# Evaluation of future developments in agrobiotechnology: The potential roles of protein nitrogen and sulfur for better crop plants

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## Abstract

Agricultural biotechnology provides a whole array of measures to improve crop plants. In addition to the commercially already successful traits of herbicide and insect resistance as well as currently developed traits of biofuel plants as renewable resources the improvement of yield and quality of crops using nutrient-based traits is an innovative approach. Balanced supply of nitrogen and sulfur in combination with transgenic traits bears the potential to improve seed nutritional value as well as to enhance defense against plant pathogens.

*Keywords: plant biotechnology, crop improvement, nitrogen, nutritional value, sulfur*

## Zusammenfassung

**Bewertung zukünftiger Entwicklungen in der Agrar biotechnologie: Das Potential von Stickstoff und Schwefel in Proteinen zur Verbesserung von Nahrungspflanzen**

Die landwirtschaftliche Biotechnologie stellt eine Vielzahl neuer und die Züchtung ergänzender Ansätze zur Verbesserung von Nahrungspflanzen zur Verfügung. Zusätzlich zu den bereits kommerziell verwendeten Merkmalen der Herbizid- und Insektenresistenz sowie den gegenwärtig entwickelten Sorten für die Erzeugung erneuerbarer Energien stellt die Entwicklung ernährungsbasierter Merkmale eine innovative Strategie dar. Die ausbalancierte Versorgung mit Schwefel und Stickstoff in Kombination mit geeigneten transgenen Merkmalen hat das Potential den Ernährungswert von Saatgut zu erhöhen und darüber hinaus die Abwehr gegen Pathogene zu verbessern.

*Schlüsselwörter: Ernährungswert, Pflanzenbiotechnologie, Pflanzenzüchtung, Schwefel, Stickstoff*

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## 1 Introduction

Nitrogen and sulfur are essential macronutrients for plants and need to be balanced to achieve optimal yield and quality. Deficiencies in nitrogen, phosphorus, potassium but also sulfur, although being the least required one of the six macronutrients, are limiting factors for plant productivity, notably in high-yield agriculture. Nitrogen and sulfur applications need to be monitored to achieve optimized nutrient use efficiency. The genetically defined nitrogen:sulfur (N:S) ratio in proteins of a plant is only sustained if vegetative growth enhanced by nitrogen fertilizers is matched by sulfur availability (Oenema and Pastma, 2003). Nutrition during vegetative or generative growth strongly affects not only grain yield but also composition and thus quality. The term quality in this context can refer either to biological or technological value. This may be illustrated by barley varieties for beer brewing that have higher carbon:nitrogen (C:N) ratio and sulfur contents compared to barley used for food and feed in order to optimize flavour development after fermentation. Sulfur supply and concomitant sulfur content of storage proteins are known to be important for baking quality of bread wheat (Zhao et al., 1999).

Traditionally, crop plants were not bred under conditions of nutrient limitation. Therefore, increased biomass production and improved protein composition as realized with new cultivars significantly enhance the demand for nutrients that is conventionally met by organic or inorganic fertilization. Such new cultivars today also include transgenic crops. All crop plants that underwent breeding processes (by crossing or laboratory methods) are genetically altered *per se.* Thus, the term 'genetically modified organism' or GMO for plants that have been engineered by gene transfer methods is avoided here (Hell and Hillebrand, 2006). Such cultivars are instead named transgenic plants. Yield increase achieved by using transgenic crop plants carrying the currently dominating traits insect resistance and herbicide tolerance (see below) can be treated in the same way as conventionally altered crops with respect to mineral nutrient requirement. Due to the fact that nitrogen applications usually exceed 100 kg/ha, reports on specific nitrogen deficiencies of transgenic crops are rare, suggesting no extra nutrient requirements for production of the currently planted varieties (Crosby, 2003; Powell, 2006). However, other nutrients, such as sulfur, may be required in particular crop improvement strategies. Breeding and biotechnology aim at the improvement of sulfur relations at several levels, but most importantly in sulfur-rich seed storage proteins. Apart from lysine and some other amino acids, cysteine and especially methionine are often limiting the nutritional value of food and feed for humans and non-ruminant animals (Tabe and Higgins, 1998; Baker, 2006). A new sulfur-related approach to stabilize yield during pathogen attack is based on the activation of sulfur metabolism and, most likely, optimized sulfur nutrition (Kruse et al., 2005; Rausch and Wachter, 2005; Bloem et al., 2007; Kruse et al., 2007). It therefore seems advisable to carefully analyze the physiology of each individual trait within the context of existing crop plant varieties to achieve optimal production. The following article will evaluate the potential of sulfur compounds containing nitrogen for seed quality and plant defense in view of the currently used biotechnological approaches.

