turn provides the templates for translational activities. These are necessary for the production of other gene products such as the transport protein that is needed for systemic invasion. Translational activities and activities such as virion assembly are often temporally and spatially separated. Translational strategies used for the expression of viral functions may vary greatly between viruses and when combined with the different transcriptional strategies provide for the wide array of genomic strategies that are so characteristic for the different virus groups.

The recombination events occurring when polymerase progeny template complexes dislodge from the replication complex and reinitiate at some later time with other possible templates provide a mechanism to include evolutionarily advantageous sequences in viruses. Virus evolution has shown clearly that incorporation of basic functional units is a means to improve evolutionary niche fit for RNA viruses.

The reason for our concern with plant virus engineered products are: i. plants carrying virus gene sequences that confer virus resistance are among the products of bioengineering that are closest to release, ii. available evidence suggests that *in vivo* recombination of plant viruses is considered a mechanism active in virus gene reassortment and evolution, and iii. the evolutionary reality of plant-virus and virus-virus relationships goes beyond our wildest dreams. The convoluted genetic relationships of the luteoviruses and the dependently transmitted umbraviruses is a case in point.

Research results from animal viruses, as well as from plant viruses indicate that recombination between viral RNAs occurs at relatively high frequencies during replication. Template switching (copy choice) seems to be generally favoured as the mechanism driving the recombination of (+)-RNA viruses.

Although transgenic plants circumvent many of the pitfalls of wholesale protective inoculation (cross protection), it can be argued that when viral gene transgenic plants become infected with related or unrelated viruses, recombination may occur between the transgenic mRNA and the replicating viral RNA. There are currently five instances of this scenario that have been described since the publication of a letter to the editor of Phytopathology written in 1991. Notwithstanding this there are colleagues who question whether viral recombination in plants is a valid subject for risk assessment or not.

Transcapsidation and phenotypic mixing should, but will not be covered here, since *sensu stricto* they do not represent recombination. They do, however, represent important *in planta* viral product exchanges with significant ecological consequences.

The work of Greene and Allison (1994) has unequivocally shown that the study of *in planta* recombination is one of the most important subjects for risk assessment, specifically because of the high frequencies of recombination (3%) in the system that they studied. Furthermore, it provides us with the rational to engineer plants such that the frequency of recombination is reduced to a point that it might lose its importance in the risk assessment that we do.

When we look at the questions that need to be addressed we may have agreements and/or disagreements about some or maybe all the arguments brought forth by the interested parties but it often seems that the disagreements leave the taste of being self-serving. If there is one thing that we must get across it is the fact that nobody is a bystander and that self-serving arguments do not further the cause. The cause is the safe use of biotechnology as it is applied in plant agriculture, in case I neglected to mention it. Let me provide you here with some of the facts, some of the fancy and our resolve.

**Facts**

1. Plus sense RNA virus evolution appears to proceed in part through the exchange of functional modules between related and possibly unrelated viruses.
2. The exchange can only be affected when the exchanging entities share a host i.e. mixed infections.
3. Evolution in higher organisms proceeds mainly along gradual lines and not normally by the exchange of functional modules as in (+)-RNA viruses.
4. Recombination occurs between replicating virus templates in a host.
5. Recombination between the transgenic message and replicating virus occurs in plants transgenic for viral nucleic acid squences.
6. The propensity of some viruses to generate DI (defective interfering)-RNA, satellite RNA and various chimeric RNAs suggests that the degree of infidelity in replication among different virus groups may differ (Tobamo viruses versus Carmo viruses) and may, therefore, influence gene recruitment from transgenic plants.
7. Viral genes can be multifunctional. Coat protein for instance has been shown to influence a range of viral functions; a. vector transmission; b. vector specificity; c. systemic invasion; d. symptom expression, and e. RNA protection to name a few.
8. Transcapsidation occurs in mixed infections and has been demonstrated to occur in Cp transgenic plants.

**Fancy**

1. There is no difference in the gene transfer affected by classical plant breeding and the transfer affected by recombinant technologies. The corollary to this is that since we mostly lack detailed functional knowledge of genes transferred in classical breeding and proceed with the process anyway, we do not need such knowledge when recombinant DNA technologies are used. The argument is for several reasons fallacious. The degree of randomness of the insertional events in classical breeding, where chromosome pairing is a requisite, differs considerably from that of genetic engineering. Gene inactivation akin to transposon mutagenesis as a result of random insertions can easily engender unexpected consequences.
2. Anything that may happen in transgenic recruitment into viruses should have happened already during evolution in mixed infections.
   The basis for this statement is not underpinned by research results. Rather, for simplicity's sake it is argued that the presentation for recombination of the transgenic messenger RNA produced in the nucleus to viral RNA present in replication complexes is similar to the presentation (to each other) of viral RNA produced in replication complexes in a mixed infection. The evidence although peripheral, does not support this contention. This proposition also suggests that as of this moment in time we have seen everything evolution has to offer.
3. Recombinant viruses in transgenic plants will be at a major selective disadvantage and will therefore be eliminated.
   In light of the fact that niche fit is a major determinant of evolutionary success of an organism, who is to say when in the process of trial and error a successful combination may occur.

It is the frequency of recombination that counts, and that should be one of the major thrusts of risk assessment.

4. Detailed information on the functional properties of viral sequences used in plant genetic engineering is necessary but often not available.
   The involvement of certain plant viral sequences in vector transmission and specificity (luteovirus, potyvirus, enamovirus) makes this an important consideration in virus resistance engineering, and argues for continued strong support for basic virus research. The latter is even more cogent when it is realized that building biological containment into transgenic plants and possibly into the transgenes will only be possible when the functions and interactions of the various viral genes are understood.
5. Detailed information on replication infidelity and recombinant potential is important information often not available for the virus genes or virus sequences that are used for bioengineering.
   The questions to be asked then are what is the frequency of such events, and by what means can their occurrence or their frequencies be limited. It is arguable that not enough data on gene flow *in planta* among viruses or their genetic modules is currently available and that such data would be useful, may be even necessary, in assessing the qualitative aspects in the risk equation. The failure to demonstrate pleiotropic effects of engineered sequences in laboratory test plants does not necessarily mean that such effects could not occur in non-target plants at some end point in the theoretical gene flow. Although this is a possibility, we might want to down play its importance in risk assessment, since it seems to be a rather untestable proposition.
6. "The amounts of Cp and transgenically expressed (+)-RNA in plants is so low that statistically the chance for recombination or transcapsidation is orders of magnitude less than in mixed infections."
   Although this might be true, it does not necessarily mean safe. Again this emphasizes that frequency is the important component of risk assessment. Furthermore, we do not know how how much Cp needs to be produced for transcapsidation to occur, nor do we know how much transcapsidation needs to occur for it to result in vector transmission or specificity changes. Unless estimates are based on facts statistical arguments are conjecture.
7. The chances for recombination to occur in large monocultures of viral sequence transgenic plants are the same as those in mixed infections in similarly sized monocultures of nonengineered plants.
   If double infection is defined as the sustenance of the production of functional modules of two or more different viruses in a plant, then all plants becoming virus infected in a viral gene transgenic monoculture are doubly infected. In monocultures of nonengineered plants, only a fraction of singly infected plants will become doubly infected. The chances (frequency of occurrence) of having a recombinant event in a large monoculture of transgenic plants are therefore inherently greater than in similar monocultures of nontransformed plants.

**Our resolve**

When science addresses the societal concerns then application of new technologies brings leadership with a social conscience and public education becomes the issue.

1. Those involved in plant biotechnology must lead the risk assessment.
2. Leadership can only be exerted if risk assessment decisions are made on the basis of research data.
3. There is a paucity of risk assessment data; this can only be remedied by additional research.

4. Small-scale environmental release permits are given when and if containment levels are optimized. Extrapolation from the results obtained in this manner are inherently flawed since release was prevented from occurring.
   This paradox is the greatest difficulty in risk assessment. The question will be whether experiments can be designed that will lead us in an unequivocal manner out of this impasse, or whether we will be relegated to experiments that can only address partial solutions leading to inferences; and whether that evidence is mild enough to proceed with environmental releases. For this purpose the following two considerations should be satisfied.
5. The quality of the data collected in risk assessment and the conclusions based on these studies should reflect the best scientific methods. The data should be accessible to both science and industry so that guiding principles can be derived and practiced.
   We may yet solve our common problems, if we keep reminding ourselves that:
6. In science, no body of tenets or speculation held by any group can outweigh scientifically gathered data in the decision making process.

**Commerce**

It is important for the success of our new technologies that commerce can take place. It is, therefore, important that we apply the same standards of safety for commerce in engineered materials as we do in commerce of other products. It remains important, however, to follow the NAS (1987) promulgated standard that it is the product and its environmental release that triggers regulation and not the manner in which the product was obtained if such regulation is necessitated by scientific and plant health considerations. It seems that quarantine services are the vehicle that can prevent introductions of exotic genes in geographic areas where they do not naturally occur.
For the sake of our discussion I will make a statement here that might be construed to be controversial: No potatoes engineered for PVYN-Cp derived resistance should be imported into the USA nor should Sharkavirus-Cp derived resistant plum trees be imported in the USA at this time.

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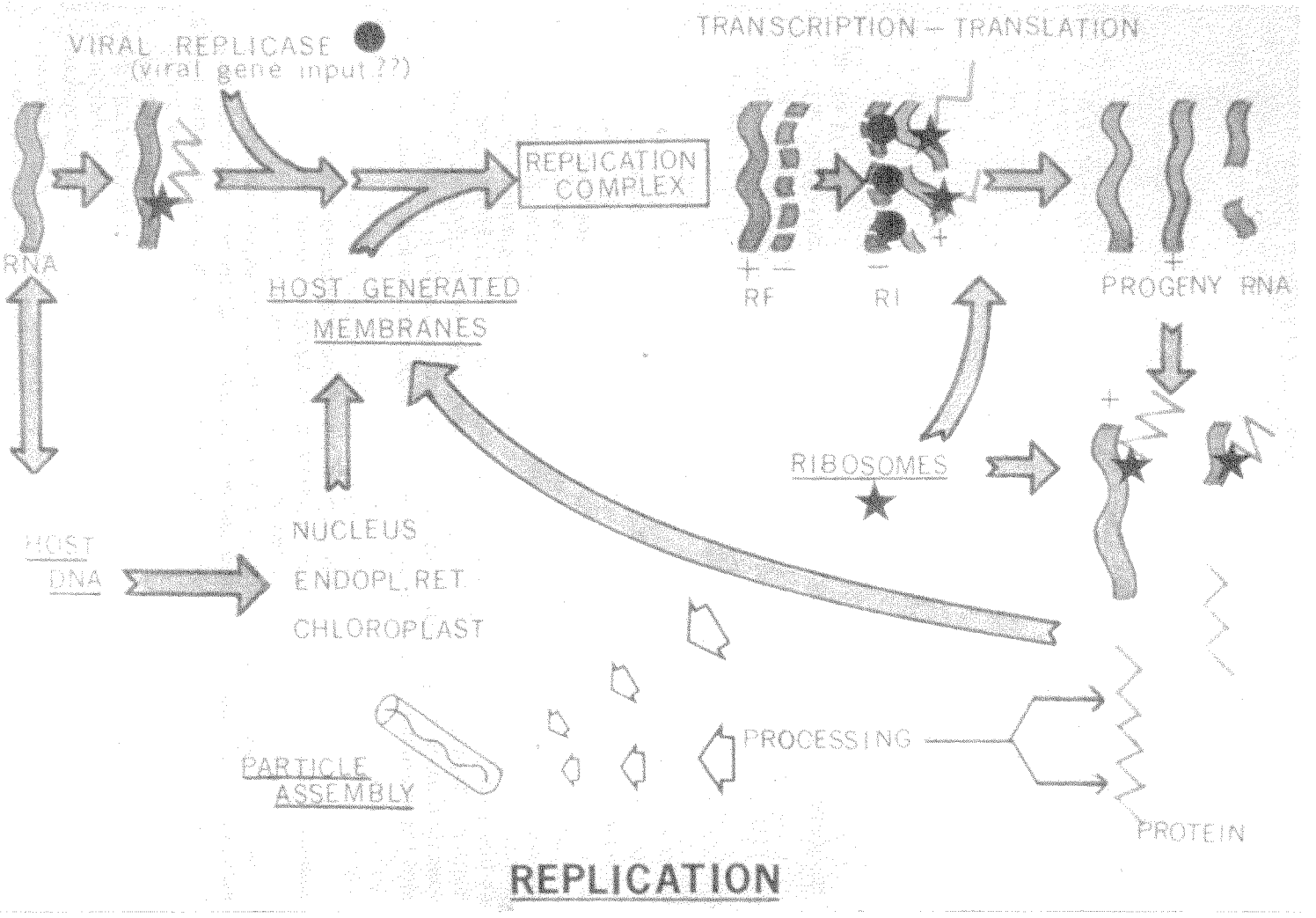
VIRAL REPLICASE
(viral gene input ??)
TRANSCRIPTION — TRANSLATION
REPLICATION COMPLEX
RNA
HOST GENERATED MEMBRANES
RF
RI
PROGENY RNA
RIBOSOMES
HOST DNA
NUCLEUS
ENDOPL. RET.
CHLOROPLAST
PARTICLE ASSEMBLY
PROCESSING
PROTEIN
REPLICATION

Figure 1

# Release of viral transgenic plants to the environment: prospects and problems

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## Introduction

Recent advances in the understanding of the molecular mechanisms of how viruses function and how they interact with plants have led to the development of non-conventional approaches to protection of plants against viruses. Many of these approaches, giving what is sometimes termed pathogen-derived protection, involve the introduction of viral or virus-based sequences into the plant's genome. Expression of these sequences then interferes with one or more of the viral functions, thus giving some protection against the virus. This topic has attracted considerable attention and has been reviewed several times previously (Beachy, 1988; Baulcombe, 1989; van den Elzen *et al.*, 1989; Gadani *et al.*, 1990; Hull, 1990a, 1990b, 1994; Loesch-Fries, 1990; Mayo and Barker, 1990; Joshi and Joshi, 1991; Harms, 1992; Hull and Davies, 1992; Wilson, 1993; Fitchen and Beachy 1993; several papers in the volume edited by Beachy, 1993; Wilson and Davies, 1994). The viral genes most frequently used to provide protection are those for the coat protein, the viral replicase and the cell-to-cell movement protein (see above reviews for details). Among those also being considered as targets are insect transmission factors and replication and expression control sequences. In the case of coat protein, protection is often given by the unmodified gene product. However, most other gene products are used in a form modified to affect normal functioning. There is increasing evidence that in some situations it is the expressed RNA and not the gene product which gives the protection.

Many of these transgenic plant lines, and especially those expressing coat protein, have reached the stage of field testing for the efficacy of protection and are even being more generally field released. This raises the question of possible risks which could arise on general field release, a topic which has previously been discussed by Hull (1990a; 1990b; 1994), de Zoeten (1991), Hull and Davies (1992) and Tepfer (1993). In spite of these discussions the issue has not fully been resolved and various other aspects are being raised. This paper is intended to air some of these issues in the context of general field release and to discuss some of the ways by which potential risks could be recognised and circumvented.

## Potential risks on field release

The two main areas of concern in general for most transgenic plants are possible adverse effects of the expression of the transgene on animals feeding on the plant and spread of the transgene into wild species. There is no evidence that the expression of plant viral sequences could be detrimental to man or animals feeding on transgenic plants; in fact, virus-infected plants have been eaten for millennia. Furthermore, the lack of recognised allergy problems due to pollen from virus-infected plants indicates that the expression of viral transgenes is unlikely to cause any problems in this area either. The spread of transgenes, and especially herbicide resistance, into wild species has focused mainly on expression enhancing the weediness of the wild species. Several points arise here in relation to transgenes giving virus resistance. Firstly, as with other types of transgenes, the wild species must be sexually compatible with the transgenic crop species for the transgene to be transferred. Secondly, it is unlikely that conferring virus resistance on a wild species will make it any more of a weed problem than it was already. Thirdly, relatively little is known about the ecological effects of virus infection on wild plant communities. That wild plant communities are virus reservoirs is well established but it is unknown if virus resistance will influence the competition between species. The movement of a resistance transgene into wild species

could be beneficial in that it would reduce or eliminate the virus reservoir. Finally, there would be no difference in effects between a natural viral resistance gene and a transgene.

The area of concern specific to viral transgenes is the potential risks on any interactions between the viral or virus-related sequences being expressed from the transgene and another virus superinfecting that plant. There are three main scenarios which are usually considered, heteroencapsidation, synergism and recombination.

**Heteroencapsidation**

This involves the superinfection of a plant expressing the coat protein of a virus, say virus A, by an unrelated virus B. Heteroencapsidation is the encapsidation of the genome of virus B by the coat protein of A thereby conferring on virus B properties of virus A. There are several examples of heteroencapsidation in transgenic plants both between viruses of the same group (Farinelli *et al.*, 1992; Lecoq *et al.*, 1993) and between unrelated viruses (Candelier-Harvey and Hull, 1993). The main property of coat protein that is considered is that of vector transmission characteristics. However, there is increasing evidence that coat proteins are involved in long distance viral movement around infected plants and heteroencapsidation could enhance the movement of a superinfecting virus which normally did not move systemically.

The discussion of heteroencapsidation has focused on superinfecting viruses. However, there is the possibility that heteroencapsidation of retrotransposons could present a problem. Retrotransposons are a major class of transposable elements whose structure resembles the integrated copies of retroviruses and which are considered to be important in evolution (see White *et al.*, 1994). The *Ty1-copia* group of retrotransposons is widespread in plant genomes (Flavell *et al.*, 1992; Voytas *et al.*, 1992; Hirochika and Hirochika, 1993) and it has been suggested that there might be horizontal transmission between species (Flavell *et al.*, 1992). Sequencing has shown that most copies of the *Ty1-copia* retrotransposons in plants are mutated so they would not be active. However, several active ones capable of retrotransposition have been described (Johns *et al.*, 1985; Grandbastien *et al.*, 1989; Varagona *et al.*, 1992; Hirochika, 1993; White *et al.*, 1994) and presumably replicate, as do all retrotransposable elements, via RNA. Among the factors which activate plant retrotransposons is tissue culture, a process involved in transformation (Hirochika, 1993). This raises the possibility that introduction of the coat protein transgene could activate retrotransposon RNA which becomes heteroencapsidated and transmitted to other species.

**Synergism**

The possible synergistic effect of a viral transgene on a superinfecting virus can have two manifestations. It could enhance the symptoms of the superinfecting virus. Such synergism between viruses is well known, for instance between potato virus X (PVX) and various potyviruses in tobacco and tomato (tomato streak). The recent report (Vance *et al.*, 1995) demonstrating that there was a synergistic effect of the expression of the 5' proximal sequence of tobacco vein mottling potyvirus as a transgene on infection with PVX highlights that this problem has to be kept in mind. An alternative synergistic effect is that the expression of the transgene could mobilize a superinfecting virus which normally would be localized to the site of infection (subliminal infection).

**Recombination**

Three sorts of recombination have been recognised (Lai, 1992), homologous with cross overs between related RNAs at precisely matched sites, aberrant homologous with cross overs between related RNAs not at corresponding sites and non-homologous with cross overs between unrelated RNAs at non-corresponding sites. There is considerable evidence for extensive recombination in RNA viruses (see Lai, 1992; Simon and Bujarski, 1994 for details) and probably all three mechanisms have been involved at one time or another. It is generally

considered that recombination plays an important role in the evolution of RNA viruses (see Goldbach and Wellink, 1988; Strauss and Strauss, 1991; Lai, 1993; Simon and Bujarski, 1994). Evidence is now forthcoming of recombination between superinfecting viral RNA and RNA expressed from a transgene (Greene and Allison 1994) through the aberrant homologous recombination mechanism. The finding of recognisable host RNA sequences within viral RNAs (Mayo and Jolly, 1991; Sano *et al.*, 1992) is suggestive of non-homologous recombination. In fact, one of the more generally accepted theories of virus evolution is that viruses comprise basic replicons to which are 'attached' cassettes of genes which adapt them to specific situations (see Strauss *et al.*, 1990; Rybicki, 1990; Hull, 1992). Several points in relation to the risks of recombination in field released transgenic plants are raised by these observations. Recombination can lead to an increase in relative 'fitness' of a virus (Fernández-Cuartero *et al.*, 1994) which presumably is one of the driving forces in virus evolution. However, little or nothing is known about the detailed molecular mechanisms of RNA-RNA recombination, especially of non-homologous recombination. As well as molecular mechanisms it is also important to have information on cellular location and whether there are any differences in recombination between two RNAs replicating in the cytoplasm and between one in the cytoplasm and one expressed in the nucleus.

**Risk assessment**

The above discussion has focused on possible risks of field release of viral transgenic plants. What is now needed is an assessment of these potential risks. Risk assessment has three aspects, hazard identification, risk estimation and risk evaluation. These terms are well defined in risk assessment and it is important to understand the definitions. A hazard is "a situation that could occur during the lifetime of a product, system or plant that has potential for human injury, damage to property, damage to the environment, or economic loss" (British Standard 4778, 1991, quoted in Warner, 1992). Risk is "the probability that a particular adverse event occurs during a stated period of time, or results from a particular challenge" (Warner, 1992).

