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## Plant molecular farming: systems and products

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**Abstract** Plant molecular farming is a new and promising industry involving plant biotechnology. In this review, we describe several diverse plant systems that have been developed to produce commercially useful proteins for pharmaceutical and industrial uses. The advantages and disadvantages of each system are discussed. The first plant-derived molecular farming products have reached the marketplace and other products are poised to join them during the next few years. We explain the rationale for using plants as biofactories. We also describe the products currently on the market, and those that appear likely to join them in the near future. Lastly, we discuss the issue of public acceptance of molecular farming products.

**Keywords** Recombinant protein · Edible vaccine · Monoclonal antibody · Pharmaceuticals

### Introduction

Similar to the evolution of new biological organisms, new technologies can appear in rapid succession once certain technical breakthroughs have been achieved. A scant 15 years after the first reported stable transformed plant (Fraley et al. 1983; Horsch et al. 1984), the use of transgenic higher plants to produce foreign proteins with economic value was being realized (Kusnadi et al. 1998). Although transgenic animals, bacteria and fungi are also utilized for the production of proteins, it is with plants that the highest economic benefit will likely be achieved.

The utility of higher plants as biofactories has been reviewed several times in the last 4 years (Hood and Jilka 1999; Daniell et al. 2001; Hood and Howard 2002). In

brief, the advantages of using higher plants for the purpose of protein production include: (1) significantly lower production costs than with transgenic animals, fermentation or bioreactors; (2) infrastructure and expertise already exists for the planting, harvesting and processing of plant material; (3) plants contain no known human pathogens (such as prions, virions, etc.) that could contaminate the final product; (4) higher plants generally synthesize proteins from eukaryotes with correct folding, glycosylation, and activity; and (5) plant cells can direct proteins to environments that reduce degradation and therefore increase stability. Some of these factors will be addressed again in later sections.

The feasibility of expressing various proteins in plants has now been demonstrated. However, only very few products have reached the marketplace to-date. We will describe the current products and those products expected to come to market within the next 5 years. The choice of plant species to be used as the production system is critical. We attempt to cover the advantages and disadvantages of several systems currently being utilized in the plant molecular farming industry. We make no pretense at writing an inclusive reference work of all molecular farming literature, rather we will focus on the above-mentioned issues.

### A brief history of molecular farming

Molecular farming activity has existed since the first higher plant was successfully transformed (Fraley et al. 1983), because any protein has the potential of being a protein product. One of the earliest marker genes that scientists have used in developing transformation systems in plants, *uidS* (Jefferson et al. 1987), is now a molecular farming product (Kusnadi et al. 1998; Witcher et al. 1998). The first report of human antibodies produced in plants was by Daring (1988) and was expanded to include secretory antibodies by Hiatt et al. (1989). The first report of a protein being produced in plants for the specific purpose of extraction, purification, and sale of that protein

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was by Hood et al. (1997), which detailed the production of avidin, an egg protein with several important properties. Aprotinin, one of the first molecularly farmed pharmaceutical proteins to be produced in plants (Zhong et al. 1999), may soon be used on medical patients for wound closure and to suppress the systemic inflammatory response during surgery.

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## Molecular farming systems

There are currently four methods of protein production from plants: (1) stable nuclear transformation of a crop species that will be grown in the field or a greenhouse, (2) stable plastid transformation of a crop species, (3) transient transformation of a crop species, and (4) stable transformation of a plant species that is grown hydroponically such that the transprotein is secreted into the medium and recovered. A recent review has made a detailed comparison of the economics, processing and regulatory constraints associated with the most common plant production systems (Nikolov and Hammes 2002).

### Stable nuclear transformation

The most common of the methods to date, nuclear transformation of a crop species, has produced all of the products available in the marketplace today. This system requires a method for transferring the foreign genes into the plant cells, usually using *Agrobacterium tumefaciens* or particle bombardment, in which the genes are taken up and incorporated into the host nuclear genome in a stable manner.

#### Advantages

When performed in a crop species such as grains, the protein product is normally accumulated in the seed, which is then harvested in a dry state and stored until processing can be accomplished (Delaney 2002). As an example, dry corn seed has a moisture content of approximately 11–15% and, at that level, the seed is metabolically inert. Thus, long-term non-refrigerated storage of the seed, if properly carried out, can allow the protein to exist without degradation for at least 2 years. This seed can then be transported to its final destination without refrigeration. Another advantage to this system is that large acreage can be utilized with the lowest possible cost. Since crops such as rice and corn are grown throughout the world, the products have the potential to be produced near the target markets.

#### Disadvantages

Some grains, such as corn, have the potential to cross with native species or food crops. There are technologies that

will prevent outcrossing, e.g., mechanical detasseling, or genetics-based male sterility. Such technology generally reduces the cost advantage of the system due to higher manual labor requirements, lower yields, and less effective genetics.

### Plastid transformation

The laboratory of Henry Daniell at the University of Central Florida (Orlando, Fla.) has put emphasis on plastid transformation for the purposes of molecular farming (Daniell et al. 2002). A system for plastid transformation was first described by Svab et al. (1990) using tobacco. Tobacco appears to be the only species in which plastid transformation has been established as routine (Svab and Maliga 1993; Daniell et al. 2002).

#### Advantages

Protein expression levels exceeding 40% on a dry weight basis have been reported when tobacco chloroplasts were transformed. Plastid genes are not usually transmitted through pollen so that outcrossing is not a major concern.

#### Disadvantages

As with any fresh tissue molecular farming system, protein stability over time will change even with refrigeration. Extraction and purification must be performed at very specific times following harvest. Tobacco is currently a highly regulated crop and is not edible. Large volume products and edible vaccines would not appear to be feasible using this system.

### Transient transformation of a crop species

This system depends on the ability of recombinant plant viruses such as tobacco mosaic virus (TMV) to infect tobacco plants and then transiently express a target protein in the plant tissue. The protein will accumulate in the interstitial spaces. The interstitial fluid can then be collected by centrifugation under vacuum.

#### Advantages

TMV can be readily manipulated genetically and the infection process is rapid. Small quantities of the target protein can be obtained within several weeks. Probably the ideal system for a large number of custom proteins that are needed in relatively small amounts.

### *Disadvantages*

Probably not suitable for any protein needed in large (kg) quantities. Product must be processed immediately as storage will cause degradation of the plant tissue. As discussed above, tobacco is a highly regulated crop and is not grown on large acreage.

Stable transformation of a plant species that is grown hydroponically

In this system, transgenic plants containing a gene coding for the target protein are grown hydroponically in a way that allows release of the desired product as part of the root exudate into the hydroponic medium (Raskin 2000).

### *Advantages*

Plants grown hydroponically are contained in a greenhouse setting and so have reduced fears of unintentional environmental release. Purification of the desired product is considerably easier since no tissue disruption is needed and the quantity of contaminating proteins is low.

### *Disadvantages*

Probably not amenable to producing large (kg) quantities of any protein product. Greenhouse/hydroponic facilities are relatively expensive to operate.

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## **Classes of proteins within molecular farming**

Proteins currently being produced in plants for molecular farming purposes can be categorized into four broad areas: (1) parental therapeutics and pharmaceutical intermediates, (2) industrial proteins (e.g., enzymes), (3) monoclonal antibodies (MAbs), and (4) antigens for edible vaccines. A brief synopsis is provided for each area.

### Parental therapeutics and pharmaceutical intermediates

This group includes all proteins used directly as pharmaceuticals along with those proteins used in the making of pharmaceuticals. The list of such proteins is long, growing, and includes such products as thrombin and collagen (therapeutics), and trypsin and aprotinin (intermediates). Practically speaking, only those proteins with high value will be considered as candidates for molecular farming. Products in this classification must generally be manufactured under stringent cGMP (current good manufacturing practices) procedures and be of high purity. MAbs can also be classified in this group but are

described separately due to their distinct characteristics (below).

### Industrial proteins—enzymes

This group includes hydrolases, encompassing both glycosidases and proteases. Oxido-reductase enzymes such as laccase, a fungal enzyme used in fiber bleaching and bioglue of wood products (Hood et al. 2003; Bailey et al. 2004), represent a separate class of industrial enzymes. Enzymes involved in biomass conversion for the purposes of producing ethanol are candidates for molecular farming. All of these products will usually be characterized by the fact that they are used in very large quantities and must therefore be produced very inexpensively (Hood et al. 1999). Regulatory hurdles as they exist today may be a major hindrance to the molecular farming of these products since very large acreage will be required. This may necessitate deregulation by federal regulatory agencies or reduced regulations for this type of product based on safety assessments.

### Monoclonal antibodies

This group includes all antibody forms (IgA, IgG, IgM, secretory IgA, etc.) and antibody fragments (Fv). They can be produced in plants in both glycosylated and nonglycosylated forms. These plant-derived MAbs (plantibodies) have the potential of alleviating the serious production bottleneck that currently exists as dozens of new MAb products attempt to reach the marketplace. Examples of plant-derived MAbs in product development include  $\alpha$ -caries for prevention of dental decay and  $\alpha$ -herpes for prevention of herpes transmission.

### Antigens for edible vaccines

Specific protein antigens can be produced in plants that will induce a humoral immune response when eaten by an animal or human (e.g., see Streatfield et al. 2003). Protection studies have shown good efficacy when these edible (or oral) vaccines have been used. In some cases, protection has actually been better with the edible vaccine than with the commercially available vaccine (Lamphear et al. 2004).

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## **Products on the market**

### Avidin

Avidin is a glycoprotein found in avian, reptilian and amphibian egg white. It is used primarily as a diagnostic reagent. The protein is composed of four identical subunits, each 128 amino acids long. The usual source for commercial quantities of avidin is from chicken egg

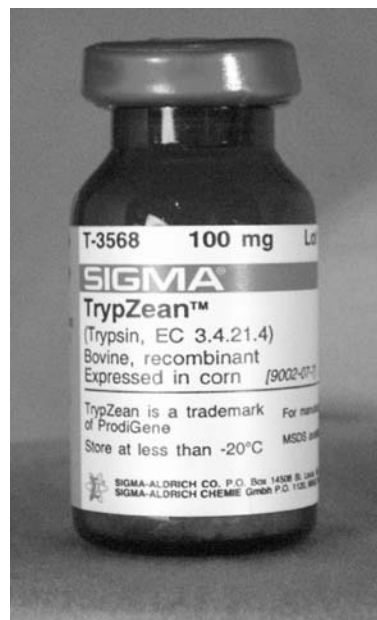
white but the resultant product is relatively expensive due to the cost of maintaining live animals. Hood et al. (1997) reported the production of chicken egg white avidin in transgenic corn using an avidin gene whose sequence had been optimized for expression in corn. The resultant avidin had properties almost identical to those of avidin from chicken egg white. The pI,  $K_i$ , and antigenic properties were identical. Both the corn-derived and the chicken egg-derived avidin were glycosylated. While the avidin apoproteins were identical, the size of corn-derived avidin was slightly less than chicken egg-derived avidin (16.8 vs 17.6 kDa) due to a less complex glycosylation composition (M. Bailey, unpublished observations). This difference did not alter the binding activity of the mature protein. Corn-derived avidin expression levels over 20% TSP (total soluble protein) have been observed (Masarik et al. 2003). Processing methodologies were developed to extract and purify this novel product from transgenic corn (Kusnadi et al. 1998). This product is currently being sold by Sigma-Aldrich (#A8706; Sigma-Aldrich, St. Louis, Mo.)

### $\beta$ -Glucuronidase

GUS ( $\beta$ -glucuronidase;  $\beta$ -D-glucuronide glucuronosylhydrolase) is a homotetrameric hydrolase (68 kDa/subunit) that cleaves  $\beta$ -linked terminal glucuronic acids in mono- and oligo-saccharides and phenols. GUS is widely used as a visual marker in transgenic plant research. It was first reported to be produced commercially in transgenic corn (Kusnadi et al. 1998, Witcher et al. 1998), where its properties were compared with GUS extracted from *Escherichia coli*, the original source of the protein. The molecular weights were identical, as were their antigenic qualities  $K_i$  and pI. Their  $K_m$  values were nearly identical (0.20 vs 0.21) and their  $V_{max}$  values for the substrate MUG (4-methylumbelliferyl- $\beta$ -D-glucuronide) were also quite similar ( $2.9 \times 10^5$  nM/h for corn-derived vs  $2.2 \times 10^5$  nM/h for *E. coli*-derived). The sequences of the two genes were similar, with the exception of the most N-terminal amino acid. It was surmised that removal of the terminal amino acid in plants was a result of the N-terminal rule in action (Hirel et al. 1989) as a result of the change in sequence. An interesting paper describing an economic evaluation of the extraction and purification of corn-derived GUS was published by Evangelista et al. (1998). Corn-derived GUS has been marketed by Sigma-Aldrich (#G2035).

### Trypsin

Although both plant-derived GUS and avidin are protein products that have been commercially available for several years, their markets have been quite small and were designed to show proof-of-concept. Maize-derived trypsin, a more recent introduction, has a significant market potential. Trypsin is a protease that is used in a



**Fig. 1** TrypZean, a corn-derived recombinant bovine trypsin product, is the first large-scale plant molecular farming product to reach the market. The worldwide market for all trypsin is estimated at US \$120 million in 2004

variety of commercial applications including the processing of some biopharmaceuticals. The availability of maize-derived bovine trypsin helps to supply the growing market for animal-free reagents. This market is being fueled by a desire by pharmaceutical companies to eliminate animal-source materials and reduce the fears of contamination of products by mammalian viruses and prions.

Trypsin is an aggressive proteolytic enzyme. Although trypsin has been expressed in a variety of recombinant systems, none of these systems has been demonstrated to be commercially viable on a large scale. Expression of this enzyme at commercially viable levels in maize (Woodard et al. 2003) was possible only by expressing the enzyme in an inactive zymogen form. Although the zymogen gene was put into plants, the active enzyme was recovered in extracts from maize seeds (Woodard et al. 2003). Zymograms and Western blots of the purified trypsin suggested that almost all of the trypsinogen was converted to trypsin in the seed, either prior to, or immediately upon, extraction of flour from the seed.

The purified maize-derived trypsin had a specific activity of 175 U/mg protein, which compared well to that of bovine pancreas-derived trypsin (166 U/mg protein). There was no discernible difference between the two sources in a variety of performance characteristics including the  $K_m$  and pH optimum. To our knowledge, corn-derived trypsin (TrypZean) is the first large-scale protein product from transgenic plant technology (Fig. 1).

**Table 1** Companies involved in molecular farming over the last several years

Company name	Location	Species for production
Meristem Therapeutics	Clermont-Ferrand, France	Maize
CropTech	Charleston, S.C.	Tobacco
PlantGenix	Philadelphia, Pa.	Various
Large Scale Biology	Vacaville, Calif.	Tobacco
Monsanto Protein Tech	St. Louis Mo.	Maize
SemBioSys	Calgary, Alberta, Canada	Safflower
Medicago	Quebec City, Quebec, Canada	Alfalfa
Ventria	Sacramento, Calif.	Rice
Epicyte Pharmaceutical	San Diego, Calif.	Maize
Planet Biotechnology	Hayward, Calif.	Tobacco
ProdiGene	College Station, Tex.	Maize

**Table 2** Molecular farming products thought to be close to or on the market in the next 5 years. *GUS*  $\beta$ -glucuronidase, *SIR* systemic inflammatory response, *TGEV* transmissible gastroenteritis coronavirus, *MAB* monoclonal antibody

Product	Company or companies	Use
Trypsin <sup>a</sup>	ProdiGene	Pharmaceutical intermediate
GUS	ProdiGene	Diagnostic reagent
Avidin <sup>a</sup>	ProdiGene	Immunological reagent
Aprotinin <sup>a</sup>	ProdiGene, Large Scale Biology	Reduce SIR and bleeding, promote wound closure, mammalian cell culture
Collagen <sup>a</sup>	ProdiGene, Medicago, Meristem Therapeutics	Gel caps, skin sealant, scar treatment (see text)
Lipase <sup>a</sup>	Meristem Therapeutics	Exocrine pancreatic insufficiency, steatorrhea, cystic fibrosis
Lactoferrin <sup>a</sup>	Ventria, Meristem Therapeutics	Natural defense protein against infections, iron repository
Lysozyme <sup>a</sup>	Ventria	Anti-viral, anti-bacterial, anti-fungal
Brazzein	ProdiGene	Natural protein sweetener
TGEV edible vaccine	ProdiGene	TGEV vaccine in swine
$\alpha$ -Caries MAB	Planet Biotechnology	Prevention of dental caries
$\alpha$ -Herpes MAB	Epicyte Pharmaceutical	Prevention of herpes transmission

<sup>a</sup> Currently obtained from animal sources

## Products close to market

Many companies have been created to produce molecular farming products. Table 1 lists 11 companies that have been involved in molecular farming over the last several years. Besides the three products described above, there are at least nine products thought to be close to reaching commercial market, i.e., in the next 5 years. Several of these proteins are normally derived from animal organs and, due to the possibility of animal pathogens being carried along with these proteins, there is a need for alternative low-cost supply. Table 2 lists these products in no particular order.

### Aprotinin

Aprotinin is a protein that inhibits serine proteases (including trypsin, chymotrypsin, kallikrein, and pepsin). This activity is known to modulate and lessen the systemic inflammatory response (SIR) associated with cardiopulmonary bypass surgery, which translates into a decreased need for blood transfusions, reduced bleeding, and decreased re-exploration for bleeding.

Current material supplied to the market is native aprotinin extracted from bovine lungs. With growing concern regarding bovine spongiform encephalopathy

(BSE) and other animal pathogens, a high market price in surgery and wound healing, and limitations on natural sources of aprotinin, a non-animal derived, lower cost and reliable source adds significant value to the user. Because of concerns over BSE, suppliers of aprotinin are limited to sources of raw materials certified as BSE-free. Consequently, limitations on supply exist. The advantages of using a plant material source include the elimination of the fear of mammalian pathogens from the source material. Unlike bovine-lung-derived material, plants do not support mammalian viruses or prions. Additionally, the scale at which grain production can be carried out will remove limitations of raw material supply.

Recombinant bovine aprotinin from transgenic corn seed was first reported by Zhong et al. (1999) using particle bombardment. They reported expression levels as high as 0.068% TSP from a line that had 20 copies of the transgene. Levels as high 8.9% TSP in transgenic corn have since been reported by Delaney et al. (2002) using *A. tumefaciens* as the vector and a seed-preferred promoter driving a maize-optimized bovine aprotinin gene. Further studies on the extraction and purification of the bovine protein from the material described by Zhong et al. (1999) have resulted in recovery of nearly 50% of the transprotein and a purification factor of 280 (Azzoni et al. 2002).

## Collagen

Collagen is a structural protein currently rendered from hooves and connective tissue of animals. Vast quantities of collagen are consumed throughout the world each year in the form of gelatin. Based on their structural roles and compatibility within the body, collagen and gelatin are commonly used biomaterials in the medical, pharmaceutical and cosmetic industries. Collagen is used commercially in the areas of arterial sealants, bone grafts, corneal implants, drug delivery, incontinence, tissue engineering (artificial skin and cartilage), and as a viscoelastic supplement. Gelatin is used in the stabilization and delivery of vaccines and drugs, in capsules and soft gels, nutraceuticals, and as plasma expanders.

The first report of human collagen produced in plants was by Ruggiero et al. (2000). The authors inserted the fibrillar collagen cDNAs  $1\alpha3$  and  $\alpha22$ , which together code for the complete human pro $\alpha1(I)$  collagen chain, into tobacco using *Agrobacterium*, and *npt2* as the selectable marker gene. The resultant protein was organized into a triple helix, which was somewhat surprising since plants do not contain the specific post-translational machinery needed for collagen assembly (Ruggiero et al. 2000). Currently several companies have reported interest in bringing human collagen to market (Table 2).

## Human gastric lipase

Human gastric lipase or lack thereof, is a protein involved in the condition known as exocrine pancreatic insufficiency (EPI). The absence of lipase results in the inability to digest food lipids. The disease mainly affects patients who have cystic fibrosis or who suffer from pancreatic disease. The current supply of gastric lipase is from porcine pancreatic tissue. Meristem Therapeutics (Clermont-Ferrand, France) is advancing a maize-derived mammalian lipase through clinical trials. Very little has been published on this product but it is described on the company website (<http://www.meristem-therapeutics.com>). The production of *canine* gastric lipase produced in transgenic tobacco has been reported (Gruber et al. 2001).

## Human lactoferrin

Human lactoferrin is a natural defense iron-binding milk protein that purportedly possesses anti-bacterial, anti-fungal, anti-viral, anti-neoplastic and anti-inflammatory properties (Samyn-Petit et al. 2001). Each natural lactoferrin molecule has two iron-binding domains, which reversibly bind iron. The bovine form is readily available, although the levels of lactoferrin are quite low in cow's milk. Recombinant human lactoferrin has been produced in *Aspergillus* sp. from which adequate levels for pharmaceutical purposes have been obtained (Conneely et al. 2001). Lactoferrin was first reportedly produced in tobacco suspension cells by Mitra and Zhang (1994).

Although the protein had lactoferrin properties and activity, it was truncated. Ventria (Sacramento, Calif.) and Meristem Therapeutics are now both attempting to commercialize lactoferrin from rice and maize seed, respectively (Samyn-Petit et al. 2001; Nandi et al. 2002; Legrand et al. 2003).

## Edible vaccines

Edible vaccines have received considerable attention from researchers in both academia and industry. Charles Arntzen (who originally coined the phrase "edible vaccine") with Hugh Mason and colleagues, now at the Arizona State University (Tempe, Ariz.), have pioneered the field with work on hepatitis B and LT-B (heat labile toxin, B subunit) in tobacco plants and potato tubers. Vaccines administered using needles (parenteral delivery) do not usually give a good immune response in the mucosal tissues of the vaccine recipient. Mucosal surfaces such as the mouth and the urogenital tract are the primary ports of entry of most disease organisms. Edible vaccines have been shown to induce excellent mucosal immune responses. Recent reviews of edible vaccines include Carter and Langridge (2002) and Streatfield and Howard (2003). The laboratory of William Langridge at Loma Linda University (Loma Linda, Calif.) has been active in developing edible vaccines against diabetes and cholera using a flexible hinge peptide between the antigens and the protein adjuvants in order to minimize steric hindrance (Arakawa et al. 1998, 2001; Yu and Langridge 2001). Korban and colleagues at the University of Illinois (Urbana-Champaign, Ill.) have reported a plant-based oral vaccine against respiratory syncytial virus (RSV) in tomato fruit (Korban et al. 2002) with the ultimate aim of moving the product into apple. HSV is a viral pathogen that causes respiratory diseases and is a leading cause of viral lower respiratory tract illness in infants and children worldwide.

Our laboratory has focused on LT-B (Lamphear et al. 2002; Streatfield and Howard 2003; Streatfield et al. 2003), hepatitis B, transmissible gastroenteritis coronavirus (TGEV) (Lamphear et al. 2002), and human immunodeficiency virus (HIV) (Horn et al. 2004) edible vaccines using corn as a delivery vehicle. A human phase I clinical trial was recently completed with our corn-based LT-B vaccine candidate (Tacket et al. 2003). Dry grain (corn, rice, wheat) may prove to be a superior delivery system for edible vaccines since the antigen will remain at a constant level for an extended period of time without refrigeration (Streatfield 2002). Vaccines require a known dose of the antigen and this would be difficult to accomplish using fresh or dried fruit such as potato, tomato or banana.

## Monoclonal antibodies

The production of antibodies or antibody fragments has been reported by a number of academic laboratories and the area has been reviewed extensively (Larrick et al. 2001; Hood et al. 2002; Schilberg et al. 2002; Stoger et al. 2002a, 2002b). As mentioned above, Klaus During described the production of MABs in his 1988 Ph.D. thesis (During 1988) and published this work in a peer-reviewed journal in 1990 (During et al. 1990). Briefly, this work demonstrated expression of the B 1–8 light chain and heavy chain in transgenic tobacco and then showed assembly of the full MAB. Unexpectedly, a high percentage of the B 1–8 MAB was found in the chloroplasts. At about this time, Hiatt and colleagues, then at the Scripps Research Institute (La Jolla, Calif.) reported that they had crossed transgenic tobacco containing gamma or kappa immunoglobulin chains and obtained progeny having fully functional MAB (Hiatt et al. 1989).

In the 14 years following, a large number of papers have been published describing a wide variety of MAB types: sIgA, IgG, IgM. Of particular note, Cabanes-Macheteau et al. (1999) of the Université de Rouen (Rouen, France) and Guy's Hospital (London, UK) reported the specific glycosylation composition of a mouse IgG expressed in tobacco plants. Frigerio and co-workers at the University of Warwick (Coventry, UK) along with the Scripps and Guy's Hospital groups, described the vacuolar accumulation of a secretory IgA in tobacco (Frigerio et al. 2000). Finally, we would like to single out the work by Verch et al. (1998) at Thomas Jefferson University (Philadelphia, Pa.) in which they used a plant virus vector (described above) to infect tobacco and produce a full-length MAB directed towards a colon cancer antigen.

One of the first plant-derived MAB products expected to reach the market is one directed against dental caries. Designated CaroRx, this secretory IgA (sIgA) inhibits the binding of the oral pathogen *Streptococcus mutans* to teeth. As reviewed by Larrick et al. (2001), the plant-derived MAB was extremely effective in reducing colonization by *S. mutans* using passive immunization, and even prevented re-colonization for up to 2 years. Phase I and Phase II clinical trials have been completed.

Another plant-derived MAB product expected to reach the market within 5 years is one directed against genital herpes. Zeitlin et al. (1998) at ReProtect (Baltimore, Md.) and Johns Hopkins University (Baltimore, Md.) reported the production of an anti-herpes humanized MAB in soybean and compared this MAB with the same MAB produced in mammalian cell culture in a mouse model. The two MABs protected the mice against herpes simplex virus-2 equally well, proving again that differences in glycosylation of transproteins do not significantly reduce efficacy in many cases. This potential product is now being produced in corn (<http://www.epicyte.com>).

## Glycosylation

The glycosylation of transproteins in plants differs slightly from those produced in transgenic animals or animal cells in vitro (Lerouge et al. 1998). The addition of xylose and change from a  $\beta 1 \rightarrow 6$  to a  $\beta 1 \rightarrow 3$  linkage of fucose are typical in plants. A significant difference with transprotein production in plants is their inability to add sialic acid to glycoproteins (Lerouge et al. 1998). This sugar has been implicated in longer clearance times for proteins in the blood and therefore is a major factor for a select group of pharmaceutical proteins. These differences have had minimal or no effect on the function of the transprotein products from plants to date (Hood et al. 1997; Samyn-Petit et al. 2001; Woodard et al. 2003).

## Public acceptance

Sales are a good measure of the public's perceived benefits of specific products. However, today's public also wants to know that not only is there a benefit for the direct end user, but that there are otherwise no significant risks to the general public. This is illustrated by the recent concerns and debates over the use of GMO products produced in plants. While the initial concern involved GMO food products, this now encompasses non-food products as well. The fear is that the non-food products may inadvertently enter the food chain and present an unintentional risk (J.A. Howard and K.C. Donnelly, A quantitative safety assessment model for non-food products produced in agricultural crops. MS submitted). On the surface, this appears to be an unprecedented use of food crops to produce pharmaceutical and industrial products. However, the use of plants to produce non-food products is not unlike the current use of other food products, such as eggs or yeast, to produce pharmaceuticals. The difference is the latter have well-established compliance programs, which are in line with the production of pharmaceutical products rather than the production of food.

Such compliance programs start with the regulatory agencies that represent the public's safety concerns. The regulatory agencies take the position that the non-food products are unsafe until proven otherwise. There is a regulatory framework in place specifically targeted toward the introduction of non-food products when using plants as the production system. There are strict rules on agronomic practices, which are targeted to keep non-food products out of the food chain. Unfortunately, in any system, plants included, it is not possible to eliminate all possibilities of unintended exposure due to unforeseen circumstances such as an accident, a natural disaster, or an act of non-compliance.

We advocate that a quantitative risk assessment be used to address these concerns (J.A. Howard and K.C. Donnelly, A quantitative safety assessment model for non-food products produced in agricultural crops. MS submitted). In the event of unintended exposures, a

quantitative assessment can be used to determine if there is cause for alarm. While some of these products may present a significant risk to the public, many of the products would not pose a hazard if they were inadvertently introduced. In fact, many of the products in the pipeline are either already in the food supply or endogenous to humans. This is not to say that we should accept these products into the food chain, but rather we are prepared for all possible situations. A quantitative risk assessment similar to that employed for other regulated products can be used to evaluate what type of measures need to be taken for specific products. This approach has proven useful to realize the benefits of new technology and to reduce or eliminate risks.

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### Future research

One of the keys to success in the future will undoubtedly be the level of expression of the recombinant protein in plants. This is the one of the most important aspects with regard to economics. The expression level affects the cost of growing, processing, extraction, purification and waste disposal. Clearly there will be a drive towards higher levels of expression and there is much more room for improvement compared to other established systems.

Expression is also a major regulatory concern. Whether or not the protein is in specific tissues will enable or nullify exposure to the environment. There has already been work to show that expression can be limited to specific tissues, thus reducing regulatory concerns. As an example, keeping the protein out of pollen can reduce inadvertent exposure to the environment. However, this does not remove the possibility that the pollen will outcross with other plants and intermix with food crops. There are physical isolation requirements imposed by the regulatory agencies to prevent this from occurring. There may be some cases where genetic control of expression is also warranted either for economic or safety concerns, depending on the product. Possibilities including male-sterile crops, induced expression, or sequences that prevent germination or the expression of the protein product in non-food products have been discussed. Some combination of these different limitations on expression will most likely find a way into future programs.

The other regulatory concern is that the pathway to commercialization for human therapeutics has not been proven. The industry will anxiously await the establishment of a clear road map detailing how this process is similar to, and/or different from, the existing protocols today. With the first approved therapeutic products will also come the realization of the many benefits of transgenic plant technology. These real benefits should also help public acceptance and open the way for a much more rapid acceptance of this technology.

### Conclusions

There is no question that plant molecular farming is starting to come into its own as a viable new industry. Important questions concerning the glycosylation, immunogenicity, accumulation, and stability of the transproteins are being answered. Academic laboratories have been instrumental in elucidating much of the science behind the potential products and will continue to do so. As the industry develops, academic laboratories will need to put more emphasis into downstream process development research. This will complement their fundamental work on protein expression and will provide the basic knowledge to fuel the industry. However, the marketing and delivery of commercial products will necessarily fall to industry. As with any new industry, there have been hurdles to overcome, both technical and regulatory. However, the experience to date has taught us much and the industry is now poised for rapid growth and profitability.

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### References

- Arakawa T, Yu J, Chong DKX, Hough J, Engen PC, Langridge WHR (1998) A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nat Biotechnol* 16:934–938
- Arakawa T, Yu J, Langridge WHR (2001) Synthesis of a cholera toxin B subunit-rotavirus NSP4 fusion protein in potato. *Plant Cell Rep* 20:343–348
- Azzoni AR, Kusnadi AR, Miranda EA, Nikolov ZL (2002) Recombinant aprotinin produced in transgenic corn seed: extraction and purification studies. *Biotechnol Bioeng* 80: 268–276
- Bailey MR, Woodard SL, Callaway E, Beifuss K, Magallanes-Lundback M, Lane JR, Horn ME, Mallubhotla H, Delaney DD, Ward M, Van Gastel F, Howard JA, Hood EE (2004) Improved recovery of active recombinant laccase from maize seed. *Appl Microbiol Biotechnol* 63:390–397
- Cabanes-Macheteau M, Fitchette-Laine A-C, Loutelier-Bourhis C, Lange C, Vine ND, Ma JK-C, Lerouge P, Faye L (1999) N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology* 9:365–372
- Carter JE III, Langridge WHR (2002) Plant-based vaccines for protection against infectious and autoimmune diseases. *Crit Rev Plant Sci* 21:93–109
- Conneely OM, Headon DR, O'Malley BW, May GS (2001) Production of recombinant lactoferrin and lactoferrin polypeptides using cDNA sequences in various organisms. *US Patent* 6 228 614
- Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci* 6:219–226
- Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends Plant Sci* 7:84–91
- Delaney DE (2002) Choice of crop species and development of transgenic product lines. In: Hood EE, Howard JA (eds) *Plants as factories for protein production*. Kluwer, Dordrecht, pp 139–158
- Delaney D, Jilka J, Barker D, Irwin P, Poage M, Woodard S, Horn M, Vinas A, Beifuss K, Barker M, Wiggins B, Drees C, Harkey R, Nikolov Z, Hood E, Howard J (2002) Production of aprotinin in transgenic maize seeds for the pharmaceutical and cell culture markets. In: Vasil IK (ed) *Plant biotechnology 2002 and beyond*. Kluwer, Dordrecht, pp 393–394



- During K (1988) Wound-inducible expression and secretion of T4 lysozyme and monoclonal antibodies in *Nicotiana tabacum*. Ph.D Thesis. Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Köln
- During K, Hippe S, Kreuzaler F, Schell J (1990) Synthesis and self-assembly of a functional monoclonal antibody in transgenic *Nicotiana tabacum*. *Plant Mol Biol* 15:281–293
- Evangelista RL, Kusnadi AR, Howard JA, Nikolov ZL (1998) Process and economic evaluation of the extraction and purification of recombinant  $\beta$ -glucuronidase from transgenic corn. *Biotechnol Prog* 14:607–614
- Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffman NL, Woo SC (1983) Expression of bacterial genes in plant cells. *Proc Natl Acad Sci USA* 80:4803–4807
- Frigerio L, Vine ND, Pedrazzini E, Hein MB, Wang F, Ma JK-C, Vitale A (2000) Assembly, secretion, and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol* 123:1483–1493
- Gruber V, Berna PP, Arnaud T, Bournat P, Clément C, Mison D, Olganier B, Philippe L, Theisen M, Baudino S, Bénicourt C, Cudrey C, Bloès C, Duchateau N, Dufour S, Gueguen C, Jacquet S, Ollivo C, Poncetta C, Zorn N, Ludevid P, Van Dorsselaer A, Verger R, Doherty A, Mérot B, Danzin C (2001) Large-scale production of a therapeutic protein in transgenic tobacco plants: effect of subcellular targeting on quality of a recombinant dog gastric lipase. *Mol Breed* 7:329–340
- Hiatt A, Cafferkey R, Bowdish K (1989) Production of antibodies in transgenic plants. *Nature* 342:76–78
- Hirel PH, Schmitter JM, Dessen P, Fayat G, Blanquet S (1989) Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci USA* 86:8247–8251
- Hood EE, Howard JA (2002) Plants as factories for protein production. Kluwer, Dordrecht
- Hood EE, Jilka JM (1999) Plant-based production of xenogenic proteins. *Curr Opin Biotechnol* 10:382–386
- Hood EE, Witcher DR, Maddock S, Meyer T, Baszczyński C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehig RJ, Hernan R, Kappel WK, Ritland D, Li CP, Howard JA (1997) Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Mol Breed* 3:291–306
- Hood EE, Kusnadi A, Nikolov Z, Howard JA (1999) Molecular farming of industrial proteins from transgenic maize. In: Shahidi F, Kolodziejczyk P, Whitaker JR, Munguia AL, Fuller G (eds) Chemicals via higher plant bioengineering. Kluwer/Plenum, New York, pp 127–147
- Hood EE, Woodard SL, Horn ME (2002) Monoclonal antibody manufacturing in transgenic plants—myths and realities. *Curr Opin Biotechnol* 13:630–635
- Hood EE, Bailey MR, Beifuss K, Magallanes-Lundback M, Horn ME, Callaway E, Drees C, Delaney DE, Clough R, Howard JA (2003) Criteria for high-level expression of a fungal laccase gene in transgenic maize. *Plant Biotechnol J* 1:129–140
- Horn ME, Pappu KM, Bailey MR, Clough RC, Barker M, Jilka JM, Howard JA, Streatfield SJ (2004) Advantageous features of plant-based systems for the development of HIV vaccines. *J Drug Targeting* (in press)
- Horsch RB, Fraley RT, Rogers SC, Sanders PR, Lloyd A, Hoffman N (1984) Inheritance of functional foreign genes in plants. *Science* 223:496–498
- Jefferson RA, Kavanaugh TA, Bevan MW (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 6:3901–3907
- Korban SS, Krasnyanski SF, Buetow DE (2002) Foods as production and delivery vehicles for human vaccines. *J Am Coll Nutr* 21:212S–217S
- Kusnadi AR, Hood EE, Witcher DR, Howard JA, Nikolov ZL (1998) Production and purification of two recombinant proteins from transgenic corn. *Biotechnol Prog* 14:149–155
- Lamphear BJ, Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. *J Control Release* 85:169–180
- Lamphear BJ, Jilka JM, Kesl L, Welter M, Howard JA, Streatfield SJ (2004) A corn-based delivery system for animal vaccines: an oral transmissible gastroenteritis virus vaccine boosts lactogenic immunity in swine. *Vaccine* (in press)
- Larrick JW, Yu L, Naftzger C, Jaiswal S, Wycoff K (2001) Production of secretory IgA antibodies in plants. *Biomol Eng* 18:87–94
- Legrand D, Salmon V, Spik G, Gruber V, Bournat P, Merot B (2003) Recombinant lactoferrin, methods of production from plants and uses. US Patent 6 569 831
- Lerouge P, Cabanes-Macheteau M, Rayon C, Fischette-Laine A-C, Gomord V, Faye L (1998) N-Glycoprotein biosynthesis in plants: recent developments and future trends. *Plant Mol Biol* 38:31–48
- Masarik M, Kizek R, Kramer KJ, Billