

Production of new/modified proteins in transgenic plants

Karin Herbers* and Uwe Sonnewald†

In recent years, plant biotechnology has almost reached maturity. Transgenic plants engineered to be herbicide- or insect-resistant are outcompeting conventional crop plants and pest managing strategies leading to a major rethinking of the chemical industry. Due to worldwide efforts to study genome function, almost any gene of interest is, or will soon be available. Thus, identification of gene function will be the major challenge of the next few years. In combination with established gene-delivery systems and desired promoter and targetting sequences, gene discovery will open a fascinating and new field of crop plant design. Transgenic plants engineered to produce superior polypeptides have already been created and the first examples are entering clinical and industrial trials.

Addresses

*SunGene GmbH and Co. KGaA, Corrensstrasse 3, 06466 Gatersleben, Germany;
e-mail: herbers@ipk-gatersleben.de

†Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstrasse 3, 06466 Gatersleben, Germany;
e-mail: sonnewald@ipk-gatersleben.de

Current Opinion in Biotechnology 1999, **10**:163–168

<http://biomednet.com/elecref/0958166901000168>

© Elsevier Science Ltd ISSN 0958-1669

Abbreviations

avr	avirulence
Bt	<i>Bacillus thuringiensis</i>
CT	cholera toxin
LT	heat-labile toxin
PAP	pokeweed antiviral protein
RIP	ribosome-inactivating protein

Introduction

Tremendous progress in plant molecular biology over the past two decades has opened ample opportunities to improve crop plants in a way not feasible a few years ago. Among other compounds, the production of foreign proteins in plants has become an attractive alternative to conventional production systems (i.e. microbial and yeast production systems). The use of plants as bioreactors is of special interest as they allow production of recombinant proteins in large quantities and at relatively low costs. In addition, formulated in seeds, plant-made enzymes have been found to be extremely stable, reducing storage and shipping costs. Furthermore, production size is flexible and easily adjustable to the needs of changing markets.

The main objectives of creating transgenic plants are attempts to engineer metabolic pathways for the production of tailor-made plant polymers or low molecular weight compounds, increased resistance towards pathogens and pesticides, improved food quality, and the production of polypeptides for pharmaceutical or technical use [1,2].

Plant-made vaccines or antibodies (plantibodies) are especially attractive as plants are free of human diseases, reducing screening costs for viruses and bacterial toxins. Production of engineered antibodies [3*] and subunit vaccines in plants [4] turned out to be very efficient and led to the first clinical trials with plant-produced vaccines and plantibodies.

In this review, we will focus on recent developments in the use of transgenic plants, paying particular attention to engineered resistance mechanisms, modified foodstuff and production of technical enzymes.

Resistance against pathogens

Insects and nematodes

Insecticidal plants have been created by the expression of *Bacillus thuringiensis* (*Bt*) δ -endotoxins, proteinase inhibitors directed against diverse proteinases, α -amylase, lectins, polyphenol oxidases and chitinase [5*]. The *Bt* toxins were the first insecticidal proteins discovered and they have been subject to extensive ameliorations for expression in plants. Strategies to improve expression in plants include an increased G/C content, the use of plant-preferred codons, and the removal of improper splice sites and polyadenylation signals [5*]. To further increase the level of *Bt* toxin in transgenic plants, the suitability of plastid transformation has been explored [6]. As in each mesophyll cell there may be as many as 100 chloroplasts, each with about 100 copies of the plastid genome, the plastidic genome provides an enormous potential for overexpression in homotransplastomic plant lines (in which all plastidic genomes have been transformed). Thus, it is not surprising that expression of unmodified *Bt* crystal protoxin (*Bt* kurstaki HD73 *cryIA*) from the plastid rRNA operon promoter resulted in an accumulation of 3–5% of total soluble protein in tobacco leaves. The tobacco plants were highly resistant against larvae of *Heliothis virescens*, *Helicoverpa zea* and *Spodoptera exigua* [6].

Currently, the use of *Bt* toxins is expanding to a vast variety of different plant species as transformation technology is improving. Recently the *Bt cryIA(c)* gene has been transformed into peanut plants resulting in improved protection against lesser cornstalk borer [7].

In addition, new proteins with insecticidal properties have been identified, including cholesterol oxidase in *Streptomyces* culture filtrates and vegetative insecticidal proteins from different *Bacillus* species. To improve pest management, new strategies will probably have to use combinations of different antipathogenic proteins to avoid easy escape from resistance by simple mutations [8].

The latter approach has recently been employed as a tool to combat nematodes by constructing dual proteinase inhibitors [9*]. Urwin *et al.* introduced cysteine and serine proteinase

inhibitors as translational fusions into *Arabidopsis thaliana*. The linkers between the inhibitors were selected to be either cleaved or not cleaved *in planta*. Analysis of cyst and root-knot nematodes revealed ingestion of the non-cleavable dual inhibitor. This dual inhibitor showed an additive effect on resistance over either inhibitor delivered individually.

Viruses and fungi

Early attempts to achieve virus-resistance were based on genes encoding viral coat proteins, replicases, movement proteins, and defective interfering RNAs and DNAs conferring pathogen-derived resistance [10]. In addition, approaches interfering with processes common to multiple plant viruses have been explored, including expression of ribosome-inactivating proteins (RIPs) and ribonucleases specific for double stranded-RNA molecules. RIPs remove catalytically an adenine residue from a specific site in the large rRNA of eukaryotic and prokaryotic ribosomes, thereby inhibiting protein synthesis. RIPs from different plant species differ significantly in their substrate specificity. A RIP isolated from *Phytolacca americana*, also called pokeweed antiviral protein (PAP), can depurinate both eukaryotic and prokaryotic ribosomes. On the other hand, a barley RIP was found to be mainly active on fungal ribosomes and to confer resistance to fungal infections [11]. Thus, it was thought that the employment of RIPs with different specificities may be a tool to create protection against different classes of pathogens. The expression of RIPs with activity also against plant ribosomes, such as PAP from *Phytolacca americana*, however, has deleterious effects on the transgenic plants. This finding resulted in a search for nontoxic PAP mutants by random mutagenesis and selection in yeast [12]. The expression of two nontoxic PAP mutants without enzymatic activity gave rise to transgenic tobacco which were indistinguishable from wild-type plants [13]. Interestingly, these plants constitutively overexpressed pathogenesis-related proteins and displayed resistance to the fungal pathogen *Rhizoctonia solani* in the absence of salicylate. These experiments showed that the mechanism conferring fungal resistance does not rely on inactivating fungal ribosomes.

Wild-type scions grafted on transgenic PAP-expressing rootstock displayed increased resistance against potato virus X and tobacco mosaic virus [14]. The wild-type portion of the graft neither showed expression of PAP and pathogenesis related-proteins nor elevated levels of salicylic acid. The mechanism by which PAP led to viral and fungal resistance, however, is not understood.

Inducible resistance-gene/avrulence-gene pairs

In many cases, resistance can be attributed to single loci (resistance genes) in crop plants matching corresponding avirulence (avr) genes on the pathogen side (incompatible reaction). The nature of bacterial avr-genes and their recognition within plant cells has recently been summarized [15]. The incompatible reaction is often associated with rapid cell death at the site of infection, referred to as

a hypersensitive response, preventing further infection by the pathogen. It has become conceivable that broad resistance can be obtained if matching pairs of resistance-genes/avr-genes are ectopically expressed. If events downstream to the initial recognition of the gene pair are conserved among different plant cultivars and plant species, however, constitutive expression of a matching pair will result in overall plant death. Thus, this strategy requires the use of chemically or pathogen-inducible promoters. The number of chemically inducible promoters is steadily increasing, making their application for conditional expression of resistance genes feasible [16]. A recent example has been provided by McNellis *et al.* [17*]. The authors cloned the avirulence gene avrRpt2 from *Pseudomonas syringae* pv. tomato behind a glucocorticoid-inducible promoter and transformed it into *Arabidopsis thaliana* harbouring the corresponding resistance gene RPS2. The transformed construct also contained the glucocorticoid-regulated transcription factor behind the constitutive 35S promoter. Induction with dexamethasone resulted in the appearance of avrRpt2 protein after about two hours and hypersensitive response after about six hours. Due to constitutive expression of the induction system the hypersensitive collapse of dexamethasone-treated plants finally led to overall plant death.