Risk estimation includes, a) the estimation of the magnitude of the specified undesirable event and b) the estimation of the probability that the specified event will occur. In the evaluation of the estimated level of risk one has to answer the following questions: a) Is the estimated risk significantly worse than the "natural" situation? If it is then: b) Does the benefit from the perceived risk outweigh the losses from it? and c) Can the perceived risk be reduced or controlled? The really important question is whether there is any significant increase in risk over what happens in the non-transgenic situation. Thus, one has to have an understanding of the "base-line" situation to be able to assess if the field release of transgenic plants is significantly increasing the risk.

**Risk reduction and control**

It is likely that it will take some time for a full risk assessment on the viral transgenic plants to be performed and commercial and other pressures will be very strong for field release. There are two approaches to risk reduction and control which can be put into effect relatively soon. One is to use biological containment (Hull, 1994). In this approach the region(s) of the transgene giving the undesirable properties are deleted while retaining those which give the desirable protection property. A good example of this approach is found in the potyviral coat protein which has an amino acid triplet (asp, ala, gly; DAG) which is involved in the interactions with the aphid vector (Harrison and Robinson, 1988; Atreya *et al.*, 1990; 1991). Mutations of this motif, or its removal (which does not affect the protection ability of the coat protein; Lindbo and Dougherty, 1992) would render heteroencapsidation with the transgene unable to confer aphid transmissibility on the superinfecting virus. Much more difficult is to avoid recombination, but targetted research on this may reveal methods.

The second approach is to design methods for monitoring the effects of field release. For small-scale releases it is relatively easy to design monitoring procedures for analyzing pollen flow into related weeds and for detecting heteroencapsidants or recombinants. This will be much more difficult, if not impossible, for large-scale releases where the approach will be to educate farmers and extension service personnel to identify any unusual events which might be associated with the transgenic plants. This will be the challenge for the future.

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## New strategies for construction of virus resistant transgenic plants

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The following two models for virus-resistant transgenic plants constructing will be discussed in my talk.
First is the "suicide model": the virus-triggered induction of virus subgenomic promoter for toxic protein production by virus-infected cells of transgenic plant. And the second is the "movement-protein model": production of the nonfunctional movement protein (MP) in transgenic plants.

I. The general idea was to develop a strategy for expression of foreign genes in virus-infected plants by constructing transgenic plants producing constitutively the chimeric minus-sense RNA containing viral sgPr in minus polarity at the 3`-proximal position and a certain toxic gene (also in minus-polarity) at the 5`-proximal position.

In our work we used sgPr of CP gene of PVX (the region of PVX RNA located upstream of CP gene and 22 amino acids of CP) and this sgPr was combined with the gene coding for the A-fragment (toxoid) of diphtheria toxin (DT) which blocks translation.
It was expected that after inoculation of transgenic plants of this type, the PVX-specific RNA replicase will appear which will recognize *in trans* sgPr, producing thereby mRNA for DT. After translation of DT RNA induced by PVX infection the toxin will block or inhibit cellular translation and in the extreme (and the best) case it will kill the primarily infected cells and localize the viral infection. Because of this reason we named this approach "suicide model".
Then the tobacco plants were made, transgenic for the construct carrying (in antisense orientation) the A-toxin gene (proximal to the 35S promoter) and sgPr of CP PVX.
Then the susceptibility of transgenics to PVX was examined hoping that even very low reaction could be detected. In these experiments the apical section of the leaves was inoculated with PVX and then the time-course accumulation of the virus was followed. It was found that accumulation of PVX in inoculated leaf was reduced (in comparison to control plants) and that translocation of PVX to noninoculated leaves was blocked in transgenic plants. It should be emphasized that at least one of these transgenic lines showed very unusual reaction on PVX inoculation: the inoculated section of the leaf yellowed and then necrotizated in about 10 days after inoculation and after that the necrosis developed along the edge of the leaf and penetrated internal regions of noninoculated sections. If the whole leaf was inoculated, it yellowed and dropped.

II. The second strategy for virus-resistant plants constructing is based on the inhibition of virus cell-to-cell movement.
It is clear now that particular virus-specific proteins, namely the transport or movement proteins (TP or MP) are responsible for the translocation of viral genome from the infected into healthy cells.
We presumed that production in transgenic plants of the MP which contains only part of functionally active domains will confer these plants a resistance to wild-type virus. It could be expected that such MPs will perform only some but not all of the steps involved in TF expression which will result in competition between the functional WT-virus-coded MP and "nonfunctional" MP produced by transgenic plants.
Transgenic plants producing the 30K temperature-sensitive (ts) transport protein (MP) tobacco mosaic virus (TMV) mutant Ni2519 (affecting cell-to-cell transport) were found to:
(i) be susceptible to wild-type TMV U1 at 24°C (a permissive temperature, for Ni2519 MP);

(ii) acquire a certain level of resistance to TMV U1 accumulation when maintained at 33° C (a non-permissive temperature for Ni2519 MP) and (iii) lost the resistance to wild-type TMV after their retransfer from 33° C to 24°C. It is suggested that reversible temperature-dependent conformational changes in Ni2519 MP are responsible for these phenomena and that production of a MP which is only partially functional in transgenic plants confers on these plants a resistance to virus owing to reduction of the level of cell-to-cell transport. Transgenic tobacco plants producing the 32K MP of brome mosaic virus (BMV) acquired a resistance to TMV U1 suggesting that BMV MP is partially functional in tobacco plants.

# Heterologous encapsidation of potyviruses in mixed infections and in transgenic *Nicotiana benthamiana* expressing the coat protein gene of plum pox potyvirus (PPV)

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## Summary

The incidence of heterologous encapsidations in mixed infections as well as in transgenic plants expressing the coat protein of plum pox potyvirus (PPV) was studied. In non-transgenic *Nicotiana benthamiana* plants which were doubly infected with PPV and potato Y potyvirus (PVY) or bean common mosaic potyvirus (peanut stripe potyvirus, BCMV), up to 7.2% or 15.8%, respectively, of the newly formed particles contained a mixture of both coat proteins indicating that heterologous encapsidations occurs to a certain frequency in mixed infections.
In greenhouse experiments transgenic *Nicotiana benthamiana* plants expressing a modified coat protein of the aphid transmissible PPV isolate PPV-AT were inoculated with various potyviruses and with viruses from other groups (tobacco mosaic tobamovirus (TMV), potato X potexvirus (PVX), and BNYVV). By means of immunogold electron microscopy PPV coat protein was reliably detected in 42.5% to 100% of the newly formed potyvirus particles, but not in the particles of the other viruses.

## Introduction

During the last years numerous plant virus genomes have been cloned and fully sequenced. The expression of plant virus genes in transgenic plants has enabled the establishment of entirely new forms of resistance, the so called pathogen-derived resistances, which are apparently due to an interference of viral gene products (Sanford and Johnston, 1985) or due to the transcription of the viral nucleic acid in the transformed cell without an expression of the viral proteins (Dougherty et al., 1994). Powell Abel et al. (1986) were the first to show that transgenic tobacco plants expressing the TMV coat protein gene show a high degree of resistance to TMV infection. Meanwhile coat protein genes of nearly all economically important viruses have been used to transform plants and to generate coat protein mediated resistances. This very successful strategy has, however, also raised concerns about the possibility of recombinations and heterologous encapsidations in transgenic plants (e.g., de Zoeten, 1991) and it has become increasingly clear that thorough investigations on potential ecological effects are necessary before transgenic plants are cultivated at a large scale. In the present study the incidence of heterologous encapsidations in mixed infections and in transgenic plants which express the coat protein of plum pox potyvirus (PPV) was followed.

The genomic RNAs or DNAs of plant viruses are normally encapsidated by their own coat protein which protects the genome against enzymatic degradation and in certain virus species has additional functions, e.g., in replication (Zuidema et al. 1983), movement (Wellink and van Kammen, 1989; Dolja et al. 1994) or vector transmission (McLean et al. 1994). Heterologous encapsidations, i.e., encapsidations of viral nucleic acids by coat proteins of other viruses, have been observed in mixed infections, especially with luteoviruses. They can lead to changes in the vector specificity of a virus (Rochow, 1970; Rochow, 1982; Creamer and Falk, 1990; Wen and Lister, 1991).

It has been shown that the viral coat proteins expressed in transgenic plants can also be used for heterologous encapsidations of viral genomes (Osbourn et al. 1990;

Farinelli et al. 1992; Candelier-Harvey and Hull, 1993). A transmission-deficient strain of a potyvirus has been shown to become aphid-transmissible in a transgenic plant supplying the coat protein of an aphid-transmissible potyvirus (Lecoq et al. 1993).

**Materials and methods**

*Nicotiana benthamiana* Domin have been transformed with a modified coat protein gene of an aphid-transmissible isolate of PPV-AT (Timpe et al. 1992) under control of the CaMV 35S promoter. The homozygous plant line 27/4 was selected for greenhouse experiments.

Virus-specific polyclonal antisera were produced by immunizing rabbits with purified virus particles or purified PPV coat protein expressed in *E. coli*. In addition the mouse monoclonal antibodies 3C8 specific for PVY and 4G12 specific for BCMV (peanut stripe potyvirus) were used to determine the heterologous encapsidation in mixed infections with PPV. For visualizing particles with mixed coat proteins, immunoelectron microscopy with gold-labeled goat anti-rabbit or goat anti-mouse antibodies as second antibodies was used. 100-500 virus particles were evaluated on the incidence of heterologous encapsidation.

**Results**

Experiments were done in order to check whether heterologous encapsidations occur in non-transgenic plants doubly infected with two potyviruses. *N. benthamiana* plants were either inoculated with PPV and PVY separately on different leaves or with a mixture of both viruses on the same leaf. Systemically infected leaves were harvested and checked two weeks after inoculation for the presence of virus particles reacting simultaneously with antibodies to both viruses. The highest percentage of particles with a mixed coat (7.2%) was found in plants which had been inoculated with a mixture of PPV and PVY on the same leaf. Considerably lower percentages of mixed encapsidations (0.3 and 1.3%) were observed when the two viruses were inoculated separately on different leaves. In these cases, the virus which has been inoculated to the younger leaf of a plant was found predominantly in the systemically infected leaves, probably due to a more rapid multiplication in the younger leaf. In addition *N. benthamiana* plants were doubly inoculated with PPV and BCMV. Up to 15.8% of the newly formed particles from systemic infected leaves reacted simultaneously with the antibodies specific to PPV and BCMV, also indicating that mixed encapsidation has occurred.

*N. benthamiana* plants expressing the coat protein of the aphid-transmissible isolate PPV-AT were first checked for their resistance to different potyviruses. Coat protein-mediated protection was only observed against infection with PPV isolates, but not against infections with any other virus. After inoculation with PPV the first 3 to 5 leaves of the transgenic plants developed symptoms identical to those shown by non-transgenic plants. However, the leaves developing later, i.e., 3 - 6 weeks after inoculation, showed no symptoms, whereas control plants displayed typical PPV symptoms. No PPV was detected by several ELISA procedures in the symptomless leaves which had developed in the later stages of the transgenic plants. A similar phenomenon of "recovery" has been reported by Lindbo et al. (1993). Recently Dougherty et al. (1994) suggested a mechanism for this type of resistance which involves a targeted elimination of RNA by the cell.

The particles of different viruses, which had been inoculated to PPV coat protein-expressing *N. benthamiana* were further studied by immunoelectron microscopy using PPV-specific antibodies in the labeling test. All tested potyviruses were able to acquire PPV coat protein from the transgenic plant (Table 1.). The percentage of potyvirus particles which were labeled ranged from 42.5% - 100%, depending on the virus species. The amount of PPV-specific antibodies sticking to the heterologously encapsidated

particles also varied with different virus species. No heterologous encapsidations were observed with viruses other than potyviruses (Table 1).

**Table 1**: Percentage of virus particles labeled with PPV-specific antibodies after propagation in non-transgenic *Nicotiana benthaminana* and in *N. benthamiana* expressing the coat protein gene of PPV-AT

| Genus | Virus | Non transgenic | 27/4 Transgenic* |
|---|---|---|---|
| Potyvirus | Potato Virus Y ($PVY^O$) | 12.0 | 93.6 |
| | Potato Virus Y ($PVY^N$) | 2.0 | 76.6 |
| | Potato Virus Y ($PVY^C$) | 26.7 | 100 |
| | Chilli Veinal Mottle Virus (ChVMV) | 12.9 | 98.4 |
| | Bidens Mottle Virus (BiMoV) | 14.6 | 83.1 |
| | Peanut Stripe Virus (PStV) | 2.5 | 65.5 |
| | Beet Mosaic Virus (BeMV) | 1.7 | 42.5 |
| | Tobacco Vein Mottling Virus (TVMV) | 76.7** | 96.7 |
| Potexvirus | Potato Virus X (PVX) | 2.0*** | 9.2*** |
| Tobamovirus | Tobacco Mosaic Virus (TMV) | 4.2*** | 8.0*** |
| Furovirus ? | Beet Necrotic Yellow Vein Virus (BNYVV) | 2.5*** | 12.0*** |

* Percentage of particles labeled with PPV-antiserum

** Strong cross reaction of PPV-antiserum with TVMV

*** Differences between transgenic and non-transgenic plants were statistically not significant. Values representing non-specific levels of labeling

**Discussion**

One of the concerns in using coat protein-mediated resistance to protect plants is the possibility of heterologous encapsidations of the genomes of viruses multiplying in transgenic plants with the foreign viral coat proteins which are expressed in these plants. Heterologous encapsidation is not an event which is restricted to transgenic plants. As it has been demonstrated for luteoviruses (Rochow, 1970; Rochow, 1982; Creamer and Falk, 1990; Wen and Lister, 1991) and also for potyviruses (Bourdin and Lecoq, 1991; this paper) heterologous encapsidations occur also in non-transgenic plants in mixed infections.

The encapsidation of a viral genome with coat protein requires the folding of the coat protein in a manner which is specific for each virus group and, in addition, apparently distinct sites on the viral RNA and their specific recognition by the coat protein (Zimmern, 1977). Therefore, heterologous encapsidations are most likely to occur with coat proteins from more or less closely related members of the same taxonomic virus group, as in the case of luteo- or potyviruses. It remains to be

shown whether heterologous encapsidations are equally frequent with viruses in other taxonomic groups. Evidence for heterologous encapsidation events between two non-related viruses has been reported by Candelier-Harvey and Hull (1993).

Heterologous encapsidations leading to an alteration of the vector specificity of a virus could possibly result in the transmission of the heteroencapsidated virus to a host that is not infected in nature. This would normally be a 'dead end' event, since in the newly infected non-transgenic host the foreign coat protein used for heterologous encapsidation would no longer be available. However, a large-scale propagation of different transgenic plants expressing various viral coat protein genes might change the situation. In this case the transmission of a virus carrying a foreign protein coat may no longer be a 'dead end' event and the epidemiological consequences are difficult to predict. A careful examination of the likelihood of heterologous encapsidations to occur should therefore be made at an early state of experimentation when the establishment of coat protein-mediated resistance is envisaged. As shown by the experiments, the likelihood for heteroencapsidations to occur may be very different for different viruses.

To circumvent potential problems connected with the use of coat protein-mediated resistance several alternative strategies are already available. The transmission of potyviruses by aphids depends on the presence of a specific amino acid motif (DAG) close to the N-terminus of the coat protein (Atreyea et al. 1991; Gal-On et al. 1992; Maiss et al. 1993). Amino acid exchanges in this motif lead to a deficiency in aphid transmission. The expression of coat proteins from non-aphid transmissible virus strains or the destruction of this motif will not influence heterologous encapsidation but will make aphid transmission impossible.

The expression of coat proteins modified in other ways can also confer resistance to virus infection to a plant, e.g. of truncated coat proteins, which lack the N-terminus, including the motif for aphid transmission (Lindbo et al. 1993). Moreover, even the expression of an untranslatable mRNA of a coat protein gene (Lindbo and Dougherty, 1992) or a coat protein gene antisense RNA (Kawchuk et al. 1991) have been reported to make plants resistant to viruses. In both cases, a foreign coat protein will no longer be expressed in the transgenic plants. Expression of viral genes coding for other proteins, for instance for viral polymerases can also confer resistance to virus infection (Golembowski et al., 1990; Braun and Hemenway, 1992; Anderson et al., 1992; Carr and Zaitlin, 1993).

The above mentioned alternatives to a coat protein-mediated resistance are nevertheless all pathogen-derived resistances for which the possibility of a recombination between the transgene and a viral nucleic acid cannot be entirely excluded (Schoelz and Wintermantel, 1993; Green and Allison, 1994). Such events could be avoided when either antibody genes (Tavladoraki et al. 1993) or natural resistance genes are transferred to plants and expressed. Unfortunately for most viruses such genes have so far not been characterized in detail.

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# Attempts to detect heteroencapsidations or other non-intended side effects in transgenic sugarbeet expressing the coat protein gene of beet necrotic yellow vein virus (BNYVV)

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**Summary**
No indications were found for a possible occurrence of heteroencapsidations or other non-intended side effects in sugarbeet expressing the coat protein gene of beet necrotic yellow vein virus (BNYVV). This might be due to the very isolated taxonomic position of BNYVV in the plant virus kingdom.

Coat protein-mediated and other forms of pathogen-derived resistances (Sanford and Johnston, 1985) have proved to be among the most promising approaches for the protection of plants against virus infections (for review see Wilson et al., 1993). However, some unintended side effects, such as heteroencapsidations (Lecoq et al., 1993; Maiss et al., 1995), viral genome recombinations (Greene and Allison, 1994) and possibly an increased spread of other viruses in the transgenic plants (Fig. 1), have raised concerns that this form of resistance may in certain cases present a biological risk.

In Germany, transgenic sugarbeets expressing the coat protein gene of beet necrotic yellow vein virus (BNYVV) have recently been released in small field trials. BNYVV is the causal agent of sugarbeet rizomania which is one of the economically most important virus diseases in Europe. Attempts to control this disease by cultural or chemical measures have more or less failed (Asher, 1993). More successful were the attempts of several breeding companies which have provided a number of tolerant or even (partially) resistant varieties. Under high infection pressure, however, the tolerance of these varieties may break down. Thus the search for new sources of resistance is continuing, and polygenic resistances interfering with the viral propagation cycle at several sites seem to be the most promising.

In order to find out whether BNYYV coat protein-mediated resistance in sugarbeet is likely to present a risk, we are presently checking whether the unintended side-effects outlined in Fig. 1 can be detected in the above mentioned field release trials and also in transgenic beets which have been artificially inoculated with other sugarbeet viruses in the greenhouse. The methods we have used for detecting possibly occurring heteroencapsidations and genome recombinations are outlined in Figs. 2 and 3.

The results of our attempts to detect heteroencapsidations by means of immunoelectron microscopy are summarized in Table 1. In field grown plants we have observed only natural infections with beet mosaic potyvirus, and at very rare occasions with beet soil-borne furovirus (BSBV). None of the virus particles showed any signs of heteroencapsidations. In order to increase the likelihood to detect heteroencapsidations we have also inoculated BNYVV coat protein-expressing sugarbeet with several other viruses in greenhouse experiments. All inoculations except with BSBV were successful. Also in the artificially inoculated plants we did not observe any heteroencapsidations. In a limited number of PCR experiments which are to be continued in the near future we also failed to detect recombinations of the BNYVV coat protein gene with the genomes of other

viruses. The low rate of field infections and our failure to infect beets with BSBV mechanically or by means of infected *Polymyxa betae* in greenhouse experiments suggested that the transgenic plants do not have an increased susceptibility for BSBV or other viruses.

Our inability to detect heteroencapsidations cannot be due to the method used. With the same method and equipment we readily detected heteroencapsidations of various potyvirus genomes with the coat protein of plum pox potyvirus expressed in transgenic *N.benthamiana* (Maiss et al., 1995). Genomes of viruses belonging to other taxonomic groups were not heteroencapsidated by plum pox virus coat protein.

The reason why we did not observe heteroencapsidations or any of the other unintended side effects outlined in Fig. 1 is possibly the taxonomically very isolated position of BNYVV which has tentatively been assigned to the furoviruses but differs from these viruses and all other viruses in many properties. Typical furoviruses have a bi- or tripartite genome whereas the BNYVV genome consists of four or sometimes five RNA species which greatly differ in size from those of the typical furoviruses. Also, BNYVV RNAs are polyadenylated in contrast to those of the typical furoviruses. Nucleotide sequence comparisons (Koonin and Dolja, 1993; Ward, 1993) place BNYVV far aside from any other plant viruses (Table 2).

