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Transgenic Plants in Therapeutically Valuable Protein Production

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ABSTRACT

Over the last 15 years a growing number of research groups worldwide have focused on plants as biofactories for the production of heterologous proteins. The reason is that plants provide a number of advantages over conventional recombinant systems including low cost, increased safety and scalable production, among others. The skepticism that received this technology when first envisaged has turned into a cautious optimism. A wide variety of proteins can be produced in plants and they are almost indistinguishable from their native counterparts. Even though there are still several issues that need refining such as boosting expression and ensuring correct post-translational processing and protein stability in plant tissues, molecular farming can potentially provide unlimited quantities of recombinant proteins for use as diagnostic and therapeutic tools. The low cost of plant-based vaccines make them ideal for large-scale programs in poor countries. Vaccinating the nearly 33 million children that each year remain unvaccinated for vaccine-preventable diseases would have profound effects on leveling the health inequities all over the world (Thanavala *et al*. 2006). It is hoped that the issue of IP does not represent an insurmountable obstacle to this end.

Keywords: antigens, edible vaccines, oral vaccination, pharmaceuticals, recombinant proteins

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INTRODUCTION

Plants have become an accepted and suitable system for large-scale production of recombinant proteins due to technological developments at many levels, including transfection methods, control of gene expression, protein targeting, the use of different crops as production platforms and modifications to alter the structural and functional properties of the recombinant product. Over the last few years, some of the limitations of plants as bioreactors such as low yields and inconsistent product quality have been overcome, which has allowed the commercial development of some plant-derived pharmaceuticals. Indeed, one of the most important driving factors has been yield improvement, as product yield has a significant impact on economic feasibility. Attention is now shifting from basic research towards commercial exploitation, and molecular farming is reaching the stage at which it may challenge established production technologies based on bacteria, yeast and cultured mammalian cells. In this review, recent progress in molecular farming will be examined. Since there have been several reviews recently (dos Santos and Wigdorovitz 2005; Rice *et al*. 2005; Streatfield 2006; Thanavala *et al*. 2006), this review will focus on very recent advances in the field.

EXPRESSION OF RECOMBINANT PROTEINS IN TRANSGENIC PLANTS

There is a high demand for production of recombinant proteins on an industrial scale because of their utility as diag-

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Invited Review

Table 1 Advantages of plant-based oral vaccines.

Lower cost of raw material for production of recombinant proteins
Rapid scale-up or down
Polyvalent vaccines are quite feasible
Plant cells provide protection for the antigen in the gut
Raw material easy to store and transport without the need for a cold chain
Reduced concerns over contamination with human pathogens in vaccine preparations
Eliminate syringes and needles and consequently medical assistance for administration
Eliminate concern over blood borne diseases through needle reuse

nostic reagents, vaccines and therapeutic agents. Recombinant DNA technology has allowed expression of heterologous proteins in different systems (Frank 1998). Originally prokaryotic hosts were the system of choice mainly because of the low overall cost and short production timescale (Terpe 2006). However, prokaryotic systems are limited about the classes of proteins that can be produced and they do not perform posttranslational modifications. Consequently, the focus turned to eukaryotic hosts: yeast, insect and mammalian cell cultures and transgenic animals. Although mammalian cell cultures and yeast have been the main expression systems employed, they have some downsides in terms of cost, scalability, risk of pathogenicity and authenticity (Balen and Krsnik-Rasol 2007). Since cost is still one of the persistent barriers for development and distribution of safe and effective new vaccines and pharmaceutical compounds to populations in dire need of them, plants represent a simple and inexpensive alternative allowing a scalable production system for recombinant proteins. Plant bioreactors have been estimated to yield over 10 kg of therapeutic protein per acre in tobacco, maize, soybean and alfalfa (Austin *et al*. 1994; Khoudi *et al*. 1999). In comparison with conventional bioreactors and mammalian cells or microorganisms, the cost of producing a protein under good manufacturing practice conditions is reduced to perhaps one-tenth (Larrick *et al*. 2001a; Daniell *et al*. 2001).

Genetically engineered plants have many advantages as sources of recombinant proteins (**Table 1**). Depending upon the promoters used, the recombinant proteins can accumulate throughout the plant, in specific organs (e.g. in seeds) or in specific organelles (e.g. chloroplasts) within a plant cell. However, considering the high content of protein, seeds have been identified as a target for recombinant protein accumulation (Takaiwa *et al*. 2007). Expression in seeds is ideal because it assures adequate storage properties and flexibility in processing management and batch production.

Furthermore, proteins produced in seed exhibit high stability; for example, enzymes and antibodies expressed in seed and stored for more than three years in the refrigerator retain full enzymatic or binding activity (Larrick *et al*. 2001a).

METHODS FOR GENE TRANSFER INTO PLANTS

There are three main approaches for expression of recombinant proteins in plants: (1) nuclear transformation and regeneration of transgenic plant lines; (2) transfer and expression of transgenes into the chloroplast genome and (3) by the use of plant viral vectors (transient expression). Each will be examined in turn.

Nuclear transformation

Successful expression of transgenes in plants is possible thanks to the unique capability of single plant cells to regenerate into whole plants while keeping all the genetic features of the parent plant. In the transgenic plant, foreign genes are stably incorporated into the plant genome, transcribed and inherited in a Mendelian fashion (Vain *et al*. 2007). In addition, it was found that gene transfer into a plant could be mediated by a plant-infecting bacterium, *Agrobacterium tumefaciens*, which is able to transfer DNA into the plant genome (Gelvin 2003). Subsequently, additional approaches such as microinjection, electroporation and microparticle bombardment were developed to deliver foreign genes into the plant genome (Vain *et al*. 2007). These procedures are based on the use of purified plasmid DNA.