From an applied point of view, a more localized induction of cell death would be required; therefore, Strittmatter *et al.* [18] have followed a different strategy. A chimeric pathogen-inducible promoter (prp1-1) from potato was used to drive the expression of the bacterial ribonuclease barnase. To minimize detrimental effects caused by basal expression of the ribonuclease, the specific RNase inhibitor barstar was simultaneously expressed under control of the CaMV 35S promoter at levels sufficiently high to inhibit the basal levels of barnase activity. Upon infection, barnase activity was found to be strongly induced at the infection site leading to a significant reduction of *Phytophthora infestans* sporulation on leaves from transgenic potato lines expressing the two-component system.

Modified food

Pharmaceutical foodstuff

A growing field of interest refers to the production of pharmaceutical polypeptides such as epidermal growth factor, erythropoietin, interferon, human protein C, human glucocerebrosidase and others [19,20], and pharmaceutical foodstuff considered for oral immunization [4].

To increase expression levels in the plant and to reduce purification costs, the coat proteins of tobacco mosaic virus and alfalfa mosaic virus have been used as carriers for the expression of antigenic peptides [21–23]. Feeding mice on spinach leaves infected with modified alfalfa mosaic virus particles containing two rabies epitopes led to the stimulation of IgG and IgA synthesis and protected 40% of the mice against challenge infection with a lethal dose of rabies virus [24].

Table 1**Important recent examples of antigens produced in transgenic plants.**

Antigen	Source	Plant	Reference
CT-B	<i>Vibrio cholerae</i>	Potato	[27,28]
LT-B	ETEC	Tobacco/potato	[25]
LT-B	ETEC	Potato	[29*]
LT-B	ETEC	Potato	[26]
Insulin linked to CT-B	Human/ETEC	Potato	[31*]
Capsid protein	Norwalk virus	Tobacco/potato	[45]
Surface antigen	Hepatitis B virus	Tobacco	[46]
Malaria epitope	<i>Plasmodium spp.</i>	Tobacco	[22]
V3 loop of HIV-1	HIV	Tobacco	[23]
Rabies virus Drg24 antigene	Rabies virus	Tobacco	[23]
Rabies virus Drg24 antigene	Rabies virus	Spinach	[24]

CT-B, subunit B of cholera toxin; ETEC, enterotoxigenic *E. coli*; LT-B, subunit B of heat-labile enterotoxin.

Other antigens that have been successfully produced in transgenic plants include the capsid protein of Norwalk virus (causal agent of acute gastroenteritis), hepatitis B surface antigen, subunit B of the heat-labile toxin (LT) of enterotoxigenic *E. coli* (causing diarrhea) and subunit B of cholera toxin (CT) (see Table 1 for recent examples). The LT- as well as the CT-toxin are composed of six subunits: an enzymatically active protein (LT-A and CT-A, respectively), which enters the epithelial cells of the gut and initiates diarrhea, and five inactive proteins (LT-B and CT-B, respectively), which allow binding to the membranes via specific interactions with the G_{M1} gangliosides of gut epithelial cells.

Already in 1995, Haq *et al.* [25] reported on the successful production of serum and mucosal antibodies against endoplasmic reticulum-targeted LT-B in mice after they had eaten recombinant potato tubers that produced the protein at a level of 2 µg/g tuber tissue. Because of low antigen expression, the authors recently designed a synthetic gene encoding LT-B by removal of the mRNA destabilizing motif ATTTA and a putative polyadenylation signal, the introduction of a plant translation initiation sequence and the optimization of the codon usage [26]. These alterations resulted in increased mRNA stability and higher levels of antigen in transgenic potato plants. Tubers of the highest expressing lines contained 7.3–17.2 µg LT-B protein/g tuber tissue. Feeding these potatoes to mice (20 and 50 µg doses) gave rise to production of anti-LT-B IgG in the serum and IgA in the mucosal gut to higher levels as compared with controls that were force-fed with 5 µg purified LT-B from recombinant *Escherichia coli*. The mice were partially protected from water loss into the gut when challenged with 25 µg active LT [26].

Arakawa *et al.* [27] reported on the expression of endoplasmic reticulum-targeted CT-B in transgenic potato plants. It was found that CT-B refolds into its native pentameric structure in potato tissue, which is a requirement for retaining its binding capacity to G_{M1}-ganglioside and full immunogenicity. Subsequently, it was shown that

mice fed with transgenic potato tubers expressing CT-B showed up to a 60% reduction in diarrheal fluid accumulation in the small intestine of these mice when challenged with cholera toxin [28]. The area of pharmaceutical food has progressed such that it has already entered the clinical phase. Very recently, feeding human volunteers with raw transgenic potato tubers expressing LT-B resulted in significant mucosal and systemic immune responses proving that the plant-produced protein remained immunogenic for humans [29*]. The overall immune response was similar to a challenge of 10⁹ virulent enterotoxigenic *E. coli* given to human volunteers. This result allows the development of strategies for oral immunisation when protective antigens for diseases such as cholera and diarrhea have been defined.

Probably because of its capacity to bind G_{M1} gangliosides in the intestine, CT-B appears to be an effective carrier molecule for the induction of mucosal immunity to polypeptides that are conjugated to it [30]. It may be that CT-B facilitates antigen delivery and presentation of conjugated peptides to the gut-associated lymphoid tissues. It has been suggested that oral administration of autoantigens may provide an approach to prevent autoimmune diseases such as type I diabetes, rheumatoid arthritis and multiple sclerosis. In order to evaluate this approach, human insulin, a major insulin-dependent diabetes mellitus autoantigen, was fused to the carboxyl-terminus of CT-B and expressed in transgenic potato plants [31*]. The fusion protein kept its pentameric structure and retained G_{M1} ganglioside binding affinity as well as antigenicity of both CT-B and insulin. Both systemic and intestinal anti-CT-B antibodies were produced in mice fed with transformed potato tubers and diabetes symptoms were delayed at doses at least 100-fold less than for the unconjugated autoantigen [31*].

Passive immunotherapy has also been envisaged with monoclonal antibodies produced in and purified from transgenic plants. Very recently, Ma *et al.* [32] have shown that colonization of teeth and gums by *Streptomyces mutans*,

Table 2**Technical enzymes produced in stably transformed plants.**

Enzyme	Origin of gene	Plant	Reference
α -amylase	<i>B. licheniformis</i>	Tobacco	[39]
α -amylase	<i>B. licheniformis</i>	<i>Vicia narbonensis</i>	[44]
α -amylase	<i>B. licheniformis</i>	<i>Pisum sativum</i>	(a)
Phytase	<i>A. niger</i>	Tobacco	[40]
Phytase	<i>A. niger</i>	Tobacco	[47]
Phytase	<i>A. niger</i>	Soybean	[41]
β (1,3-1,4) glucanase	<i>R. flavefaciens</i>	Tobacco	[48]
β (1,3-1,4) glucanase	<i>B. amyloliquefaciens/B. macerans</i>	Barley	[49]
β (1,4) xylanase	<i>C. thermocellum</i>	Tobacco	[50]
β (1,4) xylanase	<i>C. thermocellum</i>	<i>Pisum sativum</i>	(a)
β (1,4) xylanase	<i>R. flavefaciens</i>	Tobacco	[48]

(a) Saalbach I, Czihal A, Conrad B, Hofemeister P, Hofemeister J, Böumlein H, Sonnewald U, Herbers K, unpublished data.

the major cause of dental caries, could be prevented by a monoclonal secretory antibody directed against the 185 kD cell-surface adhesion protein of the bacterium.

Modification of seed quality by altered amino acid composition

Among the major crop plants, both cereals and legumes are important sources of protein for humans and their livestock. Cereal seeds typically contain 10–15% protein, whereas legumes contain 20–30% protein of which 50–60% are usually storage proteins. The major storage proteins of legumes are saline-soluble globulins, whereas the major storage proteins of cereals are alcohol-soluble prolamins. The cereal prolamins are generally devoid of lysine, whereas the legume globulins are reduced in the sulfur amino acids, methionine and cysteine [33]. Within certain limits, methionine- and lysine-poor basal diets may be compensated for by addition of methionine-rich fishmeal and lysine-rich soybean meal. The addition of 0.1% methionine equals in quality the addition of 16% soybean meal or 5.6% fishmeal. The addition of 0.1% lysine compensates for the enrichment by 3.7% soybean meal or 2.1% fishmeal [34]. Thus, there has been considerable interest for improvement of the nutritive value of seeds by breeding and genetic engineering [35•]

In order to elevate the methionine and lysine content of seeds two strategies have been employed: the respective biosynthetic pathways have been manipulated; or high-methionine or high-lysine proteins have been expressed in transgenic seeds. Three routes have been taken to follow up the latter approach: firstly, endogenous proteins were altered in their amino acid sequence; secondly, naturally occurring methionine-rich proteins from other plant species were recruited for heterologous expression; and finally, entirely synthetic genes containing high levels of methionine and lysine were created (summarized in [36]). A combination of the first and second approach has been described by Tu *et al.* [37]. To improve the methionine content of potato tubers a cDNA clone encoding the Brazil nut 2S albumin was mutagenized to increase its methionine

content by 2–7 additional methionine residues and transformed into potato plants. Irrespective of the mutation, protein content in leaves was rather low, ranging from < 0.01%–0.2% of total protein. The third approach was followed by Keeler *et al.* [38]. To increase the lysine and methionine content in seeds a synthetic protein based on an α -helical coiled-coil structure containing 31% lysine and 20% methionine (CP3–5) was designed. Driven either by the phaseolin or β -conglycinin promoter, moderate amounts of the synthetic protein accumulated in seeds harvested from transgenic tobacco plants.

Technical enzymes

Many industrial processes involving plant material face the problem of having to remove or efficiently exploit plant-specific compounds. Therefore, many approaches to produce industrial proteins in plants refer to proteins that degrade plant cell walls, starch and phytate (Table 2). The degradation of cell walls by cell-wall hydrolases, for example, plays an important role in feed and food production, and in the paper, wood and brewing industries. α -amylase is used for liquefaction of starch mainly in the food industry and in detergent manufacture. Phytate, the main phosphorus storage form in plants, needs to be hydrolyzed by phytase to improve the nutritional value of feed for monogastric animals.

So far, successful production has only been reported for technical enzymes that have been fused to signal peptides to allow for secretion via the default pathway. Protein levels varied significantly, ranging from below 1% [39] to as much as 14.4% of total soluble protein in leaves [40]. Values in the literature, however, can hardly be compared with each other because the accumulation of proteins is strongly dependent on the developmental stage of the tissue and differ with the extraction method. In addition, activities of the heterologous proteins and not their total amount are the important parameter.

Recently, the expression of plant-produced technical enzymes has been extended to crop plants that can directly

be used for their final purpose. Thus, *Aspergillus niger* phytase has been expressed in soybean and investigated as a feed supplement for broilers in comparison to commercially available fungal phytase 'Natuphos' (BASF, Mt. Olive, NJ) [41]. The investigators found that dietary phytase added to a basal diet (fed at 400, 800, 1200 U/kg raw soybeans; the unit U stands for μmol liberated phosphate per minute) linearly increased growth rate, feed intake, tibia shear force (an indirect measure for increased phosphate incorporation into skeletal elements) and energy, phosphorus digestibility and toe ash weight (a direct measure of phosphate incorporation). Except for phosphorus digestibility, which was higher in the case of phytase from the transgenic soybean seeds, there was no difference between phytase from the different sources. The specific phytase activities which were needed to improve phosphorus availability for broilers were in the range that can easily be reached by expressing heterologous enzymes in transgenic seeds. Thus, it seems feasible to use transgenic seeds in the feed industry as an alternative to microbially produced recombinant enzymes in the near future. It is noteworthy, however, that by genetic means low phytic acid mutants in barley and maize have been identified [42] which might represent an alternative strategy to phytase production.

As leguminous plants are also in widespread use as feed there is growing interest in studying the stability and performance of technical enzymes produced in alfalfa [43], narbon bean [44] and fodder pea (Saalbach I *et al.*, unpublished data). Leguminous plants, due to their symbiosis with nitrogen-fixing bacteria, need to be fertilized rarely allowing for low production costs. The expression of heat-stable *B. licheniformis* α -amylase behind the seed-specific USP promoter and of heat-stable *C. thermocellum* xylanase behind the seed-specific phaseolin promoter in transgenic homozygous pea plants resulted in activities of ~ 8000 U/kg seeds. Crossing of the homozygous lines gave rise to a F1 hybrid with activity levels of 4000 U/kg for each of the two enzymes (Saalbach *et al.*, unpublished data). This is the first example of combining independent commercial traits, showing the potential and versatility for molecular farming approaches.

Conclusions

The first generation of herbicide- and pest-resistant plants has made its way to the marketplace. In the near future, combinatorial approaches based on the expression of newly designed polypeptides with multipartite functions will lead to broad pathogen resistance. Based on encouraging developments in chemically inducible gene expression in plants, new principles such as conditional resistance will be employed to improve crop performance. Furthermore, interest in transgenic plants for the production of recombinant proteins is steadily increasing. During the past few years it has become evident that plants express, fold, assemble and post-translationally modify foreign proteins with high fidelity. Prototype vaccines can be produced in plants and they may trigger oral immunity if the plant tissues are consumed as food. In pre-clinical and first clinical

trials, it has been found that antigenic proteins may retain their antigenic properties when purified from transgenic plants and may give rise to the production of specific immune responses after immunization.

Acknowledgements

The work of the authors was partially supported by a grant (FKZ: 2191A/0085G) from the Ministry of Education of Saxonia Anhalt.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Herbers K, Sonnewald U: **Transgenic plants in biochemistry and plant physiology.** *Prog Botany* 1998, **59**:534-569.
2. Dempsey DA, Silva H, Klessig DF: **Engineering disease and pest resistance in plants.** *Trends Microbiol* 1998, **6**:54-61.
3. Conrad U, Fiedler U: **Compartment-specific accumulation of recombinant immunoglobulins in plant cells: an essential tool for antibody production and immunomodulation of physiological functions and pathogen activity.** *Plant Mol Biol* 1998, **38**:101-109.

The authors describe all types of recombinant immunoglobulins expressed in plants, their stability and yield in different cellular compartments as well as in different plant organs. Furthermore, the authors point to possible applications of antibodies in the analysis of physiological questions and in biotechnological uses.

4. Arntzen CJ: **Pharmaceutical foodstuffs – oral immunization with transgenic plants.** *Nat Med Vaccine Suppl* 1998, **4**:502-503.
5. Estruch JJ, Carozzi NB, Desai N, Duck NB, Warren GW, Koziel MG: **Transgenic plants: an emerging approach to pest control.** *Nat Biotechnol* 1997, **15**:137-141.

This review gives an extensive overview about insecticidal proteins with special emphasis on the history of the use of *Bt* toxins as pest control agents in transgenic plants.

6. McBride KE, SvabZ, Schaaf DJ, Hogan PS, Stalker DM, Maliga P: **Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco.** *Biotechnol* 1995, **13**:362-365.
7. Singsit C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P: **Expression of a *Bacillus thuringiensis cryIA(c)* gene in transgenic peanut plants and its efficacy against lesser cornstalk borer.** *Transgenic Res* 1997, **6**:169-176.
8. Fox JL: ***Bt* cotton infestations renew resistance concerns.** *Nat Biotechnol* 1996, **14**:1070.
9. Urwin PE, McPherson MJ, Atkinson HJ: **Enhanced transgenic plant resistance to nematodes by dual proteinase inhibitor constructs.** *Planta* 1998, **204**:472-479.

The authors describe the co-delivery of different anti-nematode proteins as a single transgene by translational fusions. They discuss the enhanced efficacy of stacked genes to provide defence against not only individual but also diverse types of pathogens.

10. Beachy RN: **Mechanisms and applications of pathogen-derived resistance in transgenic plants.** *Curr Opin Biotechnol* 1997, **8**:215-220.
11. Logemann J, Jach G, Tommerup H, Mundy J, Schell J: **Expression of barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants.** *BioTechnology* 1992, **10**:305-308.
12. Hur Y, Hwang DJ, Zoubenko O, Coetzer C, Uckun F, Tumer NE: **Isolation and characterization of pokeweed antiviral protein in *Saccharomyces cerevisiae*: identification of residues important for toxicity.** *Proc Natl Acad Sci USA* 1995, **92**:8448-8452.
13. Zoubenko O, Uckun F, Hur Y, Chet I, Tumer N: **Plant resistance to fungal infection induced by nontoxic pokeweed antiviral protein mutants.** *Nat Biotechnol* 1997, **15**:992-996.
14. Smirnov S, Shulaev V, Tumer NE: **Expression of pokeweed antiviral protein in transgenic plants induces virus resistance in grafted**

- wild type plants independent of salicylic acid accumulation and pathogenesis-related protein synthesis.** *Plant Physiol* 1997, 114:1113-1121.
15. Van den Ackerveken G, Bonas U: **Bacterial avirulence proteins as triggers of plant disease resistance.** *Trends Microbiol* 1997, 5:394-398.
 16. Gatz C, Lenk I: **Promoters that respond to chemical inducers.** *Trends Plant Sci* 1998, 3:352-358.
 17. McNellis TW, Mudgett MB, Li K, Aoyama T, Horvath D, Chua NH, Staskawicz BJ: **Glucocorticoid-inducible expression of a bacterial avirulence gene in transgenic *Arabidopsis* induces hypersensitive cell death.** *Plant J* 1998, 14:247-257.
- The authors investigate the glucocorticoid-inducible gene expression system to express an avirulence gene from *Pseudomonas* in *Arabidopsis* plants. These plants will be used as a tool for the dissection of avirulence gene-specified responses in resistant and susceptible plants.
18. Strittmatter G, Janssens J, Opsomer C, Botterman J: **Inhibition of fungal disease development in plants by engineering controlled cell death.** *Bio/Technology* 1995, 13:1085-1089.
 19. Goddijn OJM, Pen J: **Plants as bioreactors.** *Trends Biotechnol* 1995, 13:379-387.
 20. Cramer CL, Weissenborn DL, Oishi KK, Grabau EA, Bennett S, Ponce E, Grabowski GA, Radin DN: **Bioproduction of human enzymes in transgenic tobacco.** *Ann NY Acad Sci* 1996, 792:62-71.
 21. Hamamoto H, Sugiyama Y, Nakagawa N, Hashida E, Matsunaga Y, Takemoto S, Watanabe Y, Okada Y: **A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato.** *Bio/Technology* 1993, 11:930-932.
 22. Turpen TH, Reinl SJ, Charoenvit Y, Hoffman S, Fallarme V, Grill LK: **Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus.** *Bio/Technology* 1995, 13:53-57.
 23. Yusibov V, Modelska A, Stepiewski K, Agadjanyan M, Weiner D, Hooper CD, Koprowski H: **Antigens produced in plants by infection with chimeric plant viruses immunize against rabies virus and HIV-1.** *Proc Natl Acad Sci USA* 1997, 94:5784-5788.
 24. Modelska A, Dietzschold B, Sleysh N, Fu ZF, Stepiewski K, Hooper DC, Koprowski H, Yusibov V: **Immunization against rabies with plant-derived antigen.** *Proc Natl Acad Sci USA* 1998, 95:2481-2485.
 25. Haq TA, Mason HS, Clements JD, Arntzen CJ: **Oral immunization with a recombinant bacterial antigen produced in transgenic plants.** *Science* 1995, 268:714-716.
 26. Mason HS, Haq TA, Clements JD, Arntzen CJ: **Edible vaccine protects mice against *E. coli* heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene.** *Vaccine* 1998, 16:1336-1343.
 27. Arakawa T, Chong DKX, Lawrence M, Langridge WHR: **Expression of cholera toxin B subunit oligomers in transgenic potato plants.** *Transgenic Res* 1997, 6:403-413.
 28. Arakawa T, Chong DKX, Langridge WHR: **Efficacy of a food plant-based oral cholera toxin B subunit vaccine.** *Nat Biotechnol* 1998, 16:292-297.
 29. Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Arntzen CJ: **Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato.** *Nat Med* 1998, 4:607-609.
- The authors present the first successful clinical trial on the immunogenicity in human beings fed with raw transgenic potatoes expressing LT-B. Thus, this study provides a proof of concept for oral immunisation strategies.
30. Sun JB, Holmgren J, Czerkinsky C: **Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance.** *Proc Natl Acad Sci USA* 1994, 91:10795-10799.
 31. Arakawa T, Yu J, Chong DKX, Hough J, Engen PC, Langridge WHR: **A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes.** *Nat Biotechnol* 1998, 16:934-938.
- The authors expressed a fusion protein between insulin and CT-B in transgenic potato plants. Mice were fed with transgenic tubers containing either the fusion protein or insulin alone. The former experiment resulted in significantly less insulinitis and a delay in the progression of clinical diabetes. This approach provides a method to increase sensitivity against antigens which otherwise would only elicit immune responses at expression levels not easily attainable in transgenic plants.
32. Ma JKC, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB, Lehner T: **Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans.** *Nat Med* 1998, 4:601-606.
 33. Larkins BA, Lending CR, Wallace JC: **Modification of maize-seed-protein quality.** *Am J Clin Nutr* 1993, 58(suppl):264S-269S.
 34. Leuchtenberger W: **Aminosäuren für die Tierernährung.** *Chemie Heute* 1997/1998:76-78. [Title translation: Amino acids for animal feed.]
 35. Tabe L, Higgins TJV: **Engineering plant protein composition for improved nutrition.** *Trends Plant Sci* 1998, 3:282-286.
- This article summarizes the current status about the different biotechnological and breeding approaches and their respective success to improve protein quality in plants.
36. Müntz K, Christov V, Jung R, Saalbach G, Saalbach I, Waddell D, Pickardt T, Schieder O: **Genetic engineering of high methionine proteins in grain legumes.** In *Sulphur Metabolism in Higher Plants*. Edited by Cram WJ, De Kok LJ, Stulen I, Brunhold C, Rennenberg H. Leiden, The Netherlands: Backhuys Publishers; 1997:71-86.
 37. Tu HM, Godfrey LW, Sun SSM: **Expression of the Brazil nut methionine-rich protein and mutants with increased methionine in transgenic potato.** *Plant Mol Biol* 1998, 37:829-838.
 38. Keeler SJ, Maloney CL, Webber PY, Patterson C, Hirata LT, Falco SC, Rice JA: **Expression of de novo high-lysine α -helical coiled-coil proteins may significantly increase the accumulated levels of lysine in mature seeds of transgenic tobacco plants.** *Plant Mol Biol* 1997, 34:15-29.
 39. Pen J, Molendijk L, Quax WJ, Sijmons PC, van Ooyen AJJ, van den Elzen PJM, Rietveld K, Hoekema A: **Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction.** *Bio/Technology* 1992, 10:292-296.
 40. Verwoerd TC, van Paridon PA, van Ooyen AJJ, van Lent JWM, Hoekema A, Pen A: **Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves.** *Plant Physiol* 1995, 109:1199-1205.
 41. Denbow DM, Grabau EA, Lacy GH, Kornegay ET, Russell DR, Umbeck PF: **Soybeans transformed with a fungal phytase gene improve phosphorus availability for broilers.** *Poultry Sci* 1998, 77:878-881.
 42. Larson SR, Young KA, Cook A, Blake TK, Raboy V: **Linkage mapping of two mutations that reduce phytic acid content of barley grain.** *Theor Appl Genet* 1998, 97:141-146.
 43. Austin S, Bingham ET, Koegel RG, Mathews DE, Shahan MN, Straub RJ, Burgess RR: **An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa.** *Ann NY Acad Sci* 1994, 721:234-244.
 44. Czihal A, Conrad B, Buchner P, Brevis R, Farouk AA, Manteuffel R, Adler K, Wobus U, Hofemeister J, Bäumlein H: **Gene farming in plants: expression of a heatstable *Bacillus* amylase in transgenic legume seeds.** *J Plant Physiol* 1999, in press.
 45. Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK, Arntzen CJ: **Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice.** *Proc Natl Acad Sci USA* 1996, 93:5335-5340.
 46. Thanavala Y, Yang YF, Lyons P, Mason HS, Arntzen C: **Immunogenicity of transgenic plant-derived hepatitis B surface antigen.** *Proc Natl Acad Sci USA* 1995, 92:3358-3361.
 47. Pen J, Verwoerd TC, van Paridon PA, Beudeker RF, van den Elzen PJM, Geerse K, van der Klis JK, Versteegh HAJ, van Ooyen AJJ, Hoekema A: **Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization.** *Bio/Technology* 1993, 11:811-814.
 48. Herbers K, Flint HJ, Sonnewald U: **Apoplasmic expression of the xylanase and $\beta(1-3,1-4)$ glucanase domains of the *xynD* gene from *Ruminococcus flavefaciens* leads to functional polypeptides in transgenic plants.** *Mol Breeding* 1996, 2:81-87.
 49. Jensen LG, Olsen O, Kops O, Wolf N, Thomsen KK, von Wettstein D: **Transgenic barley expressing a protein-engineered, thermostable (1,3-1,4)- β -glucanase during germination.** *Proc Natl Acad Sci USA* 1996, 93:3487-3491.
 50. Herbers K, Wilke I, Sonnewald U: **A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified.** *Bio/Technology* 1995, 13:63-66.