Recombinant proteins from transgenic plants
Eva Franken, Ute Teuschel and Rüdiger Hain

Transgenic plants can express a wide variety of foreign genes and offer the opportunity of large-scale protein production in agricultural systems. The recombinant protein can serve both ex situ and in situ purposes. Due to significant progress in plant molecular biology, many different plant species can now be transformed and are even capable of producing very complex proteins such as antibodies or vaccines. Furthermore, recombinant proteins can mediate resistance against microbial pathogens, such as fungi or viruses, or protect transgenic plants from insect pests.

Introduction
During the past decade, fundamental changes have taken place in the field of plant molecular biology [1]. Together with improved techniques for plant genetic analysis and engineering, concepts of exploiting transgenic plants have gained increasing scientific and economic importance. Among the large number of new technologies that are available, commercial interest has focused on the ability of plants to integrate and express foreign genes and to produce recombinant proteins in bulk quantities at relatively low cost.

Plants now offer a number of expression systems for the temporally and spatially controlled production of recombinant proteins for many different purposes (Figure 1). While molecular farming focuses on the utilization of plants or cultured plant cells as bioreactors producing the protein of interest for purification and further use (ex situ application), molecular breeding exploits transgenic protein expression that renders the plant of interest resistant against herbicides, insect or microbial attack (in situ application).

A large variety of plasmid constructs are used for the transformation, integration and expression of foreign genes in transgenic plants, including different promoter and targeting sequences. The most widely used technique for the transformation of plants is the Agrobacterium Ti-plasmid binary system [2,3], where the genes of interest are first introduced into Agrobacterium tumefaciens and plant cells are then infected with the recombinant bacteria. The introduction of DNA into plant protoplasts via direct gene transfer using electroporation [4], polyethylene glycol mediated gene uptake [5], co-precipitation with calcium phosphate [6] or particle bombardment [7] facilitates the transformation of monocotyledonous plants, which are not readily infected by Agrobacterium strains.

Two major approaches have been made towards exploiting plant expression systems. Whole plants are used expressing the gene of interest and allowing recombinant protein production on an agronomical level. This strategy is especially useful in the case of proteins designated to improve the nutritional value of human or animal food plants, and for gene products causing plant resistance. In some special applications, the produced protein is directed to certain plant organs and, for example, accumulates in the fruits or seeds. On the other hand, plant cells cultured at high density in bioreactors allow the secretion of recombinant protein into the culture medium for better accessibility and easier purification.

Molecular farming
Plantibodies
There are many examples of plants that produce transgenic proteins serving a wide spectrum of purposes. An important possible application of plant expression systems originates from their ability to synthesize virtually every kind of antibody molecule. These ‘plantibodies’ can be isolated and used for ex situ purposes in diagnosis and therapy, or in situ, acting directly in the producing plant [8]. Complex antibodies consist of heavy and light chains covalently linked together by disulfide bonds. Each chain is folded into a series of variable (antigen-binding activity) and constant (secondary effector function) domains. Transgenic plants use the same pathway for the assembly of light and heavy chains as mammalian cells involving similar signal peptides and chaperones for successful folding (see [9] for a review with regard to processing and engineering of plantibodies; it also discusses potential regulatory issues for therapeutic purposes). As in mammalian cells, antibodies are secreted following assembly and post-translational processing. A difference seems to be in glycosylation patterns: plant antibodies lack N-acetyl neuraminic acid, which is the predominant terminal sugar residue in mammalian complex glycans. Single-chain Fv fragments (scFv) consist of only the variable light and variable heavy chain domains, which are covalently linked by a synthetic linker peptide (Figure 2). They bind to their antigens with similar affinities as the parental antibodies [10].

Functional recombinant scFv fragments can be produced and even stored in transgenic plants. As a model system,
Figure 1

Fields of application for transgenic plants. In situ, recombinant proteins protect plants from fungal, microbial, viral or insect attack. For ex situ purposes, recombinant proteins, such as enzymes or antibodies, can be produced by transgenic plants.

Ma et al. [14] were the first to describe the production of a functional, high molecular weight secretory immunoglobulin in transgenic tobacco. Genes encoding the heavy and light chains of murine antibody (Guy's 13 against streptococcal antigen (SA) I/II), a murine joining chain, and a rabbit secretory component were introduced into separate transgenic tobacco plants. By cross-pollination, plants were obtained that co-expressed all components and produced a functionally active secretory antibody that recognized the SA I/II cell surface adhesion molecule of *Streptococcus mutans* and *Streptococcus sobrinus*. The antibody yield from fully expanded leaf lamina was 200–500 µg per gram of fresh weight material. Until then, complex secretory monoclonal antibodies could only be generated by extremely complicated techniques like the *in vitro* conjugation of secreted dimeric immunoglobulin from mammalian plasma cells with the secretory component derived from epithelial cells. The large scale agronomic

the legumin B4 gene promoter from *Vicia faba*, which confers strict seed-specific expression, was used to express scFv in transgenic tobacco seeds [11]. The transgenic scFv was functional and had similar antigen binding affinity as an *E. coli* derived scFv and accounted for up to 0.67% of total soluble mature seed protein. These transgenic seeds could be stored at room temperature for one year without detectable loss of scFv content or activity. Increased expression levels of scFv (yielding up to 1% of the total soluble protein) targeted to the cytosol or the secretory pathway in transgenic tobacco were reported [12] when scFv was provided with the carboxy-terminal aminoacid sequence KDEL, which is a signal for retention in the endoplasmic reticulum. The affinity of plant-produced scFv was about 80-fold lower than that of the parental antibody fragment when measured by enzyme linked immunosorbent assay. This could be due to the different glycosylation patterns of plants and animals [13].

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production of such functional secretory antibodies holds considerable potential for passive immunotherapy of mucosal surfaces, where large quantities of monoclonal antibodies are required [9].

**Figure 2**

![Diagram of antibody structure](Image)

In antibodies, two heavy and two light chains are covalently linked together by interchain disulfide bonds (S-S). Both heavy and light chains contain variable regions (shown with bars) and constant regions. Single-chain Fv fragments consist of only the variable regions of a heavy and a light chain joined together by a synthetic polypeptide linker.

In addition to antibody production for ex situ techniques, in situ applications have become an important field of research. Although plants lack the mammalian antibody-triggered mechanism of antigen disposal, antibody binding can lead to agglutination of pathogens or block epitopes essential for infection. This effect is well documented for virus infection. Tobacco plants expressing a cytoplasmically targeted scFv against an epitope of the coat protein of artichoke mottled crinkle virus were less susceptible to infection with this virus [15]. Expression of a full-length heavy- and light-chain antibody against surface epitopes of the tobacco mosaic virus, possessing a signal peptide that targeted secretion to the extracellular compartment, mediated significant protection of transgenic tobacco plants [16]. More recent studies focus on the root-knot nematode *Meloidogyne incognita*. Transient expression assays with tobacco leaf protoplasts resulted in a high intracellular accumulation of functional scFv (of monoclonal antibody 6D4) directed against salivary secretions of this nematode, which are known to play a key role in the infection process [17]. No influence on root-knot nematode parasitism could be observed following expression of functional full-length heavy and light chains of 6D4 in leaves, stems, roots, and galls of *Nicotiana tabacum* cv. Xanthi tobacco [18]. As a possible explanation, the apoplastic accumulation of the plant bodies is supposed, whereas nematode stylet secretions of saliva might be injected into the cytoplasm of the parasitized cell.

**Other proteins and enzymes**

Particular attention has been paid to the production of edible vaccines by expressing immunogenic viral proteins in transgenic plants [19]. The capsid protein of Norwalk virus, a virus causing epidemic acute gastroenteritis in humans, was shown to self-assemble into virus-like particles when expressed in transgenic tobacco and potato [20]. Both purified virus-like particles and transgenic potato tubers stimulated the production of antibodies against Norwalk virus capsid protein when fed to mice. Dalsgaard et al. [21] used another approach for producing plant-derived vaccines. They inserted a DNA sequence coding for a short linear epitope from the VP2 capsid protein of mink enteritis virus (MEV) into an infectious cDNA clone of cowpea mosaic virus. When the construct was propagated in the black-eyed bean *Vigna unguiculata*, the MEV epitope was expressed on the surface of the recombinant cowpea mosaic virus, which successfully conferred protection against MEV after subcutaneous injection in mink.