Some properties which differentiate BNYVV from other viruses commonly infecting sugarbeet are listed in Table 2. Most of these viruses have a very different particle morphology and a different size of the coat protein. Beet mild yellowing virus and tobacco necrosis virus have isometric particles, beet mosaic and beet yellows virus have filamentous particles. Only BSBV and tobacco mosaic virus (TMV) have a similar gross morphology, but at least for TMV it is known that the fine structure of the particles is rather different, e.g. TMV contains 16 1/3 coat protein subunits per one turn of the nucleic acid helix, whereas BNYVV contains only 12 1/2 coat protein subunits per turn of the nucleic acid helix (Steven et al., 1981).

The main concern with heteroencapsidated viruses is that they may acquire new vector and host specificities, because coat protein is involved in the determination of the aphid and host specificities of some viruses (e.g. Lecoq et al., 1993). The question arises, if - despite of our failures to detect it - heteroencapsidation with BNYVV coat protein would occur at rare occasions, could this make other viruses transmissible by *Polymyxa betae*, and would this present a biological risk? This seems to be very unlikely. The most important other sugarbeet viruses are either already transmissible by *P. betae* (*BSBV*), or they have very efficient aphid vectors (yellowing viruses, *beet mosaic virus*) (Table 2). BNYVV coat protein alone does not mediate the transmission of BNYVV. Virus mutants which have an intact coat protein but show deletions in the 3'-terminal part of the 75K readthrough protein gene are not transmitted by *P. betae* (Tamada and Kusume, 1991). This part of the genome is not present in the transgenic plants which have been released. -Thus, even if heteroencapsidations with BNYVV coat protein would occur at rare occasions, it seems most unlikely that they would confer *Polymyxa*-transmissibility to another virus.

There are a number of alternatives to pathogen derived resistance. The most attractive approach would be the incorporaration of natural resistance genes from immune plant species into the sugarbeet genome. However, so far we do not know anything about the genes which are responsible for such immunities. An approach which is in easier reach is the expression of the reactive parts of antibodies (e.g. single chain fragments, scFv) or

other substances which would specifically block the entry, multiplication or spread of a virus in transformed plants (Tavladoraki et al., 1993; Voss et al., 1994). Such approaches are presently followed for BNYVV in our group by L. Fecker, U. Commandeur and J. Reither who have been able to express the reactive parts of antibodies specific for the coat protein and the 25K protein genes in transgenic *Nicotiana benthamiana* (Fecker et al., 1995). Such approaches are certainly much more difficult to realize than the pathogen-derived resistance, but if any, even hypothetical, risks can be avoided, the extra effort may well be worthwhile.

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**Table 1** - Unsuccessful attempts to detect heteroencapsidations in BNYVV coat protein-expressing sugarbeet by means of immunogold electron microscopy

| Virus | Number of viewing fields checked on grids precoated with a) homologous antiserum b) BNYVV antiserum | Total number of particles detected | Number of particles showing gold-labelling after treatment with BNYVV antiserum |
|---|---|---|---|
| A) Natural infections in a field release trial | | | |
| Beet mosaic virus | a) 1100<br>b) 1100 | a) 1656<br>b) 140 | a) 0<br>b) 0 |
| Beet soil-borne virus | a) 13400<br>b) 6700 | a) 10<br>b) 3 | a) 0<br>b) 0 |
| B) Experimental infections in a growth chamber | | | |
| Beet mild yellowing virus | a) 250<br>b) 250 | a) 320<br>b) 5 | a) 0<br>b) 0 |
| Tobacco necrosis virus | a) 250<br>b) 250 | a) 500<br>b) 14 | a) 0<br>b) 0 |
| Beet yellows virus | a) 250<br>b) 250 | a) 94<br>b) 10 | a) 0<br>b) 0 |
| Tobacco mosaic virus | a) 250<br>b) 250 | a) c.2500<br>b) c.20 | a) 0<br>b) 0 |
| Beet soil-borne virus | a) 250<br>b) 250 | a) 0<br>b) 0 | a) 0<br>b) 0 |

**Table 2** - Comparison of some properties of beet necrotic yellow vein virus with those of other viruses infecting sugarbeet

| Virus | Particle Morphology | Coat protein molecular weight (KD) | Transmission | Classification according to Koonin et Dolja (1993) | |
|---|---|---|---|---|---|
| | | | | Class | Order |
| Beet mosaic potyvirus | filamentous | c. 35 | aphids | I | Poty-virales |
| Beet mild yellowing luteovirus | isometric | c. 25 | aphids | I | Sobemo-virales |
| Tobacco necrosis necrovirus | isometric | 28 | fungus (surface Olpidium) | II? | Carmo-virales? |
| Beet yellows closterovirus | filamentous | c. 22 | aphids | III | Tobamo-virales |
| Tobacco mosaic tobamovirus | rodshaped | 17.8 | mechanical | III | Tobamo-virales |
| Beet soil-borne furovirus | rodshaped | 19? | fungus (inside Polymyxa betae) | III | Tobamo-virales |
| *Beet necrotic yellow vein (furo?)virus* | *rodshaped* | *21* | *fungus (inside Polymyxa betae)* | *III* | *Rubi-virales* |

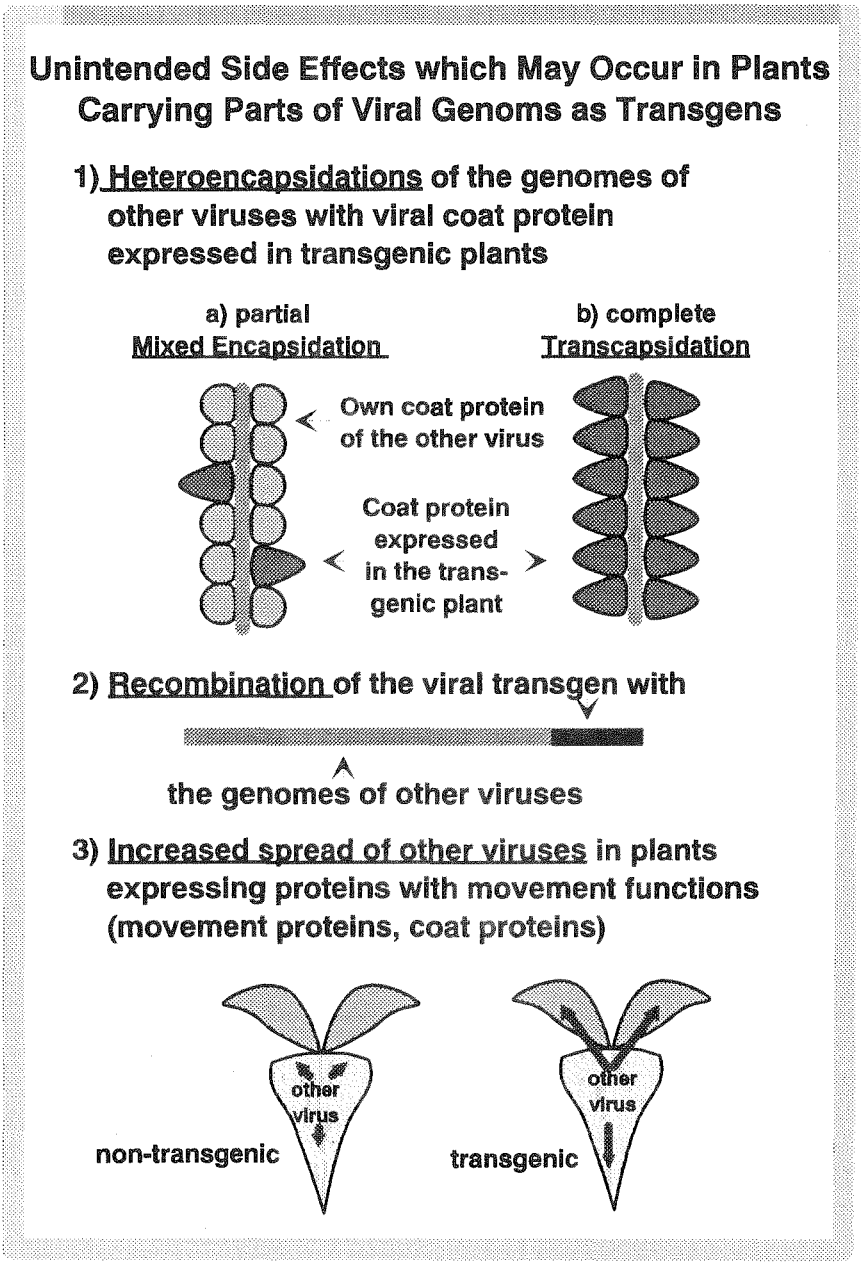
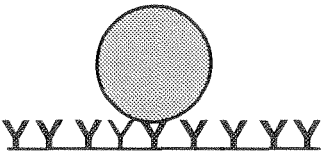

Unintended Side Effects which May Occur in Plants Carrying Parts of Viral Genoms as Transgens
1) Heteroencapsidations of the genomes of other viruses with viral coat protein expressed in transgenic plants
a) partial
Mixed Encapsidation
b) complete
Transcapsidation
Own coat protein of the other virus
Coat protein expressed in the trans-genic plant
2) Recombination of the viral transgen with
the genomes of other viruses
3) Increased spread of other viruses in plants expressing proteins with movement functions (movement proteins, coat proteins)
other virus
non-transgenic
other virus
transgenic

Figure 1

## Detection of Possibly Occurring Heteroencapsidations in BNYVV Coat Protein-Expressing Sugarbeet by Means of Immunogold Electron Microscopy

1) Trapping of virus particles on grids which have been precoated with antisera either to BNYVV or to other sugarbeet viruses *(ISEM)*



2) Identification of BNYVV coat protein in the trapped virus particles by means of BNYVV-specific antibodies and gold-labelled anti-rabbit antibodies

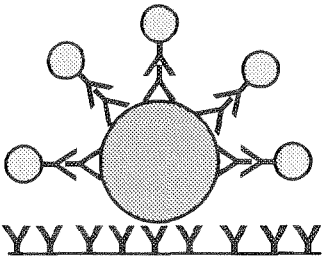
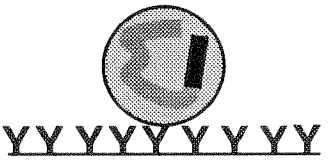


Figure 2

## Detection of Possibly Occurring Genome Recombinations in BNYVV Coat Protein-Expressing Sugarbeet by Means of Immunocapture PCR

1) Trapping of virus particles in Eppendorf tubes which have been precoated with antisera either to BNYVV or to other sugarbeet viruses



2) Detection of the BNYVV coat protein gene in the trapped virus particles by means of PCR - If this test is positive, the integration of the BNYVV coat protein gene into the genome of other viruses has to be proved by means of nucleotide sequencing

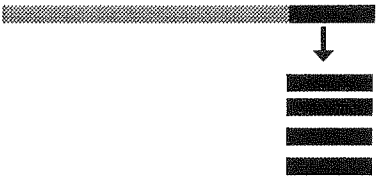


Figure 3

# Outcrossing of herbicide tolerance genes : how realistic are worst case scenarios?

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## Outcrossing - an aspect of risk assessment of the introduction of genetically modified plants

In a report prepared by the Group of National Experts on Safety in Biotechnology of the OECD, a number of issues were identified that could give rise to safety concerns when upscaling genetically modified plants (OECD 1993). "Gene transfer" headed this list including other topics such as "weediness", "trait effects", "genetic variability", "vector effects" and "worker safety". Within the scope of that document, gene transfer was focussed on movements between sexually compatible species.
As a prerequisite of the precautionary approach to environmental impacts, it is necessary to develop an understanding of the many interactions between a plant and the environment. It is clear that such an assessment cannot be limited to the introduced plant alone, but should include potential derivatives or - in a more biological sense - progeny containing the new information. It is therefore important to picture the genetic relationship and exchange possibilities between crop related species.

## Successful outcrossing involves different steps

In reviewing some arguments on the impact of genetically modified plants, it is clear that there are major confusions on the complicated process of outcrossing. In many cases pollen movement or the production of interspecific F1 hybrids are mistakenly proposed as proof of the potential for outcrossing and subsequent introgression in a wild species. While not paying too much attention to the intricate complexity of the different steps, we can at least summarize 3 major aspects :

1) Physical aspects
It may seem trivial, but the basis for any exchange between two species is that they are present and separated by a distance that is not superior to what can be covered by the pollination vector. Presence has to be interpreted in terms of space and time, and in fact even more detailed in terms of period of flowering, pollen shed, etc... Pollination vectors can be physical and/or biological (e.g. pollinating insects). In both cases weather conditions are known to influence the success of pollen movement, either by affecting the quality of pollen or the behavior of the pollinators.

2) Genetic aspects
In this aspect we deal with all the mechanisms of compatibility between species. Cases have been documented where pollen fails to germinate on the alien stigma, where pollen tube formation is arrested, where embryos are aborted, where seeds fail to germinate, etc. In general it can be said that, even if interspecific hybridization occurs, the frequency and success is much lower than the normal intraspecific fecundation. Genetic aspects do not stop at the F1 hybrid seed production. In many cases, interspecific hybrids

have been proven to be sterile, providing a dead end in an introgression scenario. Again, in few cases which revealed fertility, the frequency of seed (and progeny) production was much lower than on either wild type parent.

3) Ecological aspects
Both parental species are adapted to the environment where they are growing either as a crop or as a wild species. Interspecific hybrids - often characterized by intermediate and/or crippled phenotypes - enter into competition at any stage with the parental material. In the agronomic environment they are subject to the same management practices that are implemented. In semi-managed and non-managed environments, they have to compete with the fully adapted wild species. This competitive success will depend on the relative fitness of the interspecific hybrids, including any possible advantage introduced by the transgenes, the relative size of the transgenic population and the frequency of repeated introduction of the hybrids and the genes of interest.

In relation to risk assessment, it is important to complete the puzzle of fragmented information and realize that the distinction between species is maintained through a number of mechanisms combining these different aspects.

**The outcrossing steps have been documented for major crops**

None of the aspects discussed so far made specific reference to the transgenic nature of the plants. While highlighting the complexity of the mechanisms underlying the successful exchange of genes between species, it becomes clear that all these mechanisms are naturally occurring. They are part of the biology of the plant. Therefore, we can rely on our knowledge and practical experience in order to prepare for an assessment. This approach was followed in the OECD baseline review of "traditional crop breeding practices" (OECD 1993), which aimed at providing a background for appraising the biosafety of new technical evolutions in plant breeding.
Incorporating a survey of the State Herbarium in the Netherlands, "Botanical files" for 42 cultivated plant species were produced giving information on the cultivated plant, the occurrence of wild relatives, compatibility and observations of escapes from the field to nature (de Vries et al., 1992). Another example, that incorporates many aspects of published information on compatibility and occurrence, is a study on opportunities for gene transfer from transgenic oilseed rape to related species (Scheffler & Dale, 1994).
While these reviews combined the different aspects based on available information, other studies have tackled detailed questions. Two types of research can be recognized :

- Does outcrossing of transgenes introduce new issues ?
The overall approach was based on the similarity between interactions of transgenic crops and the non-transformed "counterpart". Some research focussed on further confirming that transgenes are inherited in a similar fashion and that - once introduced in a species - they can span the genetic relationship of that species. This type of approach has been adapted in some biosafety research programs. Crosses between potato and two related wild species (black nightshade and bittersweet) confirmed that gene flow from potato to its common wild relatives is highly unlikely in Europe (Eijlander and Stiekema, 1994). Another example involves the establishment of spontaneous hybridization between oilseed rape and weedy *Brassica campestris* (Jørgensen & Andersen, 1994).

- Can we use transgenes to document specific cases of outcrossing ?
  With the availability of easy markers - especially some herbicide tolerances - experiments were designed looking in more detail into undocumented outcrossing and into behavior of hybrids. Detailed analysis of possibilities for outcrossing of *Brassica napus* to a number of important weeds (*Raphanus raphanistrum, Brassica adpressa* & *Sinapis arvensis*) was performed by the breeding group at the INRA - Rennes (Chèvre et al., 1995). While the demonstration of outcrossing was supported by genetic markers and cytological studies, the introduced herbicide tolerance proved to be an efficient way in screening for some of the rare outcrossing events.
  Other research addressed the fate of the hybrids and their relative competitive performance upon crosses within the species (Fredshavn et al., 1994) and between species (eg. Lefol et al., 1993).

**What can we learn from worst case scenarios?**

The idea of worst case evaluation shows up in two aspects. The entire idea of risk assessment has to be seen as an *a priori* exercise to identify potential problems. When a potential problem is identified, one can then evaluate the need for appropriate management techniques to reduce the potential impact to an acceptable level. Because of the predictive, theoretical basis of this exercise, one tends to overrate the risk possibilities. Use of such worst cases is valid, but needs timely balancing based on data and experience in order to proceed to a realistic scenario.

Similarly it has to be recognized that most of the research on outcrossing relies on sophisticated design in order to demonstrate the extremely low frequencies. The researchers investigating the fate of the interspecific hybrids of potato included different approaches in identifying the mechanism that prevents the successful establishment of such hybrids. In the experiments on oilseed rape outcrossing, experiments included several types of enhancements such as ovary culture, embryo rescue, prevention of self-pollination through male sterility and self-incompatibility, growing the wild and cultivated parent in high density and in close vicinity, etc.

The results of such "worst case" help us in defining the scope of possible interactions. Yet, in view of the complexity of aspects encountered in natural situations, any determination of frequency should be regarded as an overestimation.

**There are no specific environmental problems with outcrossing of herbicide tolerance genes**

Several debates have focussed on the development of crops tolerant to a specific herbicide. While it may be a valuable topic to question the future of agriculture and the way we handle agrochemical protection of crops, no direct effect of the herbicide tolerance genes on the environment could be identified. Herbicide tolerance genes, in specific phosphinotricin tolerance and to some extent glyphosate tolerance, have been incorporated in all major biosafety research programs. They provided a suitable research system, including aspects of outcrossing. No specific issues could be revealed for these genes.

Surely the genes provide a competitive advantage when the weed control product is applied, but that is actually the intent of this development. The evaluation of the potential impact therefore seems to focus on the management of volunteers and of wild relatives having achieved the tolerance through outcrossing. The debate on the latter is sometimes confused, again, by a discussion on frequency instead of on impact.

We have now arrived at the stage of the first commercial introductions of transgenic crops, some of them incorporating herbicide tolerances. Through worst case scenarios we have pictured the framework of possible interactions in a natural gene pool. Using other worst

case scenarios on the movement of the introduced genes through the natural gene pool, no negative consequences for the environment could be identified.

**Literature**

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# Transgenic herbicide resistant crops : Problems relating to weed resistance

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Herbicide resistance can be genetically engineered by several principles:
(1) an endogenous plant gene encoding the target enzyme can be modified (mutated) causing amino acid exchange(s) which may be made at the substrate-reaction site or at the regulatory site (in case of an allosteric enzyme). Examples are the D-1 protein of the photosynthetic electron transport or the acetolactate synthase (ALS). All typical photosynthesis inhibitors bind within a 60 amino-acid stretch of the D-1 protein of photosystem II and apparently compete with the redoxactive plastoquinone. Inhibitors of acetolactate synthase bind at or close to its regulatory site.
Approach no. (2) is the introduction of a foreign (e.g. bacterial) gene into the plant genome whose gene product is insensitive to a herbicide. The enzymes produced by both the endogenous plant gene and the newly introduced one have the same function. This case is exemplified by the phytoene desaturase from the bacterium *Erwinia* spec., which contains the inventory of the carotenoid biosynthesis pathway. Its phytoene desaturase, is about 1000-fold (and more) resistant against common bleaching herbicides like norflurazon, diflufenican or fluridone as compared to the plant enzyme.
(3) A "metabolic" gene, often of bacterial origin, may be cloned, thereby allowing expression of a new enzyme in the plant catalyzing a reaction to convert a herbicide into a phytotoxically inactive analogue. The N-acetylation of glufosinate or de-nitrilation of bromoxynil are prominent examples.
Further possibilities to achieve herbicide resistance are feasible by (4) changing the transport of a xenobiotic through the plant cell membrane if a carrier is involved (as shown for paraquat), or (5) facilitating sequestration into the vacuole (as was evidenced for diclofop-type compounds). Approach no. (6) is the overexpression of the target enzyme, so that excess enzyme can titrate away the herbicide present. For (4) and (5) no genes are available at the moment, procedure no. (6) has not resulted in viable plants as yet.

Resistance relating to items 1, 2, 3 has been studied intensively in the last 10-12 years. Mutagenesis of target genes at their substrate or reaction-coding site generally, although not obligatory, leads to an altered enzyme which shows resistance to the target herbicide. Mostly the enzyme exhibits a poor catalytic performance (increased $K_m$ for phosphoenol-5-enol-pyruvylshikimate-3-phosphate synthase, EPSPS), or impaired photosynthetic electron flow through the D-1 protein. In both cases results a yield penalty of the resistant crop. On the other hand alteration of amino acids at the regulatory site of e.g. acetolactate synthase, leads to resistance against (certain) sulfonylureas or/and imidazolinones but preserves a functionally competent enzyme. Accordingly, a yield penalty of such transgenic (or mutated) crop is not apparent.