Microprojectile bombardment or biolistics (direct DNA transfer) has been a method for gene transfer into plants extensively employed (Altpeter *et al*. 2005). The method is so versatile that multiple genes (>10) coding for complex recombinant macromolecules can be transferred simultaneously into the plant genome. Interestingly, even though the genes may be delivered on different plasmids, such multiple transgenes are frequently inherited in a linked fashion (Chen *et al*. 1998). Nicholson *et al*. (2005) successfully introduced four genes coding for components of a secretory antibody into rice and approximately 20% of the resulting plants contained all four genes. This represents an advantage over alternative gene transfer methods that involve the stepwise introduction of individual components, followed by successive rounds of crossing to generate plants containing the fully assembled molecule.

Direct DNA transfer also allows the introduction into plants of minimal expression cassettes containing only the promoter, open reading frame and terminator sequences. As no vector backbone sequences are transferred, this approach increases transgene stability and expression levels considerably by preventing the integration of potentially recombinogenic sequences (Loc *et al*. 2002). The described methods are applicable to a wide range of species and this explains why the majority of plant-derived recombinant pharmaceutical proteins have been produced by nuclear transformation and regeneration of transgenic plant lines.

Transient expression

Although nuclear gene transfer is now routine in many species, the main disadvantage is the production time-scale, which has prompted the development of alternative plant-based production technologies. One consists in the vacuum infiltration of leaves with recombinant *A. tumefaciens*, resulting in the transient transformation of many cells (Fischer *et al*. 1999). High levels of protein expression can be obtained for a short time but then they decline sharply as a result of post-transcriptional gene silencing (Voinnet *et al*. 2001). Co-expression inhibitor proteins of gene silencing can increase the expression levels of recombinant proteins at least 50-fold (Moissiard and Voinnet 2004). Researchers at Medicago Inc. have described how agroinfiltration of alfalfa leaves can be scaled up to 7500 leaves per week, producing micrograms of recombinant protein each week (Fischer *et al*. 2004).

Chloroplast transformation

An alternative approach to express recombinant proteins in a stable manner is by gene transfer into the plant chloroplasts. Chloroplasts are plant cellular organelles with their own genome and transcription-translation machinery. The chloroplast genome is a highly polyploid, circular double-stranded DNA 120 kb to 180 kb in size, encoding approximately 120 genes (Maliga 2004). Each chloroplast carries a number of identical genome copies, which are attached to membranes in clusters called nucleoids. A tobacco leaf cell may contain 100 chloroplasts, with 10-14 nucleoids each, and about 10,000 copies of the genome per cell (Bock and Khan 2004). A gene may be introduced into a spacer region between the functional genes of the chloroplast by homologous recombination, targeting the foreign gene to a precise location. Gene silencing has not been observed with chloroplast transformation, whereas it is a common phenomenon with nuclear transformation. Additionally, the presence of chaperones and enzymes within the chloroplast may help

assemble complex multi-subunit recombinant proteins and correctly fold proteins containing disulfide bonds, which should eliminate expensive *in vitro* processing of recombinant proteins (Maliga 2004), thereby drastically reducing the costs of *in vitro* processing. Despite such significant progress in chloroplast transformation, this technology has not been extended to major crops. This obstacle emphasizes the need for chloroplast genome sequencing to increase the efficiency of transformation and conduct basic research in plastid biogenesis and function.

Chloroplast transformation has been achieved in several plant species (for a review see Daniell *et al*. 2005) such as carrot, tomato, *Brassica oleracea*, petunia, soybean, lettuce, the liverwort *Marchantia polymorpha* and the green algae *Chlamydomonas reinhardtii*, but transformation is routine only in tobacco. Plastid transformation in *Arabidopsis thaliana*, *Brassica napus* and *Lesquerella fendleri* has been achieved but at low efficiency (Bock and Khan 2004). The ability to transform the chromoplasts of fruit and vegetable crops represent an interesting possibility for the expression of subunit vaccines (Ruf *et al*. 2001).

There are several advantages of chloroplast transformation: high-level expression of the recombinant proteins, the recombinant proteins will accumulate within the chloroplast thus limiting toxicity to the host plant, multiple genes can be expressed in operons (Quesada-Vargas *et al*. 2005) and the absence of functional chloroplast DNA in pollen of most crops may provide natural transgene containment (Daniell 2007). Transgene expression in tobacco plastids reproducibly yields protein levels in the 5% to 20% range, however, levels up to 47% of the total soluble protein, can be achieved (Daniell *et al*. 2005).

Even though there is no protein glycosylation in chroloplasts (Tregoning *et al*. 2003). The functionality of chloroplast-derived antigens and therapeutic proteins has been demonstrated by *in vitro* assays and animal protection studies (Koya *et al*. 2005).

Expression based on plant viruses

Another tobacco transient-expression technology is based on the use of plant viruses as expression vectors. Viruses can gain entry into a plant cell where they can accumulate and then spread throughout the entire plant. They remain in the cytoplasm throughout infection and do not incorporate into the genome of the susceptible host and, thus, are not inherited by the next generation. Several features make plant viruses well suited as transient expression vectors: high-level expression of the introduced genes (up to 2 g/kg of plant tissue) within a short period (1-2 weeks after inoculation), rapid accumulation of the appropriate products and the fact that more than one vector can be used in the same plant, allowing multimeric proteins to be assembled (Verch *et al*. 1998) with the additional benefit of biological containment on the viral sequences. Post-transcriptional gene silencing, a natural mechanism of defense by the plant against viruses, can be avoided by expressing a replicase from some inducible promoters (Mori *et al*. 2001). Plant viruses have been used to produce a wide range of pharmaceutical proteins, including vaccine candidates and antibodies. Some plant viruses have a wide host range and are easily transmissible by mechanical inoculation, spreading from plant to plant, making large-scale infections feasible.