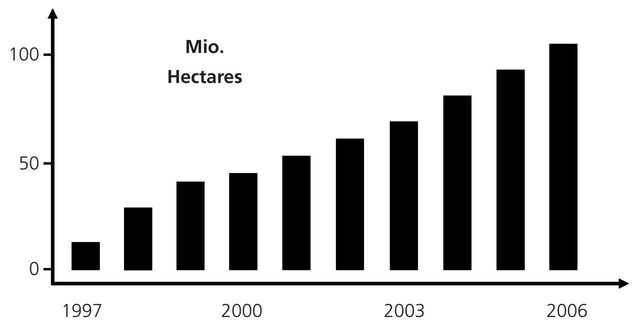
## 2 Global situation of agricultural biotechnology with respect to transgenic crops

Agrochemistry including fertilization management today forms one major option to close the gap between food and feed production and the nutrient energy requirements of a continuously growing world population of an estimated 6.6 billion in 2008. Improved agricultural techniques and breeding, particularly the so-called 'green revolution', formed a second pillar of productivity increase to close this gap in the past. Additional concepts to enhance plant production for food and feed are required to meet the challenges of sustained productivity, increasing world population and decreasing fertility of soils due to erosion and irrigation. Agricultural biotechnology is such a third option to meet these challenges using combinations of conventional breeding with marker-assisted breeding and/or gene transformation into crop plants, respectively. So far, commercial production of transgenic plants aimed at improved and stable yield using two major traits: herbicide tolerance and insect resistance or the combination of both. Reduced input levels of plant protection chemicals such as herbicides or insecticides for the existing transgenic crops at equal or higher yields per acre are meanwhile a sound contribution to sustainable agricultural practices. Crop plants with improved nutrient use efficiency for nitrate and phosphate would constitute an important contribution to further sustain and manage arable soils

Today, about 7 % of the world's acreage is planted with transgenic seeds. Commercial production of transgenic crop plants to a large extent takes place in the Americas and Asia while Europe is clearly lagging behind. The main effort is observed in Spain leading the European adoption of transgenic crops with only some 60,000 ha of Bt-maize being planted. Although the de-facto moratorium in Europe was lifted in 2004, the European Union's regulatory process for transgenic plants is very slow and complex. In Australia, the federal states of New South Wales and Victoria have revoked the moratoria to the cultivation of transgenic crop plants of 2004 and will allow the planting of oilseed rape early 2008. The two states produce about

50 % of the 1.5 million tons of oilseed rape annually produced in Australia. In Africa, depending on the country, legal regulations allow cultivation of transgenic crops.

The International Service for the Acquisition of Agribiotech Applications (ISAAA; http://www.isaaa.org), a non-profit organization for transfer of crop biotechnology applications, lists the global area planted with transgenic crops in more than 20 countries in 2006 as 102 million hectares, up from 90 million hectares in 2005 (Figure 1). Average annual increases of about 10 % were representative since the beginning of commercialization in the USA in 1997 and similar increase rates can be expected for the near future, in particular if India and China legalize large scale cash cropping of transgenics plants. The four major crop species so far were, almost exclusively, soybean, maize, oil seed rape (canola) and cotton. In 2005 71 % of these carried transgenes for herbicide resistance, 18 % for *Bacillus thuringiensis*-based (Bt) insect resistance and 11 % for the combination of Bt and herbicide resistance. Both resistances belong to the group of input traits that have been defined as mostly producer oriented and affect agricultural performance properties. Their main advantages are the improvement of plant vigor and biomass to enhance and stabilize yield. This first generation accounts for almost all currently planted transgenic crops. Future traits, including some sulfur and nitrogen use efficiency based improvements, refer to rather consumer-oriented approaches and are called output traits. These are designed to enhance mainly nutritional quality or processing characteristics.




Source: ISAAA, 2007, see text.

Figure 1:
Increase of area cultivated with transgenic plants in million hectares

## 3 Improvement of seed sulfur contents

Sulfur and nitrogen based improvements aim at the protein composition of vegetative or generative plant parts, respectively. The demand for better nutritional value of seeds is largely based on the requirement of humans and non-ruminant animals for the essential amino acid methionine. Cysteine is required for infants and can serve as substrate for the trans-sulfurylation pathway to form methionine, thus complementing a large part of methionine requirement in adults (Müntz et al., 1998). These nutritional properties are especially important for humans in geographical areas where vegetarian dominated diets based on only few staple crops for caloric intake, for example rice or lentils. For high-yield animal production (poultry and monogastric animals) the diets are mostly mixtures of grains of the legume and the cereal families. Leguminosae (e.g. soybeans) are known to be relatively low in methionine and cysteine but high in lysine. In contrast, cereals (e.g. wheat) are notoriously low in lysine but in comparison high in sulfur amino acids, although these are still regarded as targets for improvement (Wang et al., 2003). Thus, mixtures of diet components are provided to compensate for these shortcomings in amino acid composition described above. However, these measures often are still insufficient to achieve maximal animal production and give rise to supplementation by methionine, lysine and several other amino acids to enhance the biological value of feed (Müntz et al., 1998). In the past, amino acids for feed have been chemically produced but are nowadays increasingly being replaced by fermentation products (Leinfelder and Heinrich, 1995). Energetically more efficient and sustainable would be grains with enhanced contents of nutritionally valuable factors, in particular cysteine and methionine.

In such a way fortified food and feed components are expected to be provided by plant biotechnology in transgenic crops using so-called push and pull approaches. Push approaches try to enhance input or biosynthetic activity of a pathway hoping that the endproduct will accumulate. In case of cysteine and methionine it is not the concentration of free metabolites but the amount of reduced sulfur incorporated into storage protein that is relevant, since in seeds 20 - 40-times more sulfur can be bound in proteins compared to free sulfur amino acids (e.g. Chiaiese et al., 2004). Figure 2 indicates where push approaches have been attempted in the sulfur assimilation pathway. Mostly model species such as tobacco and Arabidopsis or species that are relatively easy to transform, e.g potato or rice,

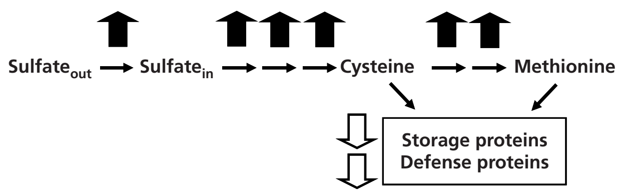



Figure 2:
Simplified scheme of transgenic approaches to enhance contents of sulfur amino acids in seeds or for defense reactions. Push approaches are shown by black arrows, pull approaches by open arrows.

have been used so far to avoid costly try-and-error experiments in transformation processes of major grain crops such as soybean and corn. In summary, these approaches resulted in moderate increases of free sulfur amino acids but did not yield to proportionally higher content in protein-bound sulfur. In summary, the results of approaches targeting the engineering of storage proteins were never anywhere near the requirements defined by food health organizations and industry (for review see Hesse et al., 2004; Sirko et al., 2004; Galili et al., 2005; Hesse and Hoefgen, 2008). In this context it is interesting to note that manipulation of mineral nitrogen uptake other than fertilization was also of little success (Hell and Hillebrand, 2001) and may thus indicate general problems of mineral uptake regulation.

In pull approaches an artificial sink in form of e.g. overexpression of sulfur-rich proteins under the control of a seed-specific promoter is introduced. So far, mostly legumes (soybean, pea, lupin, vetch) but also rice have been transformed as prominent nitrogen-rich food and feed constituents. Several experiments achieved remarkable results using sulfur-rich seed storage proteins such as the sunflower 2S albumin (SSA) that carries 7.5 % cysteine and 15.5 % methionine on weight basis: Feeding trials with rats using transgenic lupin seeds resulted in significant weight increases (Molvig et al., 1997). These pull approaches appear to be promising, but still run below the expected rates of sulfur contents (de Lumen et al., 1999). Several reasons may account for this observation. They include the plasticity of seed composition in response to nitrogen and sulfur availability (Tabe et al., 2002; Chiaiese et al., 2004), the complex processing and packing of storage proteins (Shutov et al., 2003) and the different allocation patterns of oxidized and reduced compounds used for sulfur and nitrogen transport in different plant types (Anderson and Fitzgerald, 2001). These robust developmental and metabolic programs show remarkable plasticity and have evolved over many generations to ensure survival of these plant species. They need to be studied in detail before they can be outwitted by plant biotechnologists.

## 4 Activation of sulfur-based defense against fungal pathogens

Sulfur-rich proteins in vegetative as well as seed are important components of the plant's defense reaction to pathogens. Sulfur-containing compounds in general are intimately connected to biotic stress resistance (Rausch and Wachter, 2005; Hell and Kruse, 2006). Whether or not sulfur nutrition affects a plant's ability to form protective sulfur compounds is currently under investigation (Kruse et al., 2007). The increased tolerance of oilseed rape against fungal pathogens in field experiments when optimally supplied with sulfate was termed 'sulfur-induced resistance' and was recently reviewed by Bloem et al. (2007). Controlled growth chamber experiments using the model plant *Arabidopsis thaliana* and established microbial pathovars defined the term 'sulfur-enhanced defense' (SED) and separated the resistance effect from nitrogen application effects (for review see Kruse et al., 2007). A number of sulfur compounds are probably jointly responsible for the improved tolerance against pathogens (Hell and Kruse, 2006). These observations open the possibility to engineer and select for new plant varieties. However, the well-known glucosinolates of the Brassicacea family, although rich in nitrogen and sulfur, are probably unrelated to SED. The pungent taste of the preformed glucosinolates deters feeding insects that destroy the cells. The release of the stored glucosinolates together with the degrading enzyme myrosinase results in breakdown products including isothiocyanates that exhibit toxicity to the feeding insects. While some degradation products such as sulphoraphane are classified as candidates for promotion of human health and cancer prevention and protection by activation of phase I and II detoxification enzymes (Finley, 2005), their efficiency against bacterial and fungal pathogens is seen as controversial (Burow et al., 2008). More likely involved in defense against microbes are the sulfur-containing phytoalexins of the camalexin group that are only produced upon fungal and bacterial infection in Brassiceae plants. Camalexin shows toxicity *in vitro* against both kinds of pathogens (Tsuji et al., 1992). Elemental sulfur is a taxonomically far more wide-spread defense compound in plants than camalexins (Cooper et al., 1996; Williams and Cooper, 2003). $S^0$ can exist preformed but may also be induced upon infection and accumulates in vascular tissue to prevent the spread of infections along the plant transport routes.

Sulfur-rich proteins are probably the group with strongest impact on plant pathogen defense. They comprise several classes including thionins, defensins, lipid-transfer proteins, snakins and others, according to their primary amino acid sequences (Garcia-Olmedo et al., 1998). Sulfur-rich proteins share a small size of 4 - 11 kDa, have a mostly polar amino acid composition and several disulfide bridges (2 to 6) amounting to 10 - 20 % of total residues provide a compact tertiary structure. Most of the known thionins and defensins carry predicted signal peptides for secretion into the apoplast, hence are localized at the primary infection sites in leaves and seeds. Some are preformed, but most plants contain gene families encoding thionins and defensins the expression of which is induced upon infection with fungal pathogens. It needs to be investigated whether reduced sulfur is sufficiently available for fast synthesis of sulfur-rich proteins under less than optimal sulfur supply, thereby reducing the defense potential of the plant (Kruse et al., 2007). Relatively little is known about the precise physiological functions and mechanisms of toxicity of sulfur-rich proteins but they have been tested comprehensively *in vitro* against phytopathogenic bacteria, oomycetes and fungi, showing varying degrees of activity (Bohlmann and Apel, 1990; Thomma et al., 2002; Kruse et al., 2005, for review). The thionins and defensins appear to be most important for plant defense and thus constitute attractive targets for genetic engineering approaches. A great variety of suitable fungal test strains was used to assess the biocidal properties of these proteins. A comprehensive selection is listed in patents US 5.942.663, US 5.919.018 and US 5.986.176.

Thionin or defensin genes are potentially valuable traits in crop plants, achieved either by transgenic overexpression or marker-assisted introgression into elite lines. An early example was the constitutive overexpression of a thionin that resulted in enhanced resistance of *Arabidopsis thaliana* to *Fusarium oxysporum* infection (Epple et al., 1997). A more applied approach of defensin expression was the transformation of rice with the Wasabi defensin known from Japanese Radish (*Wasabia japonica*; Kanzaki et al., 2002). The Wasabi defensin had been selected due to its toxicity against the rice blast disease, a globally acting fungal pathogen. In transgenic rice lines the Wasabi defensin caused resistance levels comparable to a rice cultivar carrying the true blast resistance gene used as a positive control in leaf lesion tests. The resistance was stable over several generations, suggesting a durable and wide-spectrum field resistance against various rice blast races (Kanzaki et al., 2002). However, despite a considerable list of successful approaches (Kruse et al., 2005), it should be cautioned that a number of experiments failed to confer resistance for unknown reasons (Brockaert et al., 1995; De Bolle et al., 1996; Epple et al., 1997).

The publicly limited availability of information on genetics, genomics, comprehensive gene expression data and metabolomics of the majority of crop plants in combination with elaborate and costly transformation protocols slow down progress in agricultural biotechnology of specialty crops. Further research into a wider variety of crop plant species and their wild relatives will greatly support progress towards the identification and exploration of additional sulfur-rich proteins and compounds with suitable defense properties.

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