In essence any weed population will eventually produce resistant individuals against a herbicide, provided the selection pressure exerted by herbicide treatment targetting the same enzyme is high and lasts long enough to screen out the naturally occurring resistant weed mutants in the field. This possibility is evident when target genes are present and operative both in the crop and in the weed. A gene transfer by cross-pollination from herbicide-resistant crops to a taxonomically related weed species may be possible, making the progeny herbicide-resistant. The wild beet (*Beta maritima)* or red rice (*Oryza*

*sativa)* are such examples, but there are only a few more. It should be reminded that also genes of crops derived from conventional breeding may be spread into weed biotopes. Nobody is worried about that. The major driving force leading to resistance problems is the selection of herbicide-resistant weeds in fields cultivated with the same (transgenic) crop and treated with complementary herbicides affecting the same (single) target over the years.

The distribution of resistant biotypes depends on several factors:
(1) on the initial frequency of naturally occurring resistant mutants in the weed population of the field at the start of the herbicide treatment, (2) the selection pressure due to the kill efficiency of the herbicide, (3) the fitness of the herbicide-resistant mutants, namely their competition *vs.* the wild-type relating to growth and reproduction, (4) on the average life span of the seeds in the soil, and (5) the years of uninterrupted herbicide treatment of the particular field. Cross-pollination and a dominant nuclear encoded "resistance" gene will greatly increase the spread of insensitive weeds. Good examples are *Kochia scoparia* in the northern Midwest of the U.S. or *Lolium rigidum* in southern Australia.

A problem in weed management is lack of crop rotation together with a long-term repetitive application of herbicides targetting one single plant enzyme. The couple corn-atrazine may be mentioned as an example. Using transgenic crops this situation may get worse if a particular herbicide resistance is genetically engineered into several major crops. It is anticipated that widespread use of herbicide-resistant crop plants almost certainly will be accompanied by a reduced number of complementary herbicides available. Development of transgenic high-yield crop varieties by seed companies is a time-consuming and costly process which will be undertaken only for a limited number of future-oriented herbicides. Such herbicides should be beneficial to environment and warm-blooded animals, should exhibit moderate persistence, no leaching and last not least perform a broad-spectrum weed control with low use rates in post- emergent application. These prospects represent the great advantage of future resistant (transgenic) crops.

Agricultural use of these crops requires a stringent weed control management. A "herbicide rotation", i.e. application of chemicals with different enzyme targets is mandatory. This can be achieved by either offering crop varieties tolerant to different herbicides, or by cloning e.g. two resistance genes into one variety. Then the probability to develop weed resistance is markedly reduced. If the occurrence of a mutation for one gene is e.g. $1{:}10^8$, the mutation for two genes in *one* individual has a chance of $1{:}10^8 \times 10^8 = 1{:}10^{16}$. Also the search for and use of herbicides capable to inhibit *two* relevant enzymes is an attractive alternative. The use of transition-state inhibitors (substrate analogues) will markedly minimize the appearance of weed resistance. Mutations at the substrate-binding site in most cases yield low-fitness biotypes or are lethal. Examples are glyphosate and glufosinate of which no weed resistance has been reported as yet. This is in contrast to ALS inhibitors whose uninterrupted application led to appearance of resistant biotypes within a few years. This depends mostly on binding of the inhibitors to the regulatory enzyme site. At such a site amino-acid exchanges (often) preserve a properly active enzyme ensuring high fitness of the resistant biotype. Furthermore, in the big ALS-gene many mutations of different amino acids are possible, all leading to resistance. Both effects cause a high number of naturally resistant biotypes present in the weed population before any selection pressure has been impacted by herbicide application.

Resistance produced by amino-acid exchanges of the target enzyme generally leads to cross-resistance. That means resistance may show up for herbicides of competing

companies. The probability of cross-resistance, however, is negligible when dealing with transition-state inhibitors, or when resistance is achieved by a specific enzymic metabolic step. Altogether the introduction of a *new foreign* gene (generally derived from bacteria) which is instrumental for crop resistance and is not an obligatory part of the plant genome is considered as the most promising approach to obtain herbicide-resistant crops (see items 2 and 3 at the start of this article). A possible weed resistance requires transfer of that gene into weed individuals either from the resistant crop or possibly from soil bacteria previous to a subsequent selection. Such a gene transfer is unlikely, and the necessary selection process in the field will take an unrealistic long time.

Details and **references** on the problems addressed can be found in P. Böger, "Mögliche pflanzenphysiologische Veränderungen in herbizidresistenten und transgenen Pflanzen durch den Kontakt mit Komplementärherbiziden", Verfahren zur Technikfolgen-abschätzung des Anbaus von Kulturpflanzen mit gentechnisch erzeugter Herbizidresistenz, Vol. 2, 1994, W. v.d. Daele, A. Pühler, H. Sukopp, editors, Publication of the Dept. Normbildung und Umwelt, Wissenschaftszentrum Berlin für Sozialforschung (WZB), Berlin.

# Volunteer management of Glufosinate resistant transgenic crops (maize, soybeans, oil seed rape, sugar beets)

**Jürgen Cremer**, Ernst Rasche, Günter Donn, Hoechst Schering AgrEvo GmbH

**Table 1**: Volunteer potential in other crops

| **crop** | **in rotation with : (most important crops)** | **probability for volunteer control** | **remarks/ survival potential of seeds in the soil** |
|---|---|---|---|
| **oil seed rape** | cereals, sugar beets, maize, potatoes, legumes, forage crops, sunflowers, flax, other crops and fallow | high | > 3 years, but 3 years after harvest negligible |
| **maize** | cereals, soybeans, oil seed rape, sugarbeets, sunflowers, legumes, forage crops, cotton, potatoes, other crops and fallow | zero in the North, medium in the South | > 3 years, 3 years after harvest negligible |
| **soybeans** | maize, cotton, rice, cereals, sugar beets, potatoes, forage crops, other crops and fallow | medium to high | > 3 years, 3 years after harvest negligible |
| **sugar beets for sugar production** | cereals, maize, oil seed rape, legumes, forage crops, flax, sunflowers, vegetables, other crops and fallow | very low | 2 years (max.), 2 years after harvest negligible |

**Table 2::** Possibility of volunteer control in other crops

| **volunteers:** ⇒ | **oil seed rape** | **maize** | **soybeans** | **sugar beets** |
|---|---|---|---|---|
| **control in:**⇓ | for example: | for example: | for example: | for example: |
| **cereals** | reg. hormons and SU`s | fenoxaprop + safener or diclofop | reg. hormons and SU`s | reg. hormons and SU`s |
| **oil seed rape** | - | all reg. fop´s and dim`s | not applicable | trifluralin or metazachlor |
| **maize** | 2,4- D, dicamba and reg. SU´s | - | 2,4- D, dicamba and reg. SU´s | 2,4- D , dicamba and reg. SU´s |
| **sugar beets** | metamitron or triflusulfuron | all reg. fop´s and dim`s | metamitron or triflusulfuron | - |
| **potatoes** | rimsulfuron or metribuzin | all reg. fop´s and dim`s | rimsulfuron or prosulfocarb | flurochloridon or metribuzin |
| **soybeans** | not applicable | all reg. fop´s and dim`s | - | imazethapyr |
| **sunflowers** | flurochloridon | all reg. fop´s and dim`s | flurochloridon | flurochloridon |
| **cotton** | not applicable | all reg. fop´s and dim`s | cyanazin + MSMA | not applicable |
| **rice** | not applicable | not applicable | MCPA | not applicable |
| **legumes** | some hormons or pendimethalin | all reg. fop´s and dim`s | some hormons | some hormons or pendimethalin |
| **flax** | MCPA or some SU´s | all reg. fop´s and dim`s | MCPA or some SU´s | MCPA or some SU´s |
| **forage crops** | not applicable | not applicable | not applicable | not applicable |
| **fallow** | mulching or cutting | mulching or cutting | mulching or cutting | mulching or cutting |

**Evaluation of the importance; ranking and comments**

**Oil seed rape (OSR) control in following crops**:

greatest importance as volunteer because of high seed production and normally high seed losses during the harvest. In general there are good volunteer management practices used by all farmers (special soil cultivation(s)) anyway:

**1. cereals** (mostly 1st year after OSR only) - no problems in control of OSR with postemergence herbicides; 2nd or 3rd year after OSR too (Tab. 2) .

**2. fallow**: until max. 2 years after OSR strong observation on emerging or flowering OSR; avoid formation of **first** pods by mulching or cutting the plant canopy; chemical fallow in North America with Glyphosate or hormons.

**3. sugar beets**: common rotation very seldom; never before the 2nd year after OSR; problems can occur until the 3rd year after OSR; if it occurs, a very good control is possible by postemergence application(s) - (Tab. 2).

**4. maize, potatoes and other arable crops**: only until the 3rd year after OSR of importance; no problems in control of OSR with a.m. herbicides (Tab. 2).

**5. arable forage crops**: without object because the crop will be cut and harvested before the first pods of OSR are formed.

**Soybean control in:**

A volunteer management will normally be practised in rotations with maize (corn belt in North America).

**1. maize**: until the 3rd year after soybeans no problems in control of soybeans with a.m. postemergence herbicides (2,4-D, dicamba, SU´s).

**2. cotton**: worse survival chances of soybean seeds in the South, but no problems in soybean control with a.m. herbicides (Tab. 2).

**3. rice and fallow**: no problems in soybean control with a.m. herbicides or chemical fallow (see OSR and Tab. 2).

**Maize control in:**

In northern regions where maize is grown as forage crop no volunteer problems can occur. A volunteer management exists in the corn belt. In Southern Europe the probability for volunteer control is very low.

**1. soybeans and all other dicot crops:** no problems in maize control with all registered fop´s and dim´s.

**2. cereals:** in spite of less importance maize can be controlled by fenoxaprop + safener - products wich are registered in cereals.

**3. fallow:** mulching or cutting or chemical fallow with Glyphosate or fop´s and dim´s.

**Sugar beet control in:**

Normally there is an extremely limited number of plants which shoot and produce seeds because the crop is a biennial one. The life time of seeds in the soil is also very limited and volunteer plants in the following year will also grow only vegetatively.

**1. cereals**: no problems in sugar beet control with a.m. herbicides (Tab. 2) .

**2. all other crops and fallow**: no problems in sugar beet control with a.m. herbicides (Tab. 2) .

The volunteer problem in rotational crops is as old as rotation itself.
In the modern agriculture, the control of volunteers is an integrated part of weed control in general, that means that the control of volunteers does not differ basically from the control of weeds.
There are some special methods to control volunteers in certain regions worldwide.
The best measurements to prevent a volunteer problem are mainly:
- a clean harvest
- suitable soil cultivation methods
- suitable rotations.

The control of volunteer transgenic crops in other conventional crops does not create any new problems and does not differ from normal volunteer control.

**Conclusions**

- The control of volunteer transgenic crops in conventional crops does not create any new problems and does not differ from normal volunteer control.
- The volunteer control can be done by herbicide applications (Tab. 2) and by mechanical or other measures.
- The control of Glufosinate resistant transgenic crops as volunteers in other Glufosinate resistant transgenic crops is possible by using alternative herbicides (Tab. 2).
- Glufosinate resistant transgenic crops do not have any selection advantage (better survival chances) compared with „normal" crops, unless Glufosinate will be used.

# Studies on the phosphionothricin acetyltransferase gene and protein

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## Abstract

The soil bacteria *Streptomyces viridochomogenes* and *S. hygroscopicus* synthesize glufosinate as a part of a natural compound (the tripeptide bialaphos). Glufosinate kills plants via inhibiting the ammonia fixation in green plant tissues as a competitive inhibitor of the enzyme glutamine synthetase.

The same soil bacteria contain a gene encoding an N-acetyl transferase that chemically modifies glufosinate, rendering it herbicidally inactive.

Glufosinate tolerant crops contain a natural and synthetic version of the phosphinothricin acetyl transferase (pat) gene which allows for a more environmentally sustainable approach to weed control not previously available in these crop plants.

The gene product is highly specific for glufosinate. Similar compounds like glutamate or all other proteinogenic amino acids are no substrate for the pat enzyme.

The concentration of the pat enzyme in transgenic crops is 0.1% or less in leaves and about 0.005% in the seeds. There is no pat expression observed in pollen.

Pat gene and protein are readily inactivated and digested in digestive fluids from different animals and in simulated human gastric fluids.

The pat protein has no homology to known allergenic or toxic peptides.

The new metabolite N-acetyl glufosinate is non toxic and rapidly degraded in soil. It is formed in soil by different species of bacteria. It is formed in the gut of animals via the normal detoxification pathway.

## Conclusions

Glufosinate tolerant crops allow for a more environmentally safe weed control. In addition to normal plants they contain a new gene and a new enzyme which leads to the formation of a new metabolite within these crops. All three compounds are known by nature. They are non toxic, non allergenic and are readily degraded in soil and in living organisms. From this glufosinate tolerant crops can be considered as environmentally safe.

# COMMUNITY IMPACT: EFFECTS ON MICROORGANISMS OR INSECTS

## Interaction of an insect tolerant maize with organisms in the ecosystem

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Insect damage to crops is responsible for substantial yield losses worldwide. The development of insect tolerant crops therefore represents an important and practical application of genetic engineering to plant breeding.
Ciba Seeds has developed maize tolerant to the European Corn Borer (ECB, *Ostrinia nubilalis*). The tolerance is due to the insertion of a synthetic, truncated version of a gene encoding the δ-endotoxin CryIA(b) from the bacterium *Bacillus thuringiensis* subsp. *kurstaki*, strain HD1. Endotoxins from *B. thuringiensis* are already used in many biological products to control insect pests. When ingested by insects, these endotoxins undergo cleavage, leading to activated proteins which bind to specific receptors present in the insect midgut. This brings about cell lysis and subsequent death of the insect by cessation of feeding. In addition, the ECB tolerant maize from Ciba Seeds contains a selectable marker, a phosphinothricin acetyltransferase from the bacterium *Streptomyces hygroscopicus*, conferring tolerance to phosphinothricin, the active moiety of glufosinate ammonium herbicide [1].

### Field evaluation of ECB tolerant maize

The production of the CryIA(b) protein in ECB tolerant maize is tissue specific, with preferential expression in green tissues and in pollen. The level of the CryIA(b) protein varies during the plant life cycle, with the highest amount detected at anthesis, about 1.5 µg CryIA(b)/gfw in leaves and 1.8 µg CryIA(b)/gfw in pollen (mean values of numerous determinations).
The survival of ECB larvae on ECB tolerant maize is greatly reduced. Extensive field evaluations with artificial infestations showed rapid mortality of the larvae, accompanied by a significant decrease in damage to the plants [1, 2]. Under strong insect infestation, yield losses are highly reduced on ECB tolerant maize compared to control maize (maize plants lacking the protection mechanism), whereas yields are similar in the absence of infestation [3].

### Specificity of the insecticidal protein expressed by ECB tolerant maize

One question raised by the development of ECB tolerant maize concerns the specificity of the insecticidal protein. Although the specificity of the native bacterial CryIA(b) protein is well documented [4], the specificity of the truncated form of the protein encoded by the plant was also verified. Three different types of studies were conducted, which are briefly outlined below.

1) *In vitro* comparison of the activity of native bacterial CryIA(b) and of CryIA(b) protein expressed by ECB tolerant maize

The susceptibility of neonate larvae of five lepidopterous insects to the native bacterial CrylA(b) protein and to the protein expressed by ECB tolerant maize were very similar and the ranking of the insects according to their susceptibility was identical (table 1). The somewhat higher activity observed with the plant protein was anticipated, as the protein expressed in maize is a truncated version of the native protein and therefore contains more active endotoxin per unit weight of protein.

**Table 1:** *In vitro* susceptibility of lepidopterous insects to the CrylA(b) protein

| | native bacterial CrylA(b): ($LC_{50}$ in ng/cm² of diet) [1)] | CrylA(b) from ECB tolerant maize: ($LC_{50}$ in ng/cm² of diet)[1)] |
|---|---|---|
| *Ostrinia nubilalis* | 24 | 4 |
| *Trichoplusia ni* | 765 | 75 |
| *Helicoverpa zea* | 978 | 187 |
| *Spodoptera frugiperda* | no mortality | no mortality |
| *Agrotis ipsilon* | no mortality | no mortality |

[1)]: the proteins were added to standard diets for each species and the $LC_{50}$ values determined (30 replicates).

These results indicate that the protein expressed by ECB tolerant maize possesses a similar activity as the native bacterial protein.

2) Field monitoring of the entomofauna present in ECB tolerant maize and control maize.
A field study conducted in Bloomington (Illinois, USA) in 1993 showed no difference in the kind and number of insects associated with ECB tolerant maize, compared to the entomofauna associated with control maize. The monitoring encompassed phytophagous and entomophagous insects, including beneficial predators and parasites, and was conducted weekly over a 10-week period. In contrast, treatment with a conventional insecticide, permethrin, showed a diminution of the coleopteran population following the treatments on all plots (ECB tolerant maize and control maize). Table 2 shows as an example the monitoring results obtained in early August.

**Table 2:** Entomofauna associated with ECB tolerant maize and control maize

| | Number of insects per trap[1)] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Chr | Coc | Oth | Dip | Thy | Hom | Hem | Hym |
| *untreated plots:* | | | | | | | | |
| ECB tolerant maize" | 178 | 4 | 31 | 44 | 2 | 51 | 6 | 15 |
| control maize 1[2)] | 142 | 3 | 35 | 46 | 1 | 47 | 4 | 15 |
| control maize 2[2)] | 163 | 2 | 33 | 31 | 1 | 45 | 6 | 20 |
| *treated plots:* | | | | | | | | |
| ECB tolerant maize | 24*[3)] | 0* | 14* | 34 | 2 | 44 | 3 | 12 |
| control maize 1[2)] | 27* | 0* | 13* | 31 | 1 | 38 | 2 | 12 |
| control maize 2[2)] | 26* | 0* | 15* | 31 | 1 | 34 | 3 | 15 |

[1)]: Chr = chrysomelidae (coleoptera); Coc = coccinellidae (coleoptera); Oth = other coleoptera; Dip = diptera; Thy = thysanoptera; Hom = homoptera; Hem = hemiptera; Hym = hymenoptera. The insects were collected using Scentry Multigard yellow sticky traps; 2 traps per plot and 6 plots per type of maize, from which 3 were treated with permethrin (Pounce) at 225 g/ha, on July 29 and August 23.

[2)]: "control maize 1" = negative segregants from ECB tolerant maize; "control maize 2" = wild type maize.

[3)]: values statistically different from the corresponding control values ($P<0.05$).

These results confirm the high specificity of ECB tolerant maize towards target pests, compared to what can be achieved using a conventional insecticide.

3) Toxicity studies on selected organisms with ECB tolerant maize
Several toxicity studies were conducted on selected organisms, from which two are presented below.

A in-hive test showed no effect of pollen from ECB tolerant maize on the development of honeybees (*Apis mellifera*). The survival of larvae and the emergence to adults were similar for honeybees receiving pollen from ECB tolerant maize or not treated, whereas honeybees treated with Carbaryl insecticide as a positive control showed high mortality (table 3). The cause for the slightly reduced emergence of honeybees treated with pollen from control maize was unclear but is likely to be due to differences in hive vigor or genetic variability.

**Table 3:** Development of honeybee larvae treated with pollen from ECB tolerant maize

| Treatment[1)] | Survival (%, days after treatment) | | |
|---|---|---|---|
| | 2 (larvae) | 9 (larvae) | 18 (adults) |
| pollen from ECB tolerant maize | 95 | 95 | 95 |
| pollen from control maize | 75 | 73 | 65 |
| Carbaryl | 11 | 5 | 4 |
| untreated | 100 | 99 | 96 |

[1)]: brood frames with young larvae were removed from the beehives, treated in the laboratory with pollen from ECB tolerant maize or from control maize, at the concentration of 1 mg in 1 drop of water per cell, and returned to the beehives after allowing time for the pollen to be consumed. Carbaryl, 200 ppm, was used as positive control (25 honeybees per hive, 4 hives per treatment).

Earthworms (*Eisenia foetida*) were selected as a second organism for a toxicity study. Extracts from leaves of ECB tolerant maize or from control maize showed no effects on the survival and development of earthworms during a 14-day toxicity study conducted in an artificial soil. All earthworms survived the end of the study, and weight gain during the test period was similar. The concentration of CryIA(b) protein used in the study (0.35 mg CryIA(b)/kg soil) represents a much higher concentration than that to which earthworms are likely to be exposed under field conditions. Calculations based on CryIA(b) concentration in ECB tolerant maize showed that the concentration used in the test is approximately 785 times higher than the expected concentration in the soil, when maize plants will be incorporated into the soil after harvest.
Additional toxicity studies carried out on various organisms supported the lack of toxicity of the CryIA(b) protein expressed by ECB tolerant maize to non-target organisms. A single study, conducted with a soil invertebrate, showed some potential activity of ECB tolerant maize. This does not represent a safety issue as the effect seen appeared only in a concentration range well above that present in ECB tolerant maize cultivated soils.

**Conclusion**
ECB tolerant maize developed by Ciba Seeds will represent a significant new method for control of ECB damage in maize, and has proven to be highly effective. Three kinds of tests were conducted to assess the specificity of the insecticidal protein expressed in ECB tolerant maize: *in vitro* dietary tests with selected lepidopterans, field monitoring of insect

populations, and toxicity studies on selected organisms. All data available today support the safe use of the Ciba Seeds' ECB tolerant maize within the ecosystem.

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# Developments in engineering baculovirus insecticides and concepts of biosafety

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## Introduction

Baculoviruses are viral pathogens, that cause fatal diseases in insects, mainly in members of the families Lepidoptera, Diptera, Hymentoptera and Coleoptera. The more than 600 baculovirus isolates (Family: *Baculoviridae*) have been categorized in two groups: the *Eubaculovirinae* comprising the nuclear polyhedrosis viruses and the granulosis viruses, and the *Nudibaculovirinae* (Murphy *et al.*, 1995). Baculoviruses are highly specific for insects and can cause epizootics which reduce the size of insect populations in nature. These viruses are therefore, recognized as attractive biological control agents of insect pests in agriculture and forestry as alternative to chemical insecticides. Baculoviruses have been successfully used in the control of a variety of pest insects, including velvetbean caterpillar, codling moth, pine beauty moth, cotton bollworm, Douglas fir tussock moth, beet armyworm, fall army worm and many other species, on all continents.

The major limitation to a more wide-spread use of baculoviruses as insect control agents is their relatively slow speed of action, in particular in crops with low damage thresholds. Upon infection insects stop feeding only after a few days, whereas immediate insecticidal effect is often required. In this respect *Bacillus thuringiensis* toxins have a clear advantage over viruses as biological insecticide. With genetic engineering it has now become possible to generate baculoviruses with improved insecticidal properties, including increased speed of action (Wood and Granados, 1991; Vlak, 1993a,b; Miller, 1995; Bonning and Hammock, 1995, for review). In addition, it would be desirable from an application point of view to extend the host range of baculoviruses to be able to combat various insect pests with the same virus. From a safety perspective the limited host specificity is an asset.

Detailed knowledge about baculovirus gene structure, function and regulation has allowed the manipulation of the viral genome and the development of the baculovirus expression vector systems (Smith *et al.*, 1983). This technology can now be exploited and tailored for the construction of baculoviruses with novel insecticidal properties to combat insect pests. The purpose of this communication is to give an overview of the progress that has been made over the years in the engineering of baculoviruses for improved insecticidal properties. *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been the model virus for most of the data obtained. Also, an assessment of the potential risks associated with release of genetically modified baculoviruses in the environment as well as strategies to improve their biosafety will be discussed.

## Improvement of insecticidal properties

Strategies to improve the speed of action involved the introduction into baculoviruses of insect-specific toxins, hormones and enzymes. Toxins derived from the bacterium *B. thuringiensis* (Martens *et al.*, 1990; Merryweather *et al.*, 1990) and the scorpion *Bupus eupeus* (Carbonell *et al.*, 1988) did not enhance the speed of action of recombinant baculoviruses. The introduction of neurotoxin genes of the scorpion *Androctonus australis* (Stewart *et al.*, 1991; McCutchen *et al.*, 1991) and the mite *Pyemotes tritici* (Tomalski and Miller, 1991) resulted in a considerable reduction in time required to cause cessation of feeding and/or to incapacitate the host insect due to paralysis. Further improvements can be made by optimizing codon usage for these toxins, by expressing them earlier after infection or by testing novel toxins.

Another strategy to improve the speed of action of baculoviruses includes the over-expression of peptide hormones regulating diuresis (diuretic hormone; Maeda, 1989), or insect metamorphosis such as eclosion hormone (Eldridge *et al.*, 1991) and prothoracicotropic hormone (O'Reilly *et al.*, 1995). The products of these genes may, when expressed at high level, interfere with the insect metabolism resulting in, among others, faster cessation of feeding. However, up to this point these strategies, although more attractive from a safety point of view, have been unsuccessful so far. Other protein factors regulating the molt, such as receptors, transcription factors, etc. are future targets for improvement of baculoviruses with insect-derived genes.

The introduction of the juvenile hormone esterase (JHE) gene from *Heliothis virescens* (Hammock *et al.*, 1990) in recombinant baculoviruses under the control of a strong baculovirus promoter resulted in a slight increase in virulence to host insects as compared to control viruses. Site-specific mutations in the JHE gene enhanced the stability of the recombinant enzyme in the insect and, hence, further increased the speed of kill (Bonning *et al.*, 1995).

Inadvertently, some other proteins cause an enhancement of the insecticidal effect, such as the overexpression of methyltransferase (Xia *et al.*, 1993), an enzyme which adds methyl groups to nucleic acids, or the maize mitochondrial protein (URF13) (Korth and Levings, 1993), which cause male sterility in plants (!). The mode of action in these cases is entirely unknown.

Another significant finding has been the identification in baculoviruses of a gene, producing ecdysteroid UDP-glucosyltransferase (*egt*) (O'Reilly and Miller, 1989; O'Reilly, 1995). Upon glycosylation this viral enzyme inactivates ecdysteroids and, hence, causes a delay in the molting of the insect. This is much to the benefit of the virus as the larva can grow larger and produce more virus. Deletion of this gene from AcNPV resulted in the abortion of this delay and in normal larval development and, hence, in a lower yield of virus. Surprisingly, this deletion also caused a quicker death of the insect (O'Reilly and Miller, 1991), which could be attributed to malfunctioning of the Malpighian tubules (Flipsen *et al.*, 1995).

**Biosafety considerations**

The combination of a highly specific insecticidal protein with a highly selective group of insect viruses presents an attractive concept for the safe use of baculovirus recombinants. If there are risks, then these are partly similar to those associated with the deliberate release of wild-type baculoviruses such as the effects on non-target hosts. Although baculoviruses have been used for over five decades in the field and have a perfect safety record, very little has been learned about the ecology of virus infections in the field. The additional risks associated with the use of genetically modified baculoviruses cannot easily be identified and quantified. These risks may involve alteration of the host range and sensitivity, the spread of the engineered virus from the field site to other ecosystems, the physical instability of the viral genome, activation of latent viruses and mobile elements (transposons), the possible exchange of genetic information, in particular the insecticidal gene, with other organisms, and toxicity of the insecticidal protein for non-target insects.

Some of these perceived risks can be experimentally tested, but others are most difficult. Genetically engineered baculoviruses can aid in these studies by performing contained releases in the environment (Bishop *et al.*, 1988, Cory *et al.*, 1994). Host range and virulence of wild-type and *A. australis*-toxin-containing baculoviruses were very similar (Possee *et al.*, 1993) suggesting that their use in the environment would not constitute a significant risk to indigenous insect pests. Toxins produced by these recombinants did not have an effect on predators or honey bees (Heinz *et al.*, 1995; McNitt *et al.*, 1995). Field releases of genetically modified AcNPV, either with neurotoxins or with *egt* deletions, are being carried out in various parts of the world. The first report of such a field study with *A. australis*-toxin-containing AcNPV indicated more effective control than wild type virus and, as expected, lower yield of progeny virus (Cory *et al.*, 1994). The presence of transposons in baculoviruses is also a

concern, as these elements can in principle transfer insecticidal genes to other baculoviruses or even cross insect borders (Jehle and Vlak, 1995).
Recently, host-range mutants have been engineered by swapping parts of the DNA helicase of *Bombyx mori* NPV into AcNPV (Maeda *et al.*, 1993; Croizier *et al.*, 1994). The DNA helicase is involved in baculovirus DNA replication (Kool *et al.*, 1994). This extended the host range of AcNPV to *B. mori*, an insect that would otherwise not be infected with AcNPV. In principle, host-ranges of baculoviruses may now be expanded and restricted at will by genetic engineering, but it also poses an additional risk if other yet undetermined host range factors are involved.
If risk is defined as exposure times hazard and when hazards are difficult to be quantified, the biosafety of genetically engineered baculoviruses is enhanced by adding genetic traits to the virus limiting its exposure to insects. In many instances described above this prerequisite is already met, since the enhanced insecticidal activity and low virus yield render these recombinants reduced recycling potential. Additional 'biological containment' can be achieved by affecting the spread and survival of the recombinants even further. Various molecular strategies are being evaluated.

**Engineering strategies for biosafety**
The ecological and environmental fitness of such genetically modified baculovirus may be reduced by various strategies. The principle is to make these viruses suicidal.

(i) co-occlusion of polyhedrin-negative recombinant viruses and wild-type viruses. In this case the insecticidal gene in the recombinant replaces the polyhedrin gene. This polyhedrin-negative recombinant is co-occluded with wild-type baculoviruses. Since this recombinant lacks polyhedra, it will be eventually inactivated in the field, whereas the wild-type virus will prevail in the end (Miller, 1988; Wood *et al.*, 1990). Field experiments indicated that this was indeed the case (Wood *et al.*, 1994).
(ii) pre-occlusion using polyhedrin-negative recombinant viruses. Here, the virus particles waiting to be occluded (pre-occluded) is formulated and used (Wood *et al.*, 1993). This recombinant will have limited survival as polyhedra are absent and thus unable to protect the pre-occluded virus from decay once it is in the environment (H.A. Wood, personal communication).
(iii) deletion mutagenesis. Several baculovirus genes have now been described, which are not essential for viral replication, but whose inactivation reduces the persistence and spread of the virus in the environment. The genes for p10 (Vlak *et al.*, 1988), chitinase (Hawtin *et al.*, 1993) and cathepsin (Slack *et al.*, 1995) are involved in insect lysis and thus enhance polyhedra release from the insect. The pp34 gene is associated with the formation of the polyhedral envelope giving protection to polyhedral decay (Zuidema *et al.*, 1989). The *egt* gene inhibits molting of the infected host and affect virus yield (O'Reilly and Miller, 1991). Single and multiple deletion mutants are now being made and will be tested for their capacity of reduced spread and survival. In case of a p10-minus recombinant it could already be demonstrated that its spread was indeed impaired (Undorf-Spahn *et al.*, 1992). Field releases in the USA with an *egt* deletion mutant of AcNPV are carried out since 1993 by American Cyanamid.
(iv) The most promising strategy involves the deletion of the p74 gene from the virus. This gene plays an essential role in the entry of the virus into the midgut epithelial cells of insect larvae (Kuzio *et al.*, 1989). P74 is not required for infection of other larval or tissue culture cells. Absence of this gene product on the outside of occluded virions aborts the infection of larvae. When polyhedra are produced in transgenic cell lines producing p74 the occluded viruses can be complemented with this protein, but are then only able to infect insects only once; progeny virus will lack the p74 gene and is thus unable to infect other insects (Wilson *et al.*, 1994).

The construction of baculovirus recombinants which cause quick cessation of feeding has been achieved. In addition, by deletion mutagenesis these recombinants can be made less persistent in the environment. The recombinants can be produced in insects, when the insecticidal action does not cause immediate mortality. Otherwise, cell culture is an attractive alternative since inexpensive media and large-scale bioreactors are now available (Vlak *et al.*, 1992). Development of additional strategies for improvement of baculoviruses requires a more detailed understanding of insect biochemistry and physiology. There is a strong quest for additional insecticidal genes for a variety of insect species in order to tailor baculoviruses further. With the pioneering work on AcNPV, it now becomes possible to engineer baculoviruses of other, economically important pest insects. So far, efforts have had limited success, mainly due to recalcitrant cell culture systems. This makes engineering of these baculoviruses difficult, but technical advances are quickly made here as well.

## Conclusion

The ideal improved baculovirus insecticide should have a broad but defined host-range, cause cessation of feeding upon infection and noncycling. Only baculovirus insecticides with these specifications are commercially attractive to develop. Recently, the AcNPV recombinant expressing the *A. australis* toxin has been tailored for optimal expression and now reduces the time to render insects ineffective in feeding in about 1.5 day (Black, 1995). This virus has the complete viral sequence and the toxin gene. This makes baculoviruses competitive with *B. thuringiensis* preparations. It remains to be seen, however, if resistance against the toxins is generated in insects as is the case against *B. thuringiensis* toxins. Strategies for biosafety as outlined above could be employed if hazards are anticipated.

The availability of genetically engineered baculovirus insecticides with genes coding for insect-specific toxins, hormones or metabolic enzymes are not likely to impose additional environmental risks. These proteins are specific for certain insect species and they are all natural elements of the insect biosphere. Recombinant baculovirus insecticides containing genes of this nature may therefore be considered as natural, insect-specific biocontrol agents producing biorational compounds. In addition, suicide strategies render these viruses nonrecycling working more as a proteinaceous insecticide. Reduced persistence and host specificity are important features to keep as they reduce the risks associated with the deliberate release of genetically modified viral insecticides in the environment. However, studies on the behaviour of these baculoviruses in microcosm, greenhouse, and field, will provide information if the perceived risks are hypothetical or real.

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# Antibiotic (kanamycin and streptomycin) resistance traits in the environment

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## Intoduction

Antibiotics have been and are being widely used in human medicine and animal husbandry, and are key antibacterial factors determining much of the success of 20th century medicine. Unfortunately, an increasing incidence of antibiotic resistance amongst bacterial isolates has invariably been noted following the introduction of each new antibiotic. In particular, the enhanced occurrence of antibiotic resistant pathogenic bacteria has often hampered antibiotic treatments (Hinton *et al.* 1986; Saunders 1984). The massive use of antibiotics has undoubtedly been crucial in this resistance development, since it provided strong selective pressure under which underlying mechanisms of bacterial adaptation could emerge. For instance, it was shown that the rate of production and application of antibiotics in medicine and farming from the fifties to the late seventies parallelled the incidence of e.g. antibiotic-resistant *Salmonella* and *Shigella* isolates (Linton 1984; van Elsas 1992).

In all likelihood, there are two mechanisms involved in the development of antibiotic resistant bacterial populations. After emergence of an antibiotic resistance trait, clonal selection due to selective pressure exerted by the antibiotic may ensue. Alternatively, spread of the resistance trait to other bacteria can be mediated by gene transfer via transformation, transduction or conjugation. The resistance determinants currently often found on plasmids have indeed been selected under conditions of antibiotic selective pressure associated with clinical use of antibiotics, since the core replicons of self-transmissible plasmids involved in antibiotic resistance gene transfer were devoid of resistance determinants in the (antibiotic sensitive) bacteria isolated in the pre-antibiotic era (Hughes and Datta 1983).

Interest in antibiotic selective pressure and the prevalence of antibiotic resistance traits in soil and aquatic environments has recently increased in the light of the use of resistance markers in genetically engineered organisms as well as the release of antibiotic-resistant bacteria into the environment via sewage and animal manure (Smalla *et al.* 1993a). In addition, the hypothesis that antibiotic producing organisms occurring in soil, such as streptomycetes and bacilli, are the sources of antibiotic resistance traits in clinical isolates (Walker and Walker 1970; Benveniste and Davies 1973) can be tested via studies in soil. This paper will discuss the possible selection in natural environments of antibiotic resistance traits and their prevalence, as evidenced by molecular techniques. The potential of the environment, in particular soil, to provide sites conducive to selection and adaptation will be examined with an emphasis on the role of selective microsites in this environment. The focus will be on the occurrence and availability of kanamycin and streptomycin and the possible selection of kanamycin and streptomycin resistance traits given that these resistances have been widely used as markers, e.g. in the form of transposon Tn*5* and the *npt*II gene (Nap *et al.* 1992; van Elsas *et al.* 1986; 1994).

## The role of environmental gene transfer in the spread of antibiotic resistance traits

Gene transfer has played a major role in the spread of antibiotic resistance traits in clinical and veterinary settings (Levy and Marshall 1988). However, its role in the open environment is less clear since transfer may be hampered by low cell densities, spatial separation of cells, low nutrient availability and unfavourable (soil) moisture content, pH and temperature (van Elsas 1992). Experimental data on gene transfer frequencies between bacteria in the environment have been reviewed (Stotzky and Babich 1986, Trevors *et al.* 1987, Levy and Marshall 1988, Stotzky 1989, Saye and Miller 1989, Fry and Day 1990, Wellington and van Elsas 1992). Both in soil and water environments, gene transfer frequencies were generally found to be low, and the main factor limiting gene transfer is supposed to be nutrient scarcity, which reduces bacterial density and activity. Higher frequencies are possible under conditions of enhanced bacterial survival and activity (Slater 1985). For the aquatic environment, this implies, next to nutrient input, favourable temperatures and the occurrence of sites promoting high cell densities such as occur in sediment (particulate matter) and on surfaces in water systems. Transfer of genes between many bacteria in the aquatic environment has been observed (Trevors *et al.* 1987; Saye and Miller 1989; Fry and Day; Levy and Miller; Wellington and van Elsas 1992). Surfaces which permit the formation of biofilms, such as river epilithon, the microbial community present on stones in the water, have been shown to promote conjugal plasmid transfer between bacteria. Physiological temperatures, between 10 and 25°C, were permissive of highest plasmid transfer rates (Fry and Day 1990). In soil, hot spots of bacterial activity can be distinguished, in which bacterial numbers and metabolic activities are clearly stimulated (van Elsas 1992). These hot spots often represent sites of enhanced nutrient availability. Bacterial gene transfer is enhanced in hot spots (van Elsas *et al.* 1988), but is still affected by other factors such as soil moisture conditions, pH, temperature and selective pressure (Stotzky and Krasovsky 1981; van Elsas *et al.* 1988a; De Rore *et al.* 1994). Sites conducive for gene transfer in soil and soil-related habitats include the rhizosphere of plants growing in soil (Van Elsas *et al.* 1988a and 1988b), root nodules (Johnston and Beringer 1975), plant vascular systems (Lacy 1978; Manceau *et al.* 1986), decomposing organic matter (plant remains) and soil animals such as insect larvae or earthworms (Jarrett and Stephenson 1990; Armstrong *et al.* 1990).

The open environment thus incidentally provides conditions conducive to gene transfer which may result in dissemination of antibiotic resistance traits. However, it is clear that the impact of such gene transfer on microbial populations in the environment is determined by the potential for their selection by antibiotic pressure.

## Release, production and site dependent availability and activity of antibiotics in soil

Antibiotics can occur in soil due to (1) anthropogenic input, i.e. the production and use of antibiotics and their release into the environment, and (2) *in situ* production by soil fungi, actinomycetes and other bacteria. Exact data on the scale of antibiotic release into the environment are difficult to find. Until the 60s, several medically relevant antibiotics were also employed in soil environments for the control of plant diseases, but this practice has now decreased. However, some antibiotics continue to be used. In particular the application of streptomycin in fireblight control in apple and pear orchards (Misato *et al.* 1977; Chiou and Jones 1991, 1993) may be of significant environmental impact, as discussed later. Antibiotics also enter the environment via soil inadvertently following their production or application. For instance, spent mycelial waste from antibiotic (including kanamycin and streptomycin) production plants containing substantial levels of antibiotics enters the open environment including soil. Moreover, following their use antibiotics can have an impact if significant amounts are released and if they are environmentally persistent. For instance, 97% of the kanamycin used in veterinary medicine was

suggested to pass the animal.gut and enter manure (Nap *et al.* 1992). A yearly amount of 30.000 kg was estimated to be used in the Netherlands. Following spread of the manure to field soils, this might result in a level of 0.13 µg per g topsoil in the top 5 cm (Nap *et al.* 1992). Since kanamycin is very stable, several years would hence be sufficient to obtain a selective force significant for microbes (roughly above 0.2 µg per ml for the most sensitive organisms) provided soil conditions would permit the antibiotic to be available (see later).

Early studies on the microbial production of antibiotics in soil were inconclusive and for long it was believed antibiotics did not exert a role in soil (Gottlieb 1976; Williams and Vickers 1986). The main hurdle in these early studies was the difficulty to unequivocally show the presence of antibiotics in natural soil, which was probably related to the adsorption of antibiotics to soil particles, and to detect possible effects on the soil biota, even when antibiotic producing microorganisms were introduced into soil (Gottlieb 1976; Rothrock and Gottlieb 1984; Weller and Thomashow 1990). Nutrient limitation in natural soil may have been a main cause of the apparent lack of production. In addition, the sensitivity of the available detection techniques was possibly too limited to detect low amounts of antibiotics in soil (Williams and Vickers 1986; Turpin *et al.* 1992). However, the occurrence and role of antibiotics in specific sites in soil is nowadays well documented. Antibiotics are produced in microhabitats where conditions for microbial activity are favourable, and exert their effect locally. In fact, early data had already shown that the antibiotic gliotoxin was produced on seed coats after introduction of the gliotoxin producer *Trichoderma viride* on seeds planted in soil (Gottlieb 1976). Gliotoxin was also produced on wheat straw in natural soil. More recently, the antibiotic phenazine-1-carboxylic acid was unequivocally shown to be produced by fluorescent pseudomonads in the wheat rhizosphere (Thomashow *et al.* 1990).

Even though antibiotics occur and do play a role in soil microhabitats, several mechanisms may limit their action. These mechanisms pertain to chemical instability, microbial degradation and adsorption to soil clays and humics (Gottlieb 1976). Such mechanisms in turn depend on the nature of the antibiotic and on soil pH, temperature, moisture content and the presence of degrading microorganisms. It is difficult to assess to what extent these phenomena affect antibiotics produced in soil microhabitats. Added or released antibiotics might be distributed in a different way, resulting in a different fate. Kanamycin, for instance, was suggested to be largely inactivated in soil due to strong interactions with soil clays (Nap *et al.* 1992). Due to this inactivation, the occurrence of strong kanamycin pressure in soil would thus seem to be unlikely. The fate and availability of streptomycin in soil are also determined by interactions with soil clays (as well as organic matter), and inactivation is possible (Pinck *et al.* 1961; Soulides *et al.* 1962); however, its interaction with soil is less pronounced and streptomycin may be available to some extent (Pinck *et al.* 1961; Soulides *et al.* 1962; Turpin *et al.*1992).

By and large, soil is unlikely to exert the strong selective pressure found in the clinical environment, particularly in view of the probably low overall antibiotic concentrations and their potential inactivation in soil. Nevertheless, microhabitats where antibiotics are produced can provide selective environments where antibiotic resistant forms are favoured, as shown in the following section for streptomycin.

**Selection of antibiotic (kanamycin and streptomycin) resistance genes in soil**

Data on the putative selection of antibiotic-resistant bacteria in soil are scarce. It is common knowledge that, much like found in different aquatic environments (Kelch and Lee 1978; Niemi *et al.* 1983; Edwards and Loutit 1984; Smalla *et al.*1993a; Trevors *et al.* 1987), low levels of antibiotic resistant forms can be found in many soils (Smalla *et al.*1993a; Scotti *et al.* 1982; Van Elsas *et al.* 1986; Henschke and Schmidt 1990). Antibiotic resistance is found in antibiotic-producing microorganisms isolated from soil, which need the mechanism for self-protection (Gray and Fitch 1983; Trieu-Cuot *et al.*

1987a). These resistance genes usually clustered with antibiotic biosynthesis genes (Chater *et al.* 1988). Antibiotic resistance determinants have also been found on plasmids in non-producer strains. Thus, *Bradyrhizobium japonicum* strains originating from different soils were resistant to neomycin, chloramphenicol and penicillin G. In some strains, these resistances were plasmid-borne (Cole and Elkan 1973; 1979).

The levels of bacterial populations resistant to streptomycin or kanamycin are often in the order $10^4$ to $10^5$ cfu per g, resulting in resistance quotients of 0.01 to 0.6% (Henschke and Schmidt 1990; Smit and van Elsas 1992; Smalla *et al.*1993a). It is unclear what the underlying mechanisms in these resistant forms are, and whether such forms have been selected for by any antibiotic selective pressure. A screening of antibiotic-resistant soil isolates for putative resistance plasmids revealed the absence of such plasmids from Gram-negative isolates, whereas plasmids were found in some Gram-positive isolates (Henschke and Schmidt 1990). Possibly, many bacteria growing on the selective plates have a resistant phenotype due to an impermeable cell envelope, hence do not possess an active resistance mechanism.

We will briefly discuss three cases of potential selection of antibiotic resistance traits in soil, two pertaining to the selection of natural populations of bacteria, and one to the selection of an introduced transposon Tn*5* containing population. Firstly, indirect evidence for antibiotic resistance selective conditions in soil was provided by the enhanced numbers of kanamycin or streptomycin resistant rhizobia found in the rhizosphere of *Phaseolus vulgaris* following liming of acid field soil (Scotti *et al.* 1982; Ramos *et al.* 1987). Since liming of soil stimulates streptomycetes, a more active antibiotic-producing population could have provided selective conditions in the *P. vulgaris* rhizosphere favouring the appearance of antibiotic-resistant rhizobia (Ramos *et al.* 1987). Alternatively or additionally, the streptomycin produced might have become more available to microbes due to the pH change. Speculatively, antibiotic-resistant bacteria occurring in animal faeces or sewage entering soil might also be selected for in such selective soil microhabitats.

Secondly, streptomycin resistant plant pathogenic bacteria such as *Pseudomonas syringae* pv. *papulans* and *Erwinia amylovora* have been increasingly found associated with plants (apple and pear trees, potato) treated with streptomycin (Burr *et al.* 1988; Chiou and Jones 1991; 1993). The genes involved in this resistance, *str*A and *str*B, were shown to be localized on a new class II Tn3-like transposon, Tn*5393*, which in turn was present on plasmid pEa34 in *E. amylovora* (Chiou and Jones 1993). The resistance genes were further completely homologous to those found on the well-known clinically-isolated streptomycin/sulfonamide resistance plasmid RSF1010 (Chiou and Jones 1993). Moreover, the transposon was shown to be present in a variety of streptomycin resistant bacterial isolates, including *E. amylovora*, *Acinetobacter* sp., *E. herbicola*, *Pseudomonas* sp., *P. syringae*, *P. fluorescens*, and *P. aeruginosa*, suggesting its involvement in spread of streptomycin resistance under selection in this environment.

Finally, the effects of kanamycin and streptomycin in soil on the population dynamics of transposon Tn*5* modified *Pseudomonas fluorescens* were recently investigated (Britto de Oliveira *et al.*1995). Transposon Tn*5* contains kanamycin (*npt*I - neomycin phosphotransferase) and streptomycin resistance determinants which are both expressed in *P. fluorescens*. Kanamycin at 180 or 18 $\mu g\ g^{-1}$ of dry soil or streptomycin at 18 $\mu g\ g^{-1}$ had no effect on the inoculant in soil and wheat rhizosphere, but streptomycin in high concentration had a consistent stimulatory effect, in particular in the wheat rhizosphere. Streptomycin exerted its effect by selecting *P. fluorescens* with Tn*5* insertion whilst suppressing the parent strain in separate and mixed inoculation studies. Modification of soil pH by the addition of $CaCO_3$ or bentonite clay resulted in an enhancement of the selective effect of streptomycin by $CaCO_3$ and its abolishment by bentonite clay. Soil liming possibly made the antibiotic more available to microorganisms whereas clay made

it less available. The addition to soil of malic acid or wheat root exudate, but not of glucose, enhanced the streptomycin selective effect on the Tn*5*-modified *P. fluorescens* strain. Hence, the effect of streptomycin in soil on inoculant Tn*5*-carrying bacteria depended on the occurrence of conditions of enhanced activity such as established by (wheat) root exudates and suitable available substrate. By and large, however, streptomycin was found to be able to exert a selective effect on the transposon Tn*5*-marked *P. fluorescens* populations in soil.

**Screening for the occurrence of transposon Tn*5* and the *npt*II gene in the environment**

In view of the widespread use of transposon Tn*5* and the kanamycin resistance gene *npt*II as markers both in molecular biology and ecology of microorganisms and plants, we performed a screening of these elements in various environmental habitats, i.e. different soil, sewage, river water and pig manure samples (Smalla *et al.*1993a). Both kanamycin resistant bacterial isolates and environmental DNA extracts were used. Molecular techniques applied were hybridization of cells in dot blots, of Southern-blotted genomic DNA extracts and of PCR amplification products. Two probes, a 2.7 kb one characteristic for the transposon Tn*5* central region and a 925 bp one specific for *npt*II, were used. The PCR systems employed, specific for *npt*II or Tn*5*. Colonies reacting with either probe were primarily found in sewage samples, whereas fewer were obtained from pig manure or river water. No probe-positive bacteria, i.e. no transposon Tn*5*- or *npt*II-carrying bacteria were found in over 550 bacterial isolates obtained from several soil samples. This result has now been extended to over 2.000 isolates from the sugarbeet rhizosphere (Smalla *et al.*1995). Transposon Tn*5* could be unequivocally demonstrated in 3 sewage isolates, identified as *Aeromonas* spp. (2x) and *Escherichia coli*, via cloning of kanamycin resistance on a characteristic HindIII DNA fragment in kanamycin sensitive *E. coli*, as well as molecular analysis of the original isolates and the transformed *E. coli* derivatives (Smalla *et al.* 1993a). Various other strains revealed the presence of *npt*II in a non-Tn*5* background.

Using specific PCR, we also assessed the putative presence of *npt*II- and Tn*5*-homologous sequences in total community DNA extracts obtained from the habitats. DNA extracts were obtained using the rapid procedure developed in our laboratories (Smalla *et al.* 1993b). Evidence for the occurrence of *npt*II was obtained for sewage, pig manure, river water (2 out of 3 types), and several soils (3 out of 6 analysed). Tn*5* detection via PCR did not provide conclusive evidence for its occurrence.

From these data, it appears that transposon Tn*5* as well as the kanamycin resistance trait *npt*II are released into the open environment via sewage and pig manure slurry. However, even with this potential input, the natural incidence of Tn*5* in soil is probably low, given the absence in the culturable bacterial fraction. *Npt*II might be present in the microbial communities in these habitats, in the non-culturable fractions.

**Concluding remarks**

It is clear that the emergence of antibiotic resistance traits amongst bacteria in the clinical and veterinary environment has been favoured by the strong selective pressure in these settings. A variety of genetic adaptation events may have endowed bacteria with antibiotic resistance determinants, including active antibiotic-modifying and efflux systems on highly flexible genetic elements (plasmids and transposons). Two sets of data support this, first the recent emergence of identical antibiotic resistance genes in different bacteria, and secondly the *in situ* experimental demonstration of transfer of antibiotic resistance elements.

Further, in the open environment antibiotic resistance traits are omnipresent. The potential for selection and transfer of such traits in soil is unclear due to uncertainties

about the extent of selective pressure. Selective force is related to the anthropogenic input, microbial production and bioavailability of antibiotics and these are not at all understood. More information should become available on the effect of environmental input of antibiotics by man, and on the natural production of antibiotics in the environment. For instance, kanamycin is suggested to be largely inactivated in soil due to interactions with soil clays, but streptomycin might be more available.

Evidence was reviewed which pointed to a role for streptomycin in the selection of natural streptomycin-resistant bacteria in orchards under streptomycin pressure and in the rhizosphere of *P. vulgaris* where streptomycetes were stimulated due to soil liming. In addition, *P. fluorescens* containing transposon Tn*5* was selectable in soil by added streptomycin, in particular in the rhizosphere of wheat.

Using molecular tools, evidence was found for the occurrence of the kanamycin resistance trait *npt*II as well as transposon Tn*5* primarily in bacteria in sewage (Tn*5*, *npt*II) and pig manure and river water (*npt*II). In soil culturable bacteria, no such evidence was obtained. One might speculate whether these observations are related to the possibility that the former habitats exert a stronger kanamycin pressure than the latter. However, the natural occurrence of *npt*II homologous sequences (and the absence of Tn*5*) in total community DNA extracts of various soils (in addition to other habitats) pointed to the presence of *npt*II, possibly non-expressed or present in non-culturable bacteria.

Since both transposon Tn*5* (or its derivatives) and *npt*II are in use as markers for ecological studies, it is important to assess their benefits and potential hazards. We have used both markers in bacteria in soil microcosms and the field, and found them to be suitable selectable and hybridization markers, allowing specific detection of our target bacteria. When combined with chromosomal rifampicin resistance, detection down to 10-100 cfu per g soil was possible. Further, there is no reason to suppose these markers are inherently unsafe. For instance, upon ingestion, *npt*II apparently does not cause any detectable harm (Nap *et al.*1992). In addition, Tn*5* (and within it *npt*II) already enters the open environment via sewage, in a largely uncontrolled fashion. The suggested absence in soil of kanamycin selection of *npt*II and the apparent (weak) selection of the Tn*5* streptomycin resistance might further warrant the use of *npt*II but argues against using the full Tn*5*.

Nevertheless, we recommend that in transgenic plants or microbial biocontrol agents intended for large-scale production and use, alternative, non-antibiotic resistance markers are employed given the undesirability of large-scale release of any antibiotic resistance traits into the environment.

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## FOOD LABELING: PRACTICABILITIES AND CONSIDERATIONS

### Do we need a declaration for genetically engineered food?

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The abstract was not available (November 1995).

# BIOSAFETY RESEARCH: NEEDS AND BASIC ISSUES

## Future aspects of ecological biosafety research

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**Abstract**
Future aspects of ecological biosafety research have to focus on ecological long-term effects in ecosystems, communities, and populations. A differentiation between the natural dynamics and the potential influence on natural system development is necessary. Direct and indirect monitoring research is presented here for the beet species (*Beta vulgaris* L.). Direct monitoring methods include the measurement of factors influencing plant spread. As an example, field tests in Germany quantified the potential ecological advantage of virus resistance under virus and non-virus infestation conditions. The introduction of biological indicators and observation of global system parameters are part of indirect monitoring methods. With beet, for example, an impact of naturalization on natural, non-agricultural habitats may appear in wild beet populations in Italian seed beet production areas. However, in coastal areas of North-Eastern Italy an increase in wild beet fitness seems improbable at the present stage, since no virus infestation was observed in 1994. Future ecological experiments must give direct quantitative data on plant multiplication to serve as the basis for risk analysis. According to Kareiva and Parker (1994), primary data on plant demography under field conditions is the best measure of 'invasiveness' and thus of ecological risk.

### 1. Ecological long-term effects in ecosystems, communities and populations

Here we would like to present a perspective on future aspects of ecological biosafety research. Our view focusses on the fundamental basis of monitoring the commercialization of transgenic plants.
As a model system we use beet (*Beta vulgaris* L.), both because it has been the subject of ecological research (Bartsch et al. 1994, Bartsch et al. 1995), and because it is an important cash crop in Europe and a common target for potential projects using recombinant DNA technology. Beets are also an important subject for invasiveness studies for two reasons. Firstly, a gene flow between cultivated sugar beets (*Beta vulgaris* subsp. *vulgaris* provar. *altissima* DÖLL) and wild beets (*Beta vulgaris* subsp. *maritima* ARCANG.) has been demonstrated, as evidenced by the introgression of the annual habit into cultivated beets (Boudry et al. 1993) and the introgression of gene form seed beet into wild beet populations (Santoni & Berville 1993). Secondly, hybridization between wild beets and sugar beet breeding plants leads to a hybrid form ("weed beet") that is able to bolt during the cultivation period among the biennial habit of sugar beet varieties. Beet breeding districts often overlap with regions supporting wild beets (Fig. 1).
It is already known that monitoring has to deal with the various dynamics in natural ecosystem development (Fig. 2). Ecosystem dynamics and evolution will probably change the system structure and processes (from level A to level B). Looking at the past, the demographic distribution of wild beets in the Netherlands has changed as seen in the reports before and after 1950 (Meenema et al. 1985, van der Meijden et al. 1989). However, we still do not know the dynamics of wild beet on sea dams.

It is necessary to differentiate the clear impact of a transgenic plant ("stress factor") e.g. the alternative system development from level A to level C (Fig. 2). Will the ecological long-term effect be the displacement of natural individuals with a loss of genetic variance in a wild beet habitat? And, more importantly, is the difference between the resulting levels B and C (Fig. 2) an ecological damage? Do we interpret a simple introgression of the genetically engineered trait in wild populations as no damage, if the genetic structure/variance has not been effected?

**Types of ecological monitoring experiments**

**I. Direct monitoring**

Direct monitoring of ecological long-term effects includes the observation of the spread of transgenic organisms and the measurement of influencing factors (Fig. 2). This applied biosafety research should give direct quantitative data on plant multiplication, serving as basis of risk analysis. According to Kareiva and Parker, "primary data on plant demography and field conditions is the best measure of 'invasiveness' and thus on ecological risk".

Our active monitoring includes the first test of invasiveness in a transgenic plant modified for an ecologically relevant trait (Bartsch et al. 1994, Bartsch et al., in press). The trait expressed by the transgenic beets in our study was resistance to the Rizomania caused by the virus BNYVV, a disease which has spread through the sugar beet fields of Europe, California, Japan, and China. The disease leads to decreased sugar beet yield and a loss of up to 30% sugar content. In places where the disease is very severe, such as some parts of northern Italy, sugar beet production has been stopped. Because of the potential for interbreeding between cultivated and wild beets, it is important to know whether transgenic virus resistance could also increase the fitness of wild beet populations.

In 1994, no significant difference was found in seed emergence (Fig. 3) between the transgenic and the susceptible control lines at the same test site. The seeds of the normal variety always had a higher emergence rate. The emergence of the genotypes in Oberviehhausen was much worse in comparison to Wetze.

We studied the competitiveness of beets in young fallow. In 1994, the biomass production of the transgenic heterozygous line is significantly higher in comparison to the heterozygous control line at Oberviehhausen, whereas no difference is seen at the virus-free site (Fig. 4). The highest biomass production is exhibited by the conventionally virus-resistant variety. The sugar beet genotypes only differ significantly in their biomass production at the Oberviehhausen site. As expected, the biomass production decreases for all sugar beet genotypes at the test sites with an increase of the *C. album* competitor. No difference was seen between the heterozygous transgenic and the susceptible control line in 1994. The competitiveness of the conventionally resistant variety was significantly higher.

The sugar beet productivity under competition with the natural weed flora lay within the range of the productivity results of beets grown in *C. album* competition. The variance of data received from plants grown with the natural weeds was up to 50% higher than those plants grown with *C. album*. This probably reflects the fact that natural weed cover varied from 5-100% of the total area in the plot. The cover of *C. album* varied from 90-100% of the total area in the plot

**II. Indirect monitoring**

Indirect monitoring methods include the active introduction of biological indicators (Fig. 2). For example, pollen-sterile cms-plants could be introduced to study pollen flow in areas, where seed production areas and wild beet habitats overlap.

Passive monitoring studies should focus on the observation and measurement of global system parameters (Fig. 2). For the beet example, we undertook a survey of wild beet

populations to try to predict the magnitude of the effect of future transgene introgression. Some levels of virus tolerance have been found in wild beets, but it is unknown whether this reflects former selection pressure from the disease. Virus resistance would be most likely to cause "ecological release" in wild populations if the disease is widespread and highly virulent. In 1994 a first investigation was carried out to locate and sample wild beet populations nearby seed production areas in North-Eastern Italy. This part of Italy, north of the river Po, was selected because it is the area where the BNYVV was first detected in the early 1950's. We assumed that if there is a wild beet area infected with the virus, it would with most probability be in the Po estuary. We only found wild beet locations north of the river Poo estuary (Fig. 5). No wild beets could be detected south of the estuary as far as Rimini. According to our ELISA results, the coat protein was detectable in the transgenics, whereas none of the 60 tested wild beet plants from 6 different locations showed a virus infestation.
A naturalization in wild beet habitats certainly could occur, but the ecological advantage of the genetically engineered trait was not observed in one of the most important coastal zones on the Adriatic Sea.
However, we need more data on the undisturbed situation in wild communities in order to detect future implications for transgenic sugar beets.

**Need for more ecological experiments**

Ecological tests of invasiveness for genetically modified organisms (GMOs) to date have investigated traits with little ecological relevance (Crawley et al. 1990, Bergelson 1994, Parker & Kareiva, in press). The fact that these studies found no additional risk of invasion was not surprising under the circumstances, and they provide us with little confidence in the statement made by many in the biotechnology industry that genetic engineering is unconditionally safe. Past ecological studies have avoided important traits primarily because of the "catch 22" of GMO regulation: If the trait is risky enough to be interesting, it is too risky for the kind of experiment that has ecological validity. However, we are now reaching a point where companies in some countries have gained permission to grow such "risky" transgenic plants on a commercial scale (Parker & Kareiva, in press) without any solid ecological evidence on the risk they pose.
The need for more ecological experiments is clearly demonstrated by Williamson (1992), Regal (1994), and Kareiva & Parker (1994). An important study was also made on the usefulness of experiments in predicting the invasiveness of GMOs (Kareiva et al., paper submitted to Ecology). The main result was that experiments have to be conducted for replication at least in three different vegetation periods.

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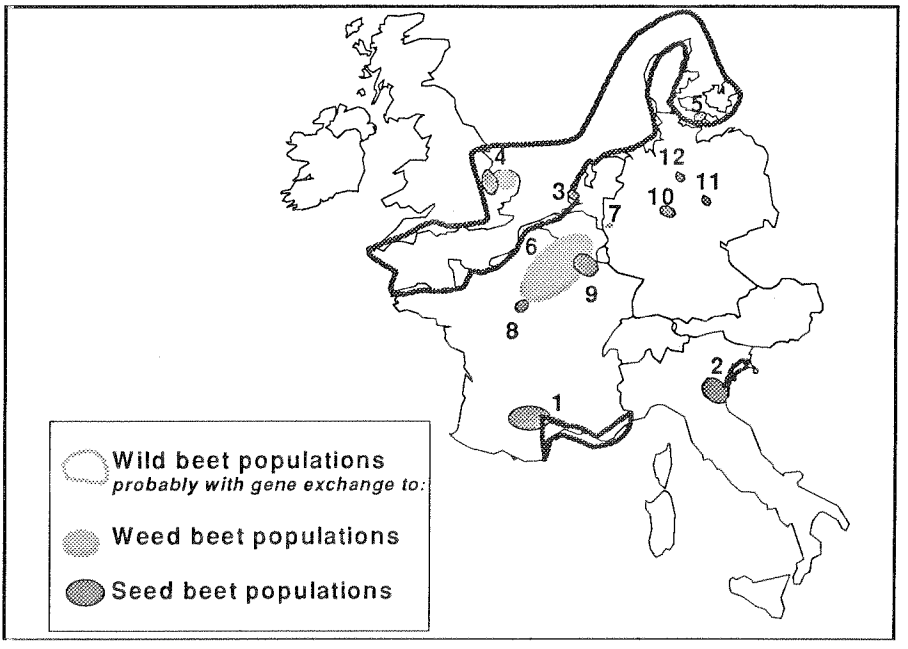
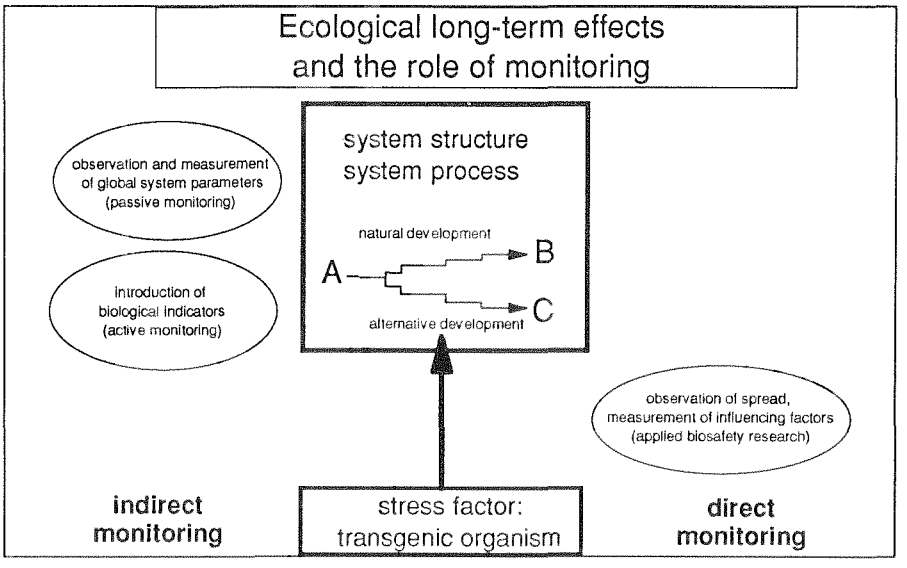
**Figure 1**



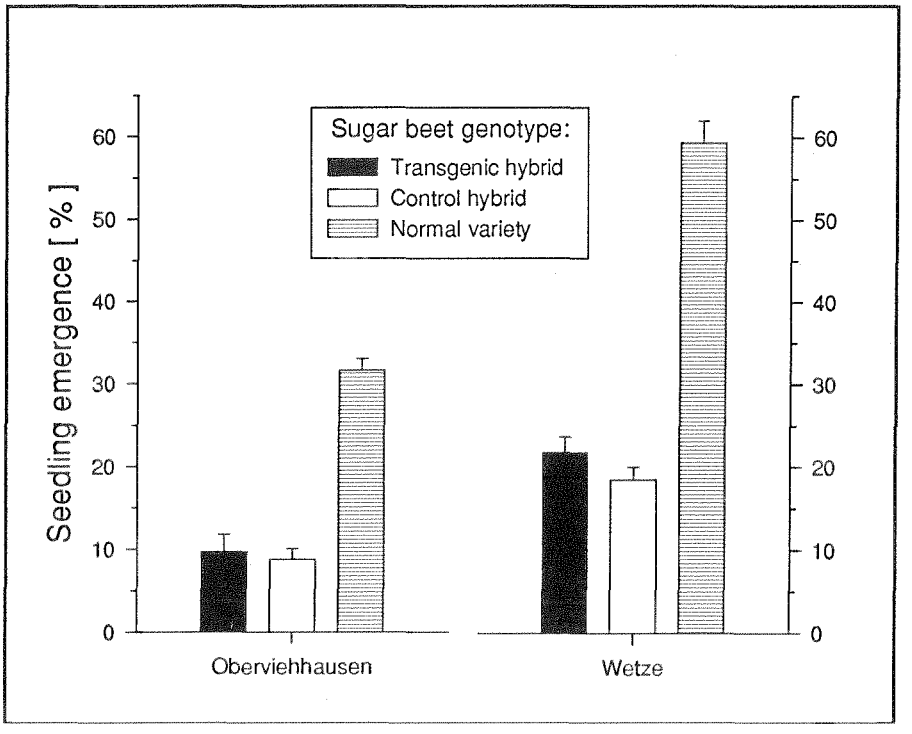

Overview: The distribution of breeding districts (with seed beet populations), agricultural areas (with weed beet populations) and natural habitats (with wild beet populations) is shown for some examples in Central Europe. An overlap of seed beet/wild beet populations is given in numbers,
1-5, some weed beet populations are illustrated in numbers 4,6-7 and a few probably isolated locations of breeding companies (with seed beet populations) are found in numbers 8-12. Data are extracted from Boudry et al. (1993), Doney et al. (1990), Frese (1991), Hornsey & Arnold (1979), Letschert (1993), Longden (1974), Longden (1989).

**Figure 2**



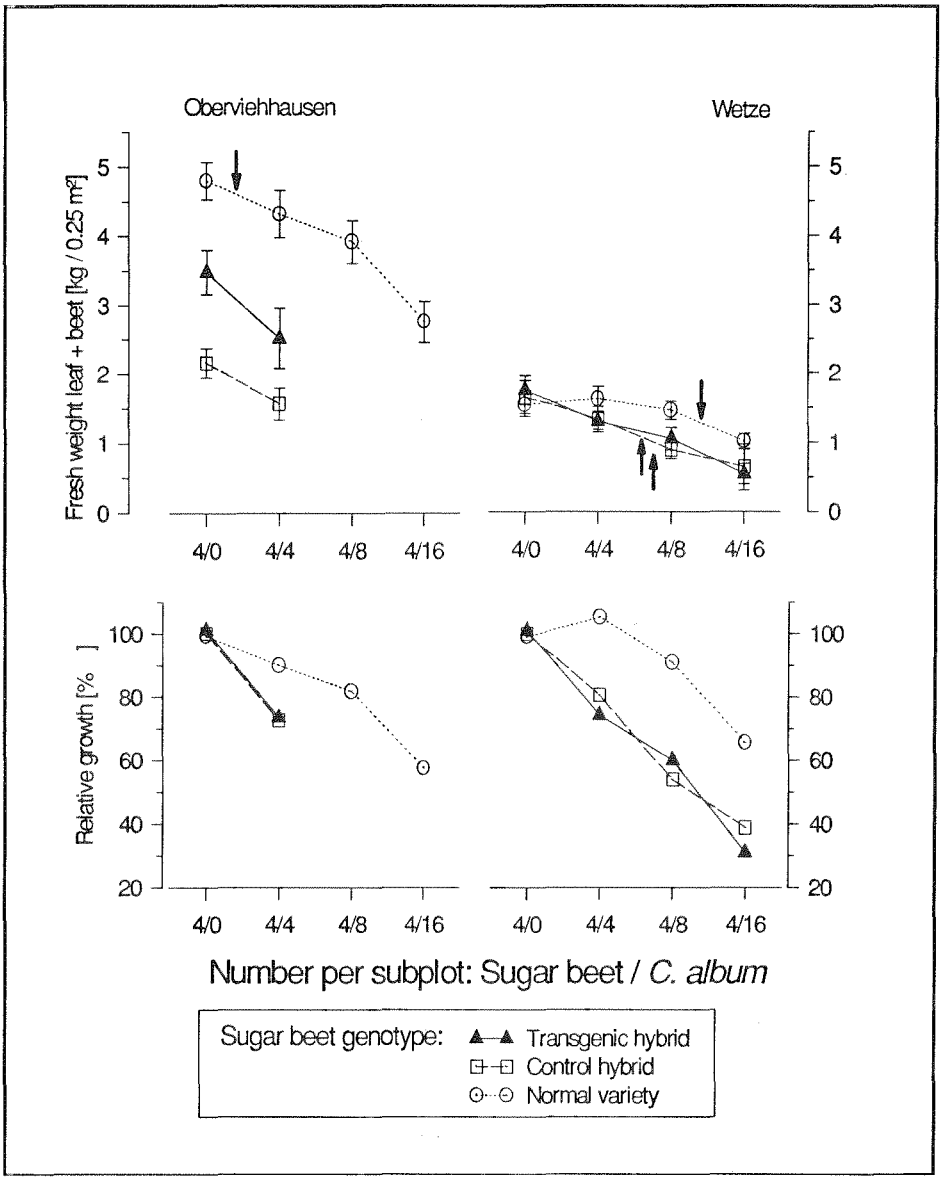

Possible influence of transgenic organisms on biological system dynamic. The natural development of a system from A to B in time may be altered to C by transgenics. Different types of monitoring are available.

**Figure 3**



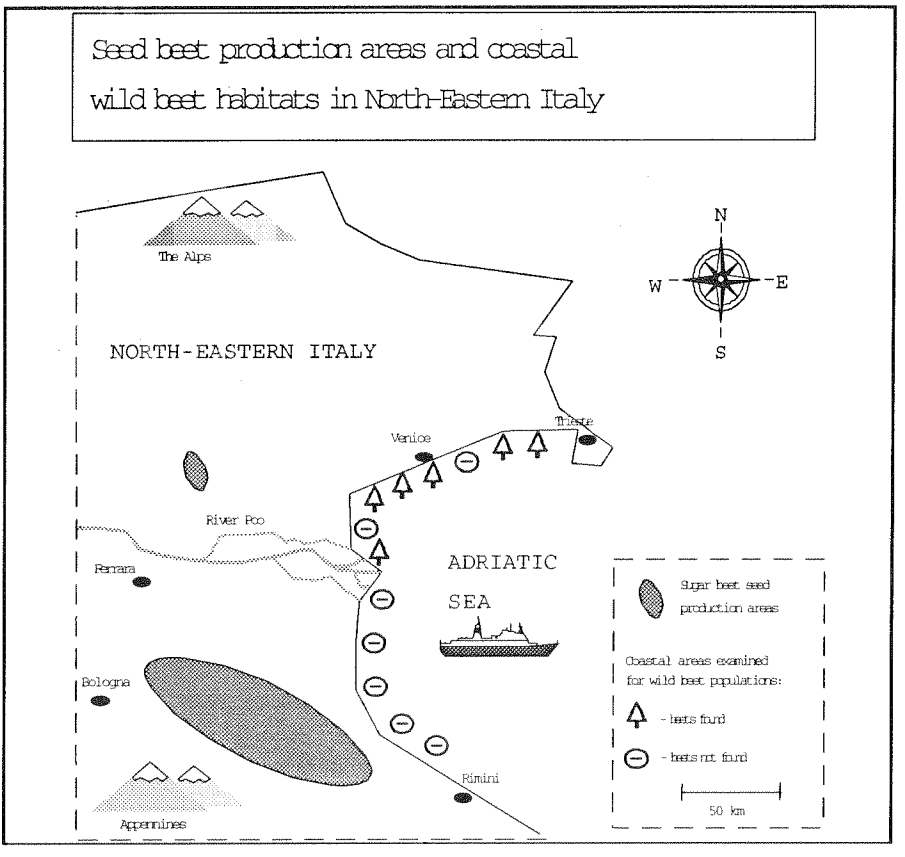

Emerged seedlings of three different sugar beet hybrids (transgenic virus-resistant hybrid, control susceptible hybrid, normal virus-tolerant variety) in the 1994 field test at a site with (Oberviehhausen) and a site without BNYV-Virus infection (Wetze). Bar lines represent the standard error from the mean (n = 100 parallels of 16 seedlings).

**Figure 4**




Absolute and relative biomass production in a 1994 field test: Three different sugar beet hybrids were sown as seeds and grown in four different competition densities with *Chenopodium album* at a site with (Oberviehhausen) and a site without BNYV-Virus infection (Wetze). Bar lines represent the standard error from the mean (n = 20 parallels, 4 plants each). Arrows symbolize the relative position of sugar beet productivity after competitive growth with the naturally occurring weeds.

**Figure 5**




An overview of breeding areas (with seed beet populations) more than 50 kilometres apart from natural habitats (with wild beet populations) in North-Eastern Italy. Although the possibility of an unintended pollen exchange cannot be excluded, an ecological advantage of virus resistance was not apparent. There was no infestation of the wild beet populations in 1994.

# Biosafety research accompanying field releases of GMOs

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Genetically modified organisms (GMOs), as well as non-GMOs, are neither inherently risky nor inherently safe; whether or not the release and commercialization of an organism poses a risk to human health or the environment depends on the characteristics of the organism and the circumstances of its application. In the context of the "Precautionary Principle", the relative lack of familiarity with GMOs justifies appropriate risk assessment for their application. The risk assessment should be focused on organisms and traits which are of commercial and social importance. These studies should aim to include field work.

As a precautionary measure, European Union and the European Member States are supporting prenormative biosafety research related to the release of genetically modified organisms. This research should contribute to a science-based development and/or adaptation of a regulatory framework.

In Germany, the Federal Ministry of Education, Science, Research and Technology (BMBF) is supporting biosafety research accompanying field releases of genetically modified organisms. The titles of the projects presently dealt with are given below.

1. The project "Ecological investigations in connection with field releases of transgenic sugar beets" (duration: 1992-1996) is performed at Aachen University, and is supported with 1.2 million DM.
2. At the Federal Biological Research Centre for Agriculture and Forestry (BBA), Braunschweig, the project "Biosafety research accompanying field releases of transgenic plants" is performed from 1993-1996. It is supported with 1.8 million DM.
3. The project "Field releases of genetically modified Rhizobia" (duration: 1993-1996) is coordinated by Bielefeld University and is supported with 3.9 million DM. The modified Rhizobia are being released at the Federal Research Centre for Agriculture (FAL) in Braunschweig.

The following projects are planned:
"Ecological investigations with transgenic sugar beets and their hybrids with mangel" by Aachen University (1995-1996), and "Field releases of transgenic potato plants expressing T4 lysozyme" (1995-1996) by the Federal Centre for Breeding Research on Cultivated Plants, Quedlinburg.

The BMBF-supported project "Biosafety research accompanying field releases of transgenic plants" worked on at the Federal Biological Research Centre for Agriculture and Forestry consists of three main topics:
- Transcapsidation and recombination of viruses in transgenic plants expressing virus genes
- Gene transfer from transgenic plants to other organisms
- Influence of foreign gene expression in transgenic plants on plant-associated microorganisms.

The transgenic plants tested in the field in 1993 and 1994 are:
- Rizomania resistant sugar beets containing the Rizomania coat protein gene and additional marker genes

- potato plants with the yeast invertase gene
- potato plants with an antisense sequence of the granule bound starch synthase gene.

The potato plants have been released in Wetze (Niedersachsen). The sugar beet plants have been tested in fields infested with Rizomania (Oberviehhausen, Bayern) as well as in non-infested fields (Wetze). The potato plants transformed with the T4 lysozyme gene were only tested in the greenhouse.

In the following, the different parts of the cooperation project are listed, including the scientists involved. The results will be reported elsewere.

"Investigations on the encapsidation, recombination, and spread of plant viruses in transgenic sugar beets that express the coat protein gene of the rizomania virus"
(R. Koenig, U. Commandeur, D.-E. Lesemann, E. Maiß and L. Fecker; 7/93-6/96)

"Transfer of partial virus genomes into crop plants: Investigations on encapsidation and recombination of plant viruses"
(E. Maiß and D.-E. Lesemann; 4/93-3/96)

"Investigations on persistence of Agrobacteria in transgenic sugar beet and potato plants"
(J. Schiemann and A. Matzk ; 7/93-6/95)
This project is closely linked with another BMBF-supported project titled "Extrachromosomal foreign DNA in transgenic plants - Investigations on the persistence of agrobacteria and on the gene transfer into endophytes"
(J. Schiemann, A. Matzk and J. Landsmann; 1/92-3/95)

"Genetic and molecular analysis of hybrids between transgenic sugar beet and related beet varieties"
(A. Dietz-Pfeilstetter; 4/93-3/96)

"Possible effects of T4 lysozyme in transgenic potato plants on associated microorganisms"
(K. Smalla and H. Heuer; 4/93-3/96)

"Investigation on horizontal gene transfer from transgenic sugar beet plants into sugar beet associated bacteria and soil bacteria"
(K. Smalla and F. Gebhard; 1/94-12/96)

"Investigations on the possible formation of a new reservoir of pathogens during cultivation of transgenic potatoes"
(F. Niepold; 7/93-12/95)

"Analysis of the effects that organisms modified by genetechnological methods, or xenobiotics may have on the activity and structure of bacterial flora"
H. Backhaus and B. Engelen; 4/93-3/96)

# Strategies of dealing with the risks of genetic engineering: Lessons from a participatory technology assessment in Germany

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## 1. The political agenda of 'risk'

The debate about the risks posed by genetic engineering is the latest in a long line of social controversies over modern technology, which have come to dominate political debate more and more in the last two decades or so. Politically, these controversies take the form of a criticism of risk, but in fact they are an expression of objections which extend far beyond the stated aim of averting danger.

As the example of the anti-nuclear movement has shown, resistance to technology is fuelled by fundamental reservations about value systems in our society, which culturally favour technological modernization, moral utilitarianism, political pragmatism, instrumental activism, and the individualism that governs economic life. The conflicts about technology address the classical themes of political struggle: social inequality, the objectives of social development, the distribution of power, and the scope of democratic participation.

This last point is worth emphasizing. Resistance to new technologies is also a protest against the feeling of being overwhelmed by innovation. In contrast to what was promised by enlightenment and modernization, we are far from gaining control over the conditions of human existence. Technological revolutions sweep over mankind like acts of God. Admittedly, the relevant decisions are taken somewhere in society, but always, it seems, somewhere else, and certainly not where those affected could themselves take action and make their voices heard. It is not open to us to renounce new technologies or opt out of current trends.

The objections to genetic engineering are similarly wide-ranging to those raised against nuclear power, in that they concern not only manifest safety problems but also question the basic ethical admissibility of the technology concerned, the threat of its abuse, and the undesirable social, political and cultural trends which it could possibly help to trigger. As in the case of nuclear power there is also a tendency to overburden the concept of risk by incorporating within it the widest possible variety of motives and critical demands.

There are a number of reasons for this tendency:

- Arguments involving risk have a special political appeal. They have per se a high expedition of consensus. Who could seriously oppose the call for better safeguards against the hazards of a technology?
- In the balancing of interests, safety considerations weigh more heavily than the interests of those wishing to introduce or utilize a new technology.
- Hazard prevention and precautionary measures against hazards are established and indisputably legitimate objectives of government policy.
- On the basis of a "fundamental right to safety", those affected by a technology are entitled to state protection from hazards, and can if necessary invoke that right in the Constitutional Court, even against a majority parliamentary decision to the contrary.
- Definitions of risk are no fixed, predetermined magnitudes, but cultural parameters negotiated within society. What constitutes a significant threat to safety, a hazard or case of damage depends on selective perceptions and sensibilities, and these perceptions may change as a result of education and protest.

It is for these reasons that resistance to new technology, and also the often fundamental objections advanced by movements within society, tend to find their practical political manifestation primarily in the form of criticism of risk. The same reasons explain why it

would be futile to reduce public perceptions of 'risks' to a single clearly defined concept, and why the classical instruments of risk regulation are not likely to really solve the issue.

## 2. Putting risk arguments to test

This paper presents results and conclusions from a technology assessment of transgenic herbicide-resistant crop plants, which was organized in Germany from 1991-1993 and provided a specific arena for public debate on the risks of genetic engineering. The procedure of the technology assessment was specific for two reasons. Firstly, it was *participatory*; it involved some 50 persons from environmental groups, industry, regulatory agencies and the scientific community, giving full representation and a fair share of resources (for commissioning expert reports) to the critics of the technology. Secondly, the procedure was *discursive*. Participants were expected to collect and discuss all available arguments in an ongoing process of communication and interaction. For that purpose they attended a series of conferences which lasted more than 10 days in all.

Discourse in such a social setting is remarkably different from the so-called "public" discourse pursued in mass communication. Participants in mass communication tend to use the rhetoric of arguments but rarely observe the disclipine of argumentation. They normally confine themselves to the statement of their own strongest points, neglecting countervailing arguments or selecting for consideration only those which they can easily refute. In contrast, the participants in our technology assessment were bound to take the rules of argumentation seriously. The presence of advocates of opposing views guarantees that the full range of arguments and counterarguments becomes visible. Selectivity cannot be maintained. The participants may well be committed to restrictive positions and strategic interests, but as long as they participate in the process of communicative interaction they can hardly ignore requests to substantiate reasons, to take objections into account, to present the empirical evidence for a statement, and to consider counter-evidence.

What happened to the rhetoric of risk prevailing in public debate, within the social context of the technology assessment? First of all, the neccessity of determining whether a risk exists and whether it is acceptable was reaffirmed. The existence of risks is a matter of empirical argument (causal links, probability of events). These arguments may be controversial and in some cases insoluble; it was, however, uncontested that they constitute in principle a proper domain of scientific inquiry and expertise. The acceptability of risks refers to normative issues (moral and political evaluation). Such issues are outside the competence of science; in a democratic society it is the (lay) citizens who take the decisions.

Discussions of risk in our technology assessment proceeded from recognizable to hypothetical risks and to the question of dealing with uncertainty in general; they focussed on the comparison between transgenic and non-transgenic plants, and they resulted in a call to shift the burden of proof for the absence of risk and to make demonstration of socio-economic need a prerequisite for the introduction of new technology. All these aspects have been raised in the public debate over the risks posed by genetic engineering. What the technology assessment shows is that there is a logical order or pattern of transformation, which the criticism of genetic engineering will follow if put to the test of argumentation.

## 3. Recognizable risks from transgenic herbicide resistant plants

Three types of recognizable risk were discussed:

(1) Unexpected toxic or allergenic substances might be induced in transgenic herbicide-resistant plants. Several mechanisms exist for this event: The substances could be formed during the metabolising of the non-selective herbicide in the plant (if the resistance is due to such metabolism), or the transgenic gene product could itself have a toxic or

allergenic potential, or it could activate a potential inherent in the host plant. It was agreed among the participants that these possibilities are real risks which could pose health hazards if the transgenic plants are for human consumption, and that they need testing and regulation. It was, however, pointed out that these risks are not specific to transgenic plants; they also exist in plants produced by conventional breeding techniques. The question, therefore, was how extensive the testing should be and whether transgenic crops should be regulated more strictly than new conventional varieties.

(2) Much the same argument was applied to the risk that herbicide resistant plants might lead to feral populations. This could come about either through mutations which increase the fitness of the cultivated plants or (most likely) through hybridization with wild relatives. These mechanisms apply both to transgenic and non-transgenic plants. The possible harm that could result from feral herbicide resistant plants seems to be limited. Such plants could become weeds in agricultural systems and would then mean financial losses for the farmer and more particularly for the herbicide manufacturer. They could also temporarily increase the herbicide load on the field - if farmers tried to kill resistant weeds by increasing the dose or the number of applications. It is unlikely, however, that wild herbicide resistant plants pose any ecological threat to natural habitats. The trait of herbicide resistance does not provide a selective advantage outside the area where the herbicide is being applied.

(3) Herbicide resistance genes might be proliferated from transgenic plants to soil bacteria through horizontal gene transfer. Although such transfer can also occur in plant genes from non-transgenic varieties, the transfer rates should be theoretically higher for certain transgenes - depending on the gene construct used. In any case horizontal gene transfer will remain a rare event. If it happens and the resistance genes are expressed in soil bacteria the foreseeable consequence would be selective growth of these bacteria during the time when the respective herbicide is employed. In addition the transformed bacteria could influence the soil chemistry by releasing metabolic products which have not yet been in that soil. Discussions in the technology assessment focused on the question of whether such consequences could really be considered a damage. Many agricultural practices which are uncontroversial, like crop rotation or ploughing, have considerable influence on the soil chemistry or induce wide fluctuations in microbial populations. Temporary selection of herbicide resistant soil bacteria is quite normal with most herbicide applications. A loss of soil functions is not to be expected.

The risks from transgenic herbicide resistant plants described do not seem dramatic if one compares them to the risks of conventional agricultural crops and practices. Basically, no risk from transgenic plants is recognizable which is not already known from non-transgenic plants. At this point the debate moved one step further: It was argued that it is not enough to consider recognizable risks which can be described and tested. The real problem with transgenic plants is rather that we do not exactly know what the risks are. We can neither foresee all the possible consequences, nor control them through preventive testing.

**4. Uncertainty, limits of foresight, hypothetical risks**

The critics of transgenic herbicide resistant plants argued that we cannot rule out physiological and ecological effects which are different from those which we know from non-transgenic crops. How could the possibility be ruled out, that the transgenic gene product induces toxic or allergenic potentials which have never been observed in the host species, or that the transfer of herbicide resistance genes has unexpected side effects which enhance the fitness of the plants? Theoretically, herbicide resistance genes which

are transmitted to wild species through hybridization could become stabilized, neutrally to selection, in these populations. It is unforeseeable what their impact on the evolution of species and ecosystems could be under changing conditions. Finally, soil bacteria transformed through horizontal gene transfer might release substances which are qualitatively different from new substances induced by changing conventional agricultural practices and hence could be more harmful than the latter.
The participants in the technology assessment agreed that uncertainty exists about the consequences which the introduction of transgenic plants might have. They disagreed about the conclusions to be drawn from such uncertainty. The critics concluded that the principle of minimization of risk required a ban on transgenic herbicide resistant plants. For them uncertainty of prognosis constituted not only an unavoidable but also an unacceptable risk. Others rejected this conclusion. They pointed out that uncertainty of prognosis is also endemic to conventional breeding practices and has never been considered a sufficient reason to ban new varieties.
In conventional breeding, too, one can neither foresee nor control what the physiological impact of new genes might be given the genetic background of the host plant. Unexpected and undesirable side effects are abundant and must be coped with through testing and selection in the further development of new varieties. The testing process is necessarily limited. One can never screen all the plant substances to detect changes which might be toxicologically relevant. Phenotypic changes in new plants will be identified through selection procedures only if they are undesirable in terms of the breeding goals. Other changes, which may still be ecologically relevant (like increased stress resistance), may go unnoticed. With respect to long-term impacts on the evolution of species and ecosystems one can only say that they are as indeterminate and unforeseeable for conventional plants as for transgenic plants.
As a result, comparison with conventional plants does not only 'normalize' the recognizable risks of transgenic plants but also the uncertainty and the hypothetical risks which might be implied in the fact that we have limited foresight of the possible consequences of such plants. This result refutes the main argument raised in public against transgenic plants, namely that such plants will confront us with new and specific risks. Not surprisingly, therefore, the question of whether the comparison of conventional and transgenic plants is legitimate became a central focus of the discussions in the technology assessment.

## 5. Is genetic engineering different? Discussions about the 'new quality' of transgenic plants

Risk comparisons are frequently used as a method of evaluation. Since the level of acceptable risk cannot be objectively determined one refers to what has actually been accepted in comparable cases in the past. However, the criteria for comparability are controversial. Arguments, for instance, which compare voluntary and involuntary risks are not considered conclusive. The same holds for the comparison of dread risks, which could lead to single catastrophies, with diffuse risks, where the same absolute amount of damage slowly accumulates through scattered events.
The comparison of transgenic and conventional new plant varieties did have an obvious legitimacy in the technology assessment, because it was the critics of genetic engineering who first introduced it. The Öko-Institut, from which a main report was commissioned referred continuously to the problems, side effects and uncertainties known from conventional plants in order to illustrate the possible risks implied in transgenic plants. This approach invited the argument that consequently the risks and uncertainties of transgenic plants must be the same as those of non-transgenic plants. To refute that argument it had to be shown that genetic engineering is 'different'. Two points were raised in this respect in the technology assessment:

(1) Genetic engineering allows transfer across species barriers. Hence metabolic pathways can be introduced into a host plant which have never belonged to that species and could not have been achieved through natural evolution or conventional breeding. Such new pathways are a specific uncertainty factor and therefore the risk of uncontrollable physiological or ecological side effects is higher in transgenic plants than in conventional new plants.

(2) The transfer of genes through genetic engineering disturbs the genomic context of the host plant. Transgenes are inserted at random in the genome. Therefore, position effects are to be expected which can induce changes in the transformed plant that are unrelated to the information coded in the transgene and unforeseeable.

In the technology assessment and the public debate, these were the key arguments for the assumption that transgenic plants pose higher risks than conventional new plants. The arguments refer to hypothetical risks. They assume that there are more unexpected side-effects in transgenic than in non-transgenic plants. There is no empirical evidence yet that these specific side effects exist, nor is it possible to describe them theoretically in more detail. However, as one critic put the argument, one can infer from the specific quality of genetic engineering that transgenic plants present a "specific type of uncertainty".
This reasoning claims that transgenic and non-transgenic plants are not in fact comparable. At the same time it reaffirms the legitimacy of the comparison in principle. If the arguments given are not sustainable, the comparison with conventional plants becomes valid, and the assumption that transgenic plants constitute a specific risk unfounded.
The argument of the possibility of context disturbances (position effects) was particularly prominent in the German discussion about the specific risks of genetic engineering. It could not be sustained in the technology assessment. Alterations of the genomic context are not specific to genetic engineering, they also occur with conventional breeding techniques and when natural transposable elements, which are known to exist in most plants, jump around in the genome. Transposons, too, are inserted at random. After a lengthy discussion, no argument remained to demonstrate why context disturbances in transgenic plants should be different. The argument that the possibility of context disturbances puts a "specific type of uncertainty" on transgenic plants was also invalidated through comparison.
The argument that new metabolic pathways which are unknown in the host plant constitute a specific hypothetical risk, was considered as valid in principle. But again, comparison weakened the argument. While it may be true that the probability of side effects is theoretically higher in transgenic plants, because (and if) new metabolic pathways are transferred, it would also be true that the probability of side effects is theoretically higher in non-transgenic plants, because (and if) with crossing techniques an uncontrolled number of undetermined genes is exchanged, all of which can interact with the existing metabolism, whereas with genetic engineering one exactly identifiable single gene product is transferred. No method exists to balance these two countervailing factors. The argument is one of side effects which are conceivable, but indeterminate and unforeseeable. The discussions in the technology assessment suggested that the assumption that transgenic plants will have more unexpected side effects than non-transgenic plants was as good in theory as the contrary assumption that transgenic plants have less unexpected side effects than non-transgenic plants. The latter assumption is in any case more plausible if the gene transfer introduces metabolic pathways which are otherwise already established in the host plant.

## 6. From hypothetical to speculative risks

The arguments in the technology assessment about the assumed qualitative distinctiveness of genetic engineering did not resolve the controversy over the specific risks of genetic engineering. It was argued that even if our present knowledge does not show relevant differences between genetic engineering and conventional breeding it is nevertheless possible that such differences do in fact exist and may become apparent later. And the possibility cannot therefore theoretically be excluded that specific and severe additional risks are associated with transgenic plants. With this argument the discussion moved from hypothetical to speculative risks and the rule that risk assumptions have to be substantiated was abandoned, which amounts to a reversal of the burden of proof.

Hypothetical and speculative risks were differentiated from one another in the technology assessment by distinguishing risk scenarios that build on known mechanisms from scenarios that presuppose the existence of new mechanisms for which we have no evidence so far. The scenario of additional side effects through transgenes that code for metabolic pathways which are new for the host plant is an example of a hypothetical risk. It refers to known mechanisms, which are as follows: The new gene product could find metabolic substrates in the host plant that differ from the substrates in the donor organism. In this case the consequences for the plant cannot be anticipated from the knowledge of the gene and its function in the donor organism. The probability that alternative substrates are found is theoretically higher when transgenic products are introduced which have never been in the plant metabolism, than with gene products which are interchanged through the crossing of plants with a broadly identical metabolism.

An example of a speculative risk is the assumption that there may be hidden differences between transgenic and non-transgenic plants which we cannot yet describe and which could lead to severe harm. Such differences may be a logical possibility, but as long as they are not supported by empirical indicators or a plausible theoretical model they are mere speculation. The same is true for worst-case-scenarios which assume that the risks which are known to be implied in releasing new plant varieties could lead to catastrophic results in the case of transgenic plants. It is speculation to assume that unexpected physiological changes induced by transgenes could possibly turn our established food plants into deadly poisons, or that transgenes for herbicide resistance could upset natural ecosystems if they are transmitted to wild plants through hybridization. No causal links are known which could trigger such consequences. In order to devise such a worst-case-scenario, hidden mechanisms must be postulated which operate specifically in transgenic plants.

Most participants in the technology assessment accepted the proposal that hypothetical risks should to a certain extent be taken into account in legal regulation, for instance by imposing additional testing or monitoring. They rejected, however, regulations for speculative risks. This position is not completely in line with the legal history of genetic engineering. The guidelines imposed after Asilomar may well be an example of precautionary measures that respond to the fear that something might happen which we do not know of and cannot yet substantiate; that is, they refer to speculative risks. Those measures must be seen as the price which has to be paid for the introduction of a revolutionary new technology. In our technology assessment the critics of genetic engineering went much further. They argued that speculative risks should be taken seriously and that the technology should be banned because its consequences are unforeseeable and one cannot exclude unknown risks. This conclusion is only convincing if the burden of proof is reversed. It presupposes that one does not have to prove the existence of risk in order to restrict a technology, but has instead to prove the absence of risk, in order to introduce new technology.

## 7. Reversing the burden of proof?

As a rule, new technology has to prove its relative safety by passing through a filter of preventive testing before it can be released into the society. The general principle is, however, that the burden of proof is with those who claim risks. Those who argue that a technology which has survived the testing is nevertheless not safe, have to demonstrate the additional risks. If they are unable to do so, the technology will be authorized. This regulation favours innovation. It is based upon the value judgement that hidden risks and unforeseeable consequences which escape our best efforts of anticipation may be a worthwhile price to pay for new technology. And it implies confidence that society will be able to cope with such risks and their consequences if they occur in the future.

Apparently, neither broad acceptance of innovation nor confidence in our ability to repair things after the fact can be taken as granted in society, and they were definitely not shared by the critics of transgenic plants in our technology assessment. The critics demanded a complete reversal of the burden of proof. Given the fact that herbicide resistance genes cannot in nature be contained or retrieved, the possible harm caused by hidden risks might be irreversible once transgenic plants are released into the environment. Therefore, it was argued, the release of such plants should not be allowed until it is proven that this implies no additional risks.

Under such a rule any kind of risk assumption becomes a conclusive objection: Remote or speculative scenarios do not have to be justified by the critics, but to be refuted by the proponents of the technology. Consequently, the proposal to reverse the burden of proof suggests itself as a strategy to maximize the impact of risk arguments.

The proposal remained controversial in the technology assessment. One argument was that it deters innovation and favours the technological status quo. Its underlying value premise is that society has little to lose when innovation is slowed down or blocked and that we are better off living with the risks and uncertainties of old technologies than with the risks and uncertainties of new technologies. This premise was opposed by many. Although technologies with which we are familiar are somewhat reassuring because we have already had some time to detect their hidden problems, we are not necessarily on the safe side when we opt for their continued use. Established practices can be fraught with unkown risks which we realize only tomorrow, and which may have consequences as severe as those of the unknown risks of new technology. The climatic change through continued burning of fossil fuel may serve as an example.

Another argument was that with a radical reversal of the burden of proof minimization of risk ceases to be a criterion to distinguish which technology should be allowed or forbidden. The unsubstantiated assumption that a new technology could have some unknown risks can easily be raised against any new technology and it can hardly be refuted. Empirical proof of a negative fact (in this case: that unknown risks do not exist) is a logical impossibility. To insist on such a proof would exclude any innovation at all. At this point in the discussion the critics of genetic engineering abandoned the general framework of risk arguments and shifted to socio-economic need as a criterion.

## 8. Socio-economic need: a fourth hurdle for innovation?

Common sense dictates that we should bear only those risks which are worth the price. Hence risk-benefit-analysis is prescribed in many regulations for new technologies, for instance, for new medicines, or according to German law also for the release of genetically modified organisms and products. So far, however, there are clear limits for such analysis: (1) It has to refer to the usefulness of the technology in general, not to concrete and real need; (2) Usefulness is required to compensate for recognizable risks, not for the uncertainty of possible hidden or unknown risks. In German legal doctrine such uncertainty is considered a "residual risk", which can legitimately be imposed on society without compensation.

Within these limits a risk-benefit-analysis cannot be used to reject a technology just because it arouses vague fears that something might happen, or because we do not really need it. That is exactly why critics of genetic engineering want these limits to be removed. Their argument in the technology assessment was: It may be impossible in principle to forego the uncertainty of unknown risks and unforeseeable consequences when one introduces new technology. Such uncertainty should, however, only be imposed on the society if there is a real need for the technology. Therefore, even residual risks cannot be justified if the new technology is unnecessary because it serves no acceptable purpose, or appropriate alternatives are available. This argument has been raised before by representatives of the Green Party in the European Parliament, under the heading of a "fourth hurdle" in the regulation of innovation: New technologies or products should not only pass tests of safety, efficacy and quality, but also the test of socio-economic need. In the technology assessment critics concluded that transgenic herbicide resistant plants had to be prohibited under the test of socio-economic need. They saw no ecological advantage and little if any agronomic use, since efficient weed control could in almost all cases be achieved with the available selective herbicides, and non-chemical methods of weed control would be the ecologically preferable alternative. Arguments as to whether transgenic herbicide resistant plants are useful and satisfy a proper need were accepted as a necessary and legitimate topic of inquiry (and controversy) in the technology assessment. The proposal, however, to make "socio-economic need" a legal prerequisite in the regulation of the technology was rejected by most.
Firstly, it was asked: Who should decide whether there is a proper need for the technology? Acceptable social demand is not an operational legal criterion. To determine such demand implies (particularly if available alternatives have to be weighed against one another) wide-ranging political choice, which is the prerogative of the legislator. Therefore, normal standards of the rule of law seem to exclude that decision-making is relegated to the regulatory agencies, and the proposed "fourth hurdle" would violate the existing constitution. The solution would have to be that the determination of socio-economic need is left to parliament or decided by referendum.
Secondly, and more significantly, it was argued that a legal test for socio-economic need replaced market mechanisms with political decision-making. Markets have become the decisive mechanisms in our society in determining whether a new technology or product is needed. Economic demand is taken as an irrefutable indicator of real need. The state may control the market to protect the moral order and to minimize risks, but it may not try to "educate" market actors by selecting which economic demand constitutes a proper need and which not. Full political control over the need for a technology is confined to decisions on subsidizing the technology, or to cases where the state has a virtual demand monopoly, as in military or infrastructure technology. Most participants in the technology assessment defended the established institutional balance between markets and political decision-making. They pointed out that the decline of the socialist countries had demonstrated that no model for an efficient economy exists in which decisions on innovation and investment are the domain of politics.

The critics conceded the problems but insisted that nevertheless some revisions of the institutional balance are necessary and that the question of whether we really need a new technology must be put on the political agenda. The controversy over this point remained unresolved in the technology assessment, as it is in the rest of the society.

### 9. The quest for democratic control of innovation

The postulate that socio-economic need should be a legal prerequisite for the introduction of new technology was the logical endpoint of the arguments raised in the technology assessment against transgenic herbicide resistant plants. The discourse had proceeded

through a number of stages: from well founded risk assumptions to hypotheses, to the mere uncertainty of unknown risks or unforeseeable consequences, to the question of whether herbicide resistant plants are useful or needed. The debate ended in open political dissent, but the issue was transformed. As at least the disinterested observer will notice, the dissent at the end of the discussion is not the same as in the beginning. It had shifted from legal arguments about risks to political arguments about the future development of society. This fact seems to indicate that no conclusive arguments against the use of transgenic herbicide resistant plants could be formulated within the framework of established risk regulation, and that the real issue is the quest for democratic control of the process of technological innovation.
The critics of genetic engineering were not, however, prepared to ratify these findings as a conclusion of the participatory discourse in the technology assessment. They withdrew their participation at the beginning of the final conference, at which they were expected either to accept the proposed conclusions or to reject them, giving additional reasons why they considered them incorrect. The critics justified their withdrawal by citing procedural flaws in the technology assessment. One can assume that other reasons played a role. Obviously, it would have been difficult for the critics to declare explicitly that the conflict is not about risks, but about social and political goals, after they had committed themselves categorically to the rhetoric of risk, and used it successfully in the mobilization of general public. Furthermore, they would probably lose a powerful resource if they confined themselves to a controversy over conflicting goals. In that case it would be less easy to argue that the dominant policy is irresponsible and offends against generally accepted values. When risk arguments do not play a major role, it seems more legitimate to apply the majority rule in the choice of conflicting goals.
When we conclude that the controversy over the transgenic herbicide resistant plants is in fact more about democratic control than about relevant risks, this is an evaluation from the perspective of an observer of the technology assessment; it is not the consensus opinion of the participants. Occasionally there were indications, however, that the critics may indeed share this view. One participant conceded at the end of a discourse in which he was pressed to deliver arguments for the alleged risks: "We would not have to discuss the possible risks so much if we had appropriate political institutions for a meaningful discussion about the path of technological development our society should follow."

* The structure and the history of the technology assessment is described in: *Wolfgang van den Daele*: Technology Assessment as a Political Experiment. In: *Wolfgang van den Daele, Alfred Pühler, Herbert Sukopp (eds.)*: Verfahren zur Technikfolgenabschätzung des Anbaus von Kulturpflanzen mit gentechnisch erzeugter Herbizidresistenz. Wissenschaftszentrum Berlin 1994 (discussion paper FS II 94-319).

The reports and proceedings of the technology assessment are available (in German) as discussion papers FS II 94-302 - 318. A Summary Report in English will be published in 1995.
Requests for discussion papers should be addressed to the author.

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