Plant-virus genomes can be composed of DNA or RNA but the main virus systems from which efficient expression systems are being developed mainly consist of positive-sense RNA genome, single-stranded DNA geminiviruses and double-stranded non-integrating DNA pararetroviruses (Porta and Lomonossoff 1996) but RNA viruses can multiply to very high titres in infected plants, which makes them better suited vectors for protein expression vectors. For genetic manipulation, viral RNA genomes are reverse transcribed *in vitro* and cloned as full-length cDNAs. There are at least three approaches for insertion of foreign genes into plant viral genomes: (1) gene replacement, when non-essential viral genes, like those coding for coat proteins, are replaced by the gene of interest. Since some viruses have limitations as to the size of the molecules than can be incorporated into their genomes this would be the best strategy. (2) gene insertion, where the gene of interest is placed under the control of an additional promoter; this approach would be advisable where large coding sequences have to be expressed and (3) gene fusion, when the gene of interest is translationally fused with a viral gene; the use of coat-protein genes has allowed an efficient method for presentation of foreign peptide sequences on the surfaces of viral particles (Johnson *et al*. 1997). Even though the coat protein of *Tobacco mosaic virus* (TMV) has been the most utilized system for expression of antigenic epitopes, other viruses such as *Alfalfa mosaic virus*, *Plum pox virus*, *Potato virus X* and *Tomato bushy stunt virus* have also been extensively employed (Johnson *et al*. 1997).

One of the reasons for the use of different plant viruses was to overcome the apparent size limitation (<1 kb) that prevented inclusion of large peptides and inhibited virus assembly (Avesani *et al*. 2007). However, Werner *et al*. (2006) have recently shown that large inserts (>1 kb) can be fused to the coat protein and expressed, provided that suitable linkers are included. Numerous reports have confirmed that plant viruses can be effective vectors for expression of antigens and can provide complete protection in challenge trials (Dalsgaard *et al*. 1997).

Based on the approach described above for gene replacement, Icon Genetics (Halle, Germany) has developed viral replicons that can be delivered through infection with *Agrobacterium*, a process termed 'magnifection' (Gleba *et al*. 2005). Using this system, foreign protein can be transiently expressed at up to 80% of total soluble protein, including oligomeric proteins (Giritch *et al*. 2006).

PRODUCTION OF ANTIGENS IN PLANTS

Vaccines, the most effective medical intervention to prevent disease, have been based on live, attenuated organisms, purified antigens (subunit vaccines) or DNA coding for specific antigens. Purified antigens are usually delivered at a set dose and have constituted a relatively simple and uniform material for administration, generally prepared from recombinant sources. Considerable progress has been made since Charles Arntzen first envisaged the idea of transgenic plant vaccines in the early 1990s. There are now a number of examples demonstrating the successful expression of subunit candidate vaccines both for humans and animals in transgenic plants (**Table 2**). These include antigens from bacterial and viral sources that infect humans, domestic or wild animals and representing secreted toxins and cell or viral surface antigens.

All along, the main goal has been to attain high levels of expression but, usually, levels of expression vary greatly depending on the protein expressed and the species of plant used to achieve expression. Different approaches have resulted in high levels of expression of several antigens but it is difficult to make comparisons since specific antigens have rarely been tested in multiple systems. Exceptions include the subunit B of the cholera toxin and the closely related heat-labile enterotoxin from *E. coli*, which have been expressed in multiple plant systems with a range of expression from 0.2% up to 12% of total soluble protein (Streatfield 2006). High level of antigen expression will, likely, result in downstream processing and purification at low cost. In the case of the heat-labile enterotoxin from *E. coli* expressed in corn, the plant tissue contained a sufficiently high concentration of the antigen such that a 1 mg dose, estimated to be the ideal oral dosing, corresponded to approximately 2 g of edible tissue (Lamphear *et al*. 2002). A reduction in the amount of material to be consumed is anticipated by increasing the yield, because usually, oral immunization requires higher doses of antigen than parenteral immunizations (Ogra 2003).

Table 2 Antigens recently expressed in transgenic plants.