With the aim of improving the nutritional status of agronomically important pasture legumes, a sulfur-rich seed albumin from sunflower was expressed in the leaves of transgenic subterranean clover [22]. By targeting the recombinant protein to the endoplasmic reticulum of the transgenic plant leaf cells, an accumulation of transgenic sunflower seed albumin of up to 1.3% of total extractable protein could be achieved.

Another approach exploits oleosins as carriers for foreign proteins. Oleosins are highly lipophilic proteins that accumulate on the surface of plant seed oil-bodies and are easily co-separated from the rest of the cellular extract by centrifugation. By constructing fusions with *Arabidopsis* oleosin genes it was possible to target recombinant protein, with approximately 80% of the activity partitioning with oil-bodies of *Brassica napus* [23]. The transgenic seeds could be stored for more than one year without significant loss of recombinant protein activity. Purification of the recombinant protein would involve endopeptidase cleavage of the fusion protein. Alternatively, for example, in the case of an enzyme, the oil-body could be used as an immobilization matrix. As a first example of this approach, hirudin was produced by exploiting oleosin partitioning [24]. The oleosin–hirudin fusion protein accounted for up to 1% of the total seed protein and was biologically active after proteolytic release. Thus, exploiting oleosins as carriers for foreign proteins provides for easier purification and long-term storage of recombinant proteins.

**Cultured plant cells**

In recent years, the production of high-value proteins by cultured plant cells has gained considerable attention. Cultured plant cells are especially suitable when the protein of interest has to undergo complex post-translational processing, which cannot be accomplished by recombinant
microbial systems and animal cell culture may be too costly. As intact transgenic plants require cultivation, harvesting, and extraction, recombinant protein production in plant cell cultures could provide significant economic advantage. Particularly high yields can be achieved in systems where the recombinant protein does not inhibit cell growth by accumulating in the cells but is continuously secreted into the culture medium. Culture conditions can considerably influence the yield of recombinant protein. For example, by the addition of the protein-stabilizing agent polyvinylpyrrolidone to the culture medium, the level of secreted mouse monoclonal antibody heavy chain increased by 35-fold to 0.36 µg protein/ml culture medium in suspension culture of transgenic tobacco cells [25]. The use of a perfusion air-lift bioreactor for high density Anchusa officinalis cell cultivation yielded cell densities of 27.2 g/l dry weight and total extracellular protein concentrations of 1.1 g/l in contrast to only 12.6 g/l dry weight and 0.47 g/l extracellular protein for batch culture [26*].

Molecular breeding

In addition to recombinant protein production, gene expression in transgenic plants for in situ purposes can solve agricultural problems. This is particularly true when the expressed foreign protein enables important crop plants to cope with microbial or insect attack. There are a number of ways to achieve pest resistance by transgenic plants, involving a variety of naturally occurring resistance-mediating proteins, and more recently exploiting synthetic resistance genes.

In addition to the common approach of engineering virus resistance by expressing viral coat proteins, severable other translatable viral sequences are currently being exploited as pathogen-derived resistance genes (reviewed in [27,28]). Other systems exploit transgenic plants expressing naturally occurring antiviral genes. Trichosanthin, a ribosome-inactivating protein (RIP) from the Chinese medicinal herb Trichosanthes kirilowii, accounted for up to 0.1% of the total soluble protein in transgenic tobacco leaves, and the plants were found to be completely resistant to mechanical inoculation of turnip mosaic virus [29]. In a similar approach, the RIP dianthin was inducibly expressed using the virion-sense promoter from African cassava mosaic virus in order to avoid the toxic effects of constitutive RIP expression on the transgenic plant. When inoculated with the African cassava mosaic virus, transgenic Nicotiana benthamiana plants exhibited attenuated systemic symptoms of virus infection, from which they recovered [30*]. Expression of the human antiviral RNA-decay-pathway enzymes 2–5A synthetase alone and RNase L alone was insufficient to protect transgenic tobacco plants. As demonstrated by activation experiments of RNase L in the transgenic plant leaves, both enzymes are required for antiviral activity [31].

Resistance of transgenic plants against fungal pathogens could be achieved by constitutively expressing enzymes that can hydrolyze fungal cell walls, like chitinase [32], β-1,3-glucanase [33], or both [34]. Cysteine-rich antimicrobial peptides from Mirabilis jalapa and Amaranthus caudatus were expressed in transgenic tobacco [35]; although they were functional in in vitro studies, they did not enhance plant resistance. Redirected targeting of the peptides from the extracellular spaces into an intracellular compartment also had no effect on the severity of the disease symptoms. A possible explanation for the ineffectiveness of the antimicrobial peptides to enhance plant resistance could be seen in their low level of expression or the sensitivity of these disulfide-linked peptides to the ionic strength of their environment.

Several approaches have been made towards insect resistance of important crop plants. The constitutive expression of cowpea trypsin inhibitor conferred on transgenic rice resistance against the yellow stem borer Scirpophaga incertulas and the striped stem borer Chilo suppressalis, the two major insect pests of rice in Asia [36]. While snowdrop lectin or trypsin inhibitor expressed in transgenic potato plants had a significant antifeedant effect on the tomato moth in growth room trials, bean chitinase was insufficient for plant protection [37]. Since the late eighties, the insecticidal crystal toxin genes from Bacillus thuringiensis have been used to engineer insect resistance in transgenic plants [38]; however, due to different codon usage by plants and bacteria, in most cases the level of expression was too low for efficient protection. Modifications of the toxin genes, such as removal of A+T-rich sequences that may cause mRNA instability or aberrant splicing, adjustment of the codon usage to make it more similar to that of the host plant, or truncation of the coding sequences have considerably increased toxin expression levels. Thus, the more recent approaches utilize modified synthetic crystal toxin genes for an effective insect pest control in rice [39], canola [40], soybean [41], alfalfa and tobacco [42]. Ten years after the first publication [43], transgenic B. thuringiensis-plants (e.g. Bollgard® and Newleave®) have now been introduced into the US market.

Conclusions

Molecular plant breeding is developing at a breath-taking rate. The first successful introduction of a fragment of foreign DNA into a plant cell in vitro was reported only 14 years ago [44]. Yet, the first products of this technology are already fully developed. Some transgenic products are already on the market, others are soon to be introduced. Regulatory considerations for the exploitation of transgenic plants will have to include their genetic stability, the purity of recombinant proteins, especially when they are determined for use in humans, and their environmental impact [45*]. Elaborated methods in plant molecular genetics allow the transformation of virtually every plant species and the expression of a large variety of genes. Thanks to these new technologies, we now have
the ability to use transgenic plants cultivated on an agricultural scale as potentially safe and cost-effective sources to produce almost limitless amounts of recombinant protein.

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


The author describes different transformation technologies and their applicability for the genetic engineering of major crop plants. He compares and evaluates the effectiveness of different transformation methods, and gives detailed examples for the efficient transformation of maize, rice, wheat, barley, soybean, bean and cotton. He summarizes important field trials with transgenic plants (maize, rice, soybean, cotton and tomato).


10. Fiedler U, Conrad U: Of outstanding interest of special interest

11. Fiedler U, Conrad U: Of outstanding interest of special interest

12. Bird KE, Hardman KD, Jacobsen JW, Johnson S, Kaufman B: An approach towards the large-scale economical exploitation of what are still mostly lab-scale expression techniques in plant cell culture. The bioreactor itself is described in some detail and the paper may give interesting clues to readers who are thinking of establishing large-scale plant cell culture.


An alternative approach to [20°] where oral immunization is not possible. The production and purification of the vaccine is facilitated by engineering a plant virus that carries a short animal virus epitope. By using a plant virus, the accidental shedding of virulent virus with the vaccine is abolished, and as the expressed epitope occurs in several different animal virus species, the same transgenic plant virus may be used to vaccinate several viral hosts.


16. Ma JKC, Hein B: Of outstanding interest of special interest


The authors present an elegant way of controlling the expression of dianthin, an antiviral protein, in transgenic plants. They use a promoter that is trans-activated by the product of a viral gene. Upon infection with the virus, the production of antiviral protein is induced only in the infected plant cells. Thus, constitutive expression of recombinant protein is avoided, and the regeneration of phenotypically normal plants is facilitated.


This is an interesting paper for scientists who are considering the practical application of their research. The author discusses practical problems like purity or product comparability as well as environmental concerns. For the United States, he provides some addresses where guidance documents can be obtained.