Antigen	Plant system	Expression levels	Reference	Protection assays
Yersinia pestis F1, V, and F1-V antigens	*Nicotiana benthamiana*	1 mg/g	Santi *et al*. 2006	Yes
Yersinia pestis F1 and LcrV antigens	*Nicotiana benthamiana*	120-380 µg/g	Mett *et al*. 2007	Yes
Cholera toxin B subunit protein	Tomato	0.081%	Jiang *et al*. 2007	Yes
Cholera toxin B subunit protein	Rice	30 µg of CTB per seed	Nochi *et al*. 2007	No
Human secreted alkaline phosphatase	Tobacco NT1 cell	27 mg/L	Becerra-Arteaga *et al*. 2006	No
Major structural protein VP60 of rabbit hemorrhagic disease virus	Potato	0.30%	Gil *et al*. 2006	No
VP1 protein, an epitope and the coat protein of Enterovirus 71	Tomato	N.R.	Chen *et al*. 2006	No
ApxIIA, a bacterial exotoxin *Actinobacillus pleuropneumoniae*	Tobacco	0.1%	Lee *et al*. 2006	Yes
Heat labile enterotoxin from *E. coli*	Soybean	2.4%	Moravec *et al*. 2007	No
Heat labile enterotoxin from *E. coli*	Lettuce		Kim *et al.* 2007	
Rotavirus VP7 protein	Potato	3.6- 4.0 µg/mg	Li *et al*. 2006a	No
M. tuberculosis ESAT6 antigen	*Nicotiana benthamiana*	800 µg/g	Dorokhov *et al*. 2007	No
VP1 antigen from foot-and-mouth disease virus	Tobacco, alfalfa	2-3%	Li *et al*. 2006b	No
Hepatitis B virus surface antigen	*Nicotiana benthamiana*	7.14%	Huang *et al*. 2006	No
Tetanus toxin C fragment-specific monoclonal antibody fused with the tetanus toxin C fragment	Tobacco	0.8%	Chargelegue *et al*. 2005	Yes
Fusion protein of Newcastle disease virus	Maize	0.95-3%	Guerrero-Andrade *et al*. 2006	Yes
Fimbrial FanC from *E. coli*	Soybean	0.08%	Garg *et al*. 2007	No
Fimbrial adhesin FaeG from *E. coli*	Potato	1%	Liang *et al*. 2006	No
Protective antigen of *Bacillus anthracis*	Tobacco	14.2%	Koya *et al*. 2005	Yes
HIV-Tat	Tomato	1 µg/mg dry weigth	Peña Ramirez *et al*. 2007	No
HIV-ENV and GAG fused to the surface protein antigen of hepatitis B virus	Tomato	~0.3 µg/g	Shchelkunov *et al*. 2006	No
HPV16 E7	*Nicotiana benthamiana*	0.4 µg/g	Massa *et al*. 2007	Yes
Rabies nucleoprotein	Tomato	1-5%	Perea *et al*. 2007	Yes
Rotavirus VP2 and VP6 proteins	Tomato	1%	Saldaña *et al*. 2006	No
AB5 toxin from *E. coli*	Tobacco NT-1 cells	6.5- 8.2 µg/g	Wen *et al*. 2006	Yes
Fusion protein gene of Newcastle disease virus	Potato	0.25-0.55 g/100 g of	Yang *et al*. 2007	No
Heat-shock protein A from *H. pylori*	Tobacco	<1%	Zhang *et al*. 2006a	No
Gal/GalNAc lectin of *Entamoeba histolytica*	Tobacco	6.3%	Chebolu and Daniell 2007	No
Two T-cell protective cancer epitopes	Tobacco	NR	McCormick *et al*. 2006	Yes
Multiple T-cell epitopes	Rice	NR	Takaiwa 2007	Yes
Human serum albumin	Tobacco	8%	Fernandez-San Millan *et al*. 2007	No
Spike protein (S1) of the severe acute respiratory syndrome coronavirus	Tobacco	NR	Li *et al*. 2006c	No
Sweet protein monellin	Tobacco	2%	Roh *et al*. 2006	No
Human epidermal growth factor	Tobacco	NR	Wirth *et al*. 2006	No
Human alpha-L-iduronidase	Tobacco	360 pmol/min/mg	Kermode *et al.* 2007	No
Core neutralizing epitope of porcine epidemic diarrhea virus	Tobacco (Expression based on Tobacco mosaic virus)	5.0%	Kang *et al*. 2006	No
15 amino acids of the poliovirus peptide (PVP)	Tobacco (Expression based on Tobacco mosaic virus)	0.05%	Fujiyama *et al*. 2006	No
Hep. B virus core antigen	Potato and cowpea (Expression base on Potato virus X and cowpea Mosaic virus)	10 µg/g	Mechtcheriakova *et al*. 2006	No
Norwalk virus capsid protein	Tobacco NT1 cell (Expression based on Bean Yellow Dwarf Virus)	up to 1.2% TSP	Zhang *et al*. 2006b	No
Human cytotoxic T-lymphocyte antigen 4-immunoglobulin	Rice cell suspension culture	31.4 mg/L	Lee *et al.* 2007	No

Expression levels are as reported in the literature and indicate percentage of total soluble protein (TSP) unless indicated otherwise. NR, not reported.

The importance of virus-like particles

Antigen presentation to the mucosa associated lymphoid tissue is a key element in the response of the mucosal immune system (Ogra *et al*. 2001). One major obstacle in the development of mucosal vaccines is to be able to induce systemic as well as mucosal responses and one way to overcome it has been by the use of adjuvants, which can promote the generation of antibodies to an antigen following immunization. However, many of these adjuvants do not enhance priming of cytotoxic T lymphocytes (Gilbert 2001).

Recombinant virus-like particles (VLPs) represent an attractive alternative for antigen presentation since they are more immunogenic than recombinant proteins alone and are able to stimulate both the humoral and cellular arms of the immune system (Grgacic and Anderson 2006). These self-assembling, non-replicating viral core structures consisting of one or more viral coat proteins, can act as an adjuvant by carrying peptide sequences inside the antigen presenting cells and feeding into the endogenous processing pathway (Schirmbeck *et al*. 1995), a phenomenon known as "cross-priming" (Schwarz *et al*. 2005). The efficacy of immunization with VLPs is best illustrated by the success of the HBV-like particles produced in *Saccharomyces cerevisiae*, which was the first recombinant vaccine developed (Maclean *et al*. 1984).

VLPs are especially interesting from a mucosal vaccine point of view as they offer the opportunity to deliver the

virus employing the natural route of transmission (Gilbert *et al*. 2001). Oral delivery of VLP has been shown to induce both systemic and mucosal IgA responses both to the virus particles and to foreign epitopes expressed as chimeric proteins on the VLP surface, without the need for external adjuvants (Niikura *et al*. 2002). VLPs have been produced from the capsid or envelope components of a wide variety of viruses to study virus assembly and for development of vaccines (Grgacic and Anderson 2006). Vaccines from HBV and HPV VLPs have been successful, but VLPs from pathogens affecting immune cells and those that successfully evade the immune system, such as HIV-1 and hepatitis C virus have proven to be more challenging.

Production of properly folded VLPs in plants has been extensively reported. In most cases, they have been shown to mimic the immunological properties of native VLPs and stimulate antibody and T cell responses in mice (Santi *et al*. 2006). Antigens having the ability to assemble into VLPs have been generally employed such as the surface (Sunil Kumar *et al*. 2003; Shchelkunov *et al*. 2006) and core antigens of Hepatitis B virus (Huang *et al*. 2006), the capsid protein of Norwalk Virus (Huang *et al*. 2005), the L1 protein of human papillomavirus (Maclean *et al*. 2007), the haemagglutinin/neuraminidase of paramyxoviruses (Guerrero-Andrade *et al*. 2006), two capsid proteins from rotavirus (Saldaña *et al*. 2006) and chimeric HIV (ENV and GAG). In fact Hepatitis B VLPs have been successfully obtained in a variety of plants using the surface antigen (Kumar *et al*. 2007). Many of these antigens as well as several capsid or core proteins from various plant viruses have also been employed as carriers to express a wide variety of antigenic peptides. However, not all antigens may form VLPs in plants. The hepatitis E virus capsid protein, which assemblies readily into VLPs in a baculovirus system, did not assembly adequately into VLPs in potato and this lead to a failure to elicit detectable antibodies in mice serum (Maloney *et al*. 2005). Clearly formation of VLPs is essential for immune recognition.

Chimeric VLPs offer enormous potential in specific, multi-epitope presentation but their success will be dependent on a judicious selection of the most relevant epitopes for vaccine efficacy. Although research has mainly focused on oral delivery of minimally processed plant material, purification of VLPs for parenteral delivery is also a highly realistic approach.

Human clinical trials with oral plant-based vaccines

The functionality of plant-based antigens has been tested in a number of experiments in mice and other animals. In the vast majority of the cases, plant-based antigens have been able to elicit a strong immune response and to confer protection against challenge with the pathogen. These results have paved the way for several clinical trials aimed to assessing human immune responses to plant-produced recombinant proteins. So far, three plant-produced antigens have been tested in Phase I human clinical trials, the heat-labile enterotoxin from *E. coli*, the capsid protein from Norwalk virus and the surface antigen from the Hepatitis B virus.

The heat-labile enterotoxin from *E. coli* has been tested twice in human clinical trials, in the first trial, 14 adult volunteers ingested three doses of transgenic potatoes (containing 3.7-15.7 μg/g of antigen) or control wild-type potato. Serum antibody responses were detected in 10 out of the 11 volunteers and eight out of the 11 developed neutralization titers of more than 1:100 (Tacket *et al.* 1998). This trial was the proof-of-principle that humans could develop serum and mucosal immune response to antigen delivered in transgenic plants. Recently, the same antigen was delivered to volunteers in transgenic corn with seven out of the nine volunteers developing increases in serum IgG and four of the volunteers also developing stool IgA (Tacket *et al*. 2004).

In another trial, 20 human volunteers ate two or three doses of transgenic potatoes expressing 215-751 μg of the Norwalk virus capsid protein (Tacket *et al*. 2000). Out of the 20 volunteers, 19 developed significant increases in the numbers of specific IgA antibody-secreting cells, four developed serum IgG and six developed specific stool IgA, although the levels of serum antibody were not high. This variation in immune response was probably due to the inconsistent assembly of the antigen into virus-like particles and to the possible presence of pre-existing antibodies to the antigen, which might have had an effect.

The surface antigen from the Hepatitis B virus was utilized in a randomized, placebo-controlled, double-blind trial conducted in volunteers previously immunized, 1-15 years earlier, with the licensed hepatitis B vaccine (Thanavala *et al*. 2005). A total of 42 subjects were enrolled in the study and received either three doses of placebo potatoes or transgenic potatoes (two or three doses). None of the volunteers who ingested control potatoes had any change in their antibody titers during the study. On the other hand, 63% of volunteers (10 out of 16) who consumed three doses of HBs Ag-containing transgenic potatoes showed marked increases in antibody titers compared with titers at day 0 (Thanavala *et al*. 2005). Thus, an antigen from a nonenteric pathogen, was capable to elicit a immune response with no buffering of stomach pH and without the presence of a mucosal adjuvant. The same antigen had been expressed in lettuce and fed to three seronegative volunteers (Kapusta *et al*. 1999). The volunteers received about 0.2-1 μg of antigen in a 200 g dose of lettuce. In comparison, the commercial Hepatitis B vaccine contains 10 μg of antigen per adult dose (Tacket 2004). Two of the three volunteers produced short-lived anti-HBs antibody titers, which were detectable 2 weeks after the second immunization and were no longer detectable after the additional 2 weeks.

Mucosal immunologists recognize that the heat-labile enterotoxin from *E. coli* and its relative the B subunit of cholera toxin are particular antigens with highly immunogenic properties. The fact that two different antigens were also immunogenic in humans after oral administration was rather encouraging.

PRODUCTION OF BIOPHARMACEUTICAL PROTEINS IN PLANTS

The large demand for many pharmaceutical proteins poses a burden on any transgenic production system to meet the demand. Transgenic plants could be rapidly scaled up to field scale cultivation. For example, the worldwide demand for human serum albumin (about 550 metric tons per year) could be met by 30,000 hectares of land (assuming an expression level of 1% TSP in tobacco), which is less than one thousandth of the total cultivated soil in the USA (Fischer and Emans 2000). On the other hand, transgenic animals are limited by the time needed to raise a herd of animals producing the recombinant protein.

A number of proteins of pharmaceutical and industrial importance have been produced in transgenic plants, including glucocerebrosidase and granulocyte–macrophage colony stimulating factor two of the world's most expensive drugs (Giddings *et al*. 2000).

Various proteins of industrial interest such as the human milk proteins lactoferrin (Chong *et al*. 2000) and β-casein (Philip *et al*. 2001) have been produced in transgenic plants to be employed as a supplement for infant formulas to enhance nutrition, digestibility and antimicrobial properties.

Recently, tobacco was modified with the human collagen I gene *pro1α(I)* (Ruggiero *et al*. 2000). Collagens are very important molecules employed in the cosmetics, medical and food industries. They are generally extracted from animal tissues and may represent a contamination risk if the tissue is infected. Several expression systems have been developed that produce procollagens, but these have to be chemically modified *ex vivo* to produce mature collagens (Ruggiero *et al*. 2000). The procollagen produced in tobacco cells was spontaneously processed into mature collagen during extraction, which represented a significant advantage

for large-scale, low-cost production of collagen.

Plants are also being increasingly seen as a source for biofuels, a highly controversial approach, of which methanol is currently the most popular (Wyman 2007). There have been several biodegradable polymers such as polyhydroxyalkanoates as well as a protein-based polymers produced in tobacco (Poirier 1999). One of them is a polymer similar to elastin, one of the strongest natural fibres (Guda *et al*. 2000). These polymers might be used as transducers, super-absorbents and biodegradable plastics, or in various medical applications such as tissue reconstruction surgery (Staub *et al*. 2000).

There are a number of proteins with important roles in the stimulation or modulation of the immune responses such as cytokines, chemokines, etc. Many of these compounds have been synthesized successfully in plants such as the human granulocyte-macrophage colony-stimulating factor (Lee *et al*. 1997), human interferon α and β (Kusnadi *et al*. 1997), interleukin 12 both human (Gutierrez-Ortega *et al*. 2004) and murine (Gutierrez-Ortega *et al*. 2005), etc. Similarly, some human hormones such as the growth hormone (Leite *et al*. 2000), erythropoietin (Cramer *et al*. 1996) and insulin (Arakawa *et al*. 1998) have been produced in plants.

One area where plant-based recombinant proteins are having a dramatic impact is in the area of diagnostics reagents and avidin is a case study. Avidin is normally found in egg white, from which it is purified. The cDNA was expressed in maize and could be reproducibly produced at 230 mg per kg of maize seed, which was, in the authors' estimation, 10-fold less expensive than avidin extracted from eggs (Hood *et al*. 1997). The maize avidin is fully functional and now commercially available (Sigma-Aldrich product #A8706). Similarly, plant-based β-glucuronidase and aprotinin are now also commercially available (Witcher *et al*. 1998; Zhong *et al*. 1999). It is estimated that the costs of producing aprotinin in plants are comparable with extracting it from its natural source, bovine lung.

PRODUCTION OF ANTIBODIES IN PLANTS (PLANTIBODIES)

Antibodies were the first bioactive molecules expressed in transgenic plants (Hiatt *et al*. 1989). Since then, numerous antibodies have been produced in a variety of plants, and expressed in different tissues and different sub-cellular compartments. This is not surprising, considering the wide range of uses for antibodies in the diagnostic, industrial, medical and research fields.

Plants were first envisaged as systems to produce antibodies for one reason: cost. Transgenic plants represent the most productive and economical system for making recombinant antibodies (Wycoff 2005). The cost of producing monoclonal antibodies in plants is significantly less than by traditional fermentation methods. Smith and Glick (2000) have made an estimate that assuming a best-case yield of two grams of recoverable protein/L of bacteria, the minimum cost of production (assuming $2/L for the growth medium and purification costs) would be approximately $1000/kg, not including salaries. Since multimeric proteins, such as antibodies, are assembled with very low efficiency in bacteria, the yield could be much lower which would have a direct impact on costs. It is not surprising, then, that the cost of antibodies produced using traditional microbial fermentation can be as high as $1000/g (Potera 1999). Production in plants is expected to reduce the cost of antibodies dramatically. The land, infrastructure, and expertise necessary for harvesting and processing large volumes of plant material already exist, which would result in drastically reduced capital costs.

The costs of producing an IgG from alfalfa grown in a 250 m^2 greenhouse are estimated to be within US$500-600/g, compared with US$5000 per gram for the hybridoma produced antibody (Khoudi *et al*. 1999). In another study, the cost of producing one gram of purified IgA in plants was estimated to be well below US$50/g, which compares favorably with the costs of cell culture (US$1000/g) or transgenic animal production systems (US$100/g) (Larrick *et al*. 2001b).

Corn has been the system of choice for production of many recombinant proteins including antibodies. The main reason is the high protein content of the corn kernel, which accounts for approximately 10% of the dry weight. Even if the antibodies accounted for only 0.5% of the total grain protein, it would cost less than US$200 to produce one kilogram of antibody in corn kernels. However, it has already been shown that corn can produce recombinant proteins up to 5.7% of the total protein (Kusnadi *et al*. 1998). An additional advantage is that plantibody production could be scaled up or down depending on the demand by increasing or decreasing the acreage of the antibody-producing plants. Plant-produced antibodies are predicted to cost as little as $10–100/g, approximately 10-100 times less expensive than antibodies produced in bacteria (Potera 1999).

In all likelihood, the biggest component of cost of antibodies will be purification. In theory, purification of plantibodies should be straightforward, using standard procedures. Protein degradation that may occur during extraction can be minimized by the addition of protein stabilizers and proteinase inhibitors, although proteolysis *in planta* represents a challenge (Sharp and Doran 2001). Stevens *et al*. (2000) had suggested that proteolytic degradation in leaves is, in part, linked to the natural process of senescence. This indicates that the physiological state of the plant may have an impact on antibody integrity.

On the other hand, it is conceivable that for some uses the plantibody would not even need to be purified. Delivery of the antibody could be achieved by direct consumption of the plant tissue containing the plantibody (Ma and Hein 1995). This is very important from the standpoint of safety as plants do not serve as hosts for human viruses or prions, unlike hybridomas.

There are no plantibodies yet in commercial production, however there are several candidates that are potentially useful as human therapeutics. The most advanced is a chimeric secretory IgG–IgA antibody called CaroRx*TM*, against a surface antigen of *Streptococcus mutans*, the bacteria that causes tooth decay, which prevents it from binding to teeth, thereby reducing cavities (Ma *et al*. 1998). Unexpectedly, application protects from recolonization and this may last for up to 2 years, although the antibody was applied for only 3 weeks and functional antibody was detected on the teeth for only 3 days following the final application of the antibody. This antibody has reached a pilot Phase II trial. Phase I/II confirmatory clinical trials are underway.

Another antibody, a humanized anti-herpes-simplex virus (HSV) antibody prepared in soybean, was effective in the prevention of vaginal HSV-2 transmission in mouse (Zeitlin *et al*. 1998). A third antibody was developed in rice and wheat against a carcinoembryonic antigen (Stoger *et al*. 2000). This antigen, a cell-surface glycoprotein, is one of the best-characterized tumor-associated antigens and antibodies against it are usually employed for diagnostic and therapy. Levels of the antibody in seeds did not show a significant decline after storage at room temperature for six months (Stoger *et al*. 2000).

Finally, a tumor-specific vaccine was prepared in tobacco for the treatment of lymphoma using a modified plant virus (Savelyeva *et al*. 2001). The antibody genes for expression of an scFv were derived from a mouse B-cell lymphoma. Mice were immunized with the plant-produced scFv and anti-idiotypic antibodies (antibodies against the binding portion of the antibody) were generated. The mice were protected against infection by the lymphoma that produced the original antibody (Savelyeva *et al*. 2001).

Plant viral vectors have also been employed to produce therapeutically useful antibodies in plants, including an antibody against the colorectal cancer-associated antigen GA733-2 (Verch *et al*. 1998), which upon immunization in mice elicited a comparable humoral response to that using antigen produced in insect cell culture (Verch *et al*. 2004).

Although plant antibodies are normally found to be properly folded and functional (Ma and Hein 1995), differential glycosylation by the plant still remains a major constraint for applications in human healthcare. There is still concern over the potential immunogenicity of plant-specific complex N-glycans, which are present on the heavy chain of plant-derived antibodies (see section 8). There have been several approaches to prevent addition of complex N-glycans to recombinant antibodies when glycosylation-dependent effector functions are not needed. One approach is the removal of peptide recognition sequences for N-glycosylation; Another is the addition of a ER retention C-terminal (KDEL in amino acid code) sequence which avoids Golgi-mediated modifications (Stoger *et al*. 2002). A final approach is the humanization of plant glycans, and to this end, human β-1,4-galactosyltransferase was stably expressed in tobacco plants which were crossed with plants expressing a murine antibody which resulted in a plantibody with partially galactosylated N-glycans (Bakker *et al*. 2001). Interestingly, the glycosylation profile of endogenous proteins and of a recombinant immunoglobulin in tobacco leaves also seems to be affected by senescence (Stevens *et al*. 2000).

Recently a novel strategy was reported involving the plant-based production of a fusion molecule of an antigen and the corresponding antibody (Obregon *et al*. 2006). The HIV HIV-p24 antigen was expressed as a genetic fusion with two constant region sequences from human Igα-chain and targeted to the endomembrane system. This allowed to increase the expression approximately 13-fold higher than with HIV p24 expressed alone and to enhance the immunological properties. The fusion elicited T-cell and antibody responses in immunized mice (Obregon *et al*. 2006).

ISSUES REGARDING PROTEIN PRODUCTION IN PLANTS

Even though plant-based compounds present a number of benefits, some potential issues of concern have been identified. These issues differ depending on whether the vaccine candidate is to be purified from plant tissues prior to formulation and delivery or whether it is to be administered orally as recombinant plant material.

Downprocessing

If purification is involved there are several approaches that could be implemented; One is to engineer the protein, to be secreted into the culture medium. Secretion systems are convenient because no disruption of plant cells is necessary during protein recovery, hence, release of phenolic compounds is avoided. Nevertheless, the recombinant proteins might be unstable in the culture medium. Another approach is the use of affinity tags to facilitate the recovery of proteins as long as long as the tag be removed after purification to restore the native structure of the protein. In either case, good manufacturing practices will be needed and possible lot-to-lot variability will need to be closely monitored. By contrast, if the recombinant antigen is to be delivered in a processed plant product as an oral vaccine, production would be based on food processing technology rather than protein purification schemes, but good manufacturing practices will still apply.

Consistency of product (homogeneity) is very important for plant-based vaccines as well as for purified antigens. Therefore, rather than administering whole plant organs (fruits, or grains) directly, as it was originally envisaged, it may be better to process the plant material into a uniform state and to be stable to the food processing technology. This has been assessed using recombinant corn expressing the B subunit of the heat labile toxin, and the antigen has been shown to be stable to milling and modified extrusion conditions and to be evenly distributed in the products (Streatfield 2006). This will be very important is the product is to be combined with non-transgenic material or with transgenic material expressing different antigens, or even protein adjuvants, for even dosing.

Stability of antigens over time in processed food products stored at different temperatures will also need to be assessed. The B subunit of the heat labile toxin and the S glycoprotein of transmissible gastroenteritis virus have been shown to be stable for at least a year, even when stored at ambient temperatures (Lamphear *et al*. 2002). This emphasizes the redundancy of a cold chain during storage and distribution of plant-based products. This feature is particularly important in poor countries with limited resources to provide a cold chain and the equipment and personnel needed for injections. The low cost of plant-based vaccines make them ideal for large-scale programs in poor countries.

Oral tolerance

Since oral tolerance is the usual result when the mucosal system encounters food proteins, there is some concern that oral tolerance may be induced by consumption of the recombinant protein contained in a transgenic plant. Although the mechanisms of oral tolerance remain unclear, it is likely that this concern is more relevant for pathogens transmitted by the parenteral route (for instance Hepatitis B and malaria) than pathogens whose natural route of transmission is via the gastrointestinal tract (Tacket 2004). Immune tolerance to parenterally administered proteins can occur after multiple small oral doses of the protein (Tacket 2004), therefore it is unlikely that oral vaccination with plant-based antigens would result in tolerance to parenterally administered protein since the number of doses of the oral vaccine would be very small. As the Phase I studies have demonstrated, the plant-based antigen is recognized and processed as an antigen and elicits an immune response. The possibility of incorporating mucosal adjuvants may considerably improve the immune response (Lavelle and O'Hagan 2006).

Glycosylation

Since the general eukaryotic protein synthesis pathway is conserved between plants and animals, folding and assembly, as well as transfer of an oligosaccharide precursor to *N*-glycosylation sites can be correctly accomplished in transgenic plant systems (Rayon *et al*. 1998). However, addition of plant-specific residues α-1,3-fucose and β-1,2-xylose, might pose problems for the production of fully functional therapeutic proteins, even though this may not seem not to occur with all plant-based recombinant proteins (Sriraman *et al*. 2004).

Both full-sized antibodies and various functional antibody derivatives have been produced successfully in plants, including Fab fragments, scFvs, bispecific scFvs, single domain antibodies and antibody fusion proteins. Some studies have shown that while there were some differences in the glycan groups present on plant-based recombinant antibodies, neither the antibody nor the glycans were immunogenic (Balen and Krsnik-Rasol 2007). In an elegant study, Chargelegue *et al*. (2005) tested the immunogenic effect in mice of plant glycans of transgenic murine monoclonal IgG antibodies and horseradish peroxidase. Because the same mouse strain was used for generating the original mAb, the study specifically compared the immunogenicity of a self-protein and a plant protein displaying foreign plant glycans, with the self-protein displaying mammalian glycans. Encouragingly, the plant glycans of both the self and foreign (horseradish peroxidase) proteins were poorly immunogenic even when parenterally administered with alum adjuvant.

Boosting levels of expression

Production costs are the key issues for recombinant protein production and they include the generation, growing and harvesting of the recombinant material and downstream processing and purification, which tend to increase with tis-

sue complexity. If the recombinant product is obtained in high levels, the amounts of biomass required and the processing and purification of the product can be greatly reduced. Thus, the achievement of high expression levels is a major goal in all systems. Expression levels for several proteins are already sufficiently high for economic production but further improvements in expression are necessary before other vaccine candidates can be considered practical. Several strategies have been developed to increase the levels of recombinant proteins and they have focused on transcription, transcript stability and translation. To this end, strong, tissue-specific promoters, matrix attachment regions, plant or viral 5′ non-translated regions, plant intron sequences, codon-optimization, removal of predicted mRNA secondary structures that might hinder translation and fusion to proteins that have been shown to be stably expressed at high levels in plants, have all been employed to enhance the levels of expression (Streatfield 2007). Probably stacking of several of the available tools discussed above will probably be required to produce commercial products, although this can be limited by access to the necessary intellectual property (IP). This is an issue that sooner or later will have to be resolved. However, when implementing new strategies and combining currently available approaches, care must be taken to minimize potential negative influences, in particular gene silencing (Yu *et al*. 2003).

Commercial aspects

Considering that a wide variety of recombinant proteins can be produced in a safe and inexpensive manner, it comes as no surprise the interest that the technology has generated from a commercial standpoint. Several companies have been founded based on this technology and others are actively engaged investigating the potential. There are already on the market several plant-produced proteins employed in the area of diagnostics (Witcher *et al*. 1998; Zhong *et al*. 1999) including one at large scale (Hood *et al*. 1997). Several plant-derived recombinant pharmaceutical proteins are reaching the final stages of clinical evaluation, and more are in the development pipeline. However, there is some regulatory uncertainty, particularly for proteins requiring approval for human use (Kirk and Webb 2005). It is important that these regulatory issues be resolved before pursuing product licensure. So far, only one plant-derived vaccine targeting a viral disease of poultry has reached licensure (Dow Agrosciences, USA). Although vaccines intended for use in humans will probably require a more laborious path to licensure, this milestone establishes a credible foothold for plant-derived vaccines (Thanavala *et al*. 2006).

FUTURE PROSPECTS

Plants as bioreactors for the production of foreign proteins in plants with a view to commercial production have attracted considerable attention over the past decade. Even though only a few products have reached the market so far, there are more approaching commercialization, after meeting the technological challenges and clearing the regulatory hurdles. In the short term, enzymes for large-scale industrial processes and antigens for oral animal vaccines are the most likely plant-expressed products to be commercially viable as the first ever licensure of a plant-derived vaccine targeting a viral disease of poultry described before has confirmed. Attention will be required to ensure correct post-translational processing and protein stability in plant tissues. Advances are required to boost expression further and stacking of many of the available tools discussed above will probably be required to produce commercial products.

As with all biotechnological developments, the technology for plant-derived vaccines has been patented in industrialized countries. Poor countries, which usually have a high disease burden, often have poor or inexistent IP protection rules and lack of adequate knowledge and infrastructure to protect and commercialize a biotechnological product. It has been postulated that plant-derived vaccines may be approved in an industrialized country and then be more broadly used in poor countries (Thanavala *et al*. 2006). It remains to be seen how this approach would be implemented.

In many reviews and articles on plant made-pharmaceuticals a point is made about the need (some authors have even called it a ¨moral imperative¨ (Ma *et al*. 2005) to provide low-cost medicines and vaccines to poor countries. This need is used as an important justification for the development of plant-based vaccines. Plant-based pharmaceuticals may offer a new model for vaccine development, which may allow a wider participation, beyond the well-established multinational pharmaceutical companies (Ma *et al*. 2005). Poor countries would potentially be involved, although it is still not well defined how, and the focus could be on specific regional diseases that do not feature in current drug development programs. It is hoped that this technology will eventually help those who needed it the most and that the issue of IP does not represent an insurmountable obstacle. Putting the collective benefit ahead of the personal gains, will be the key for the full realization of this technology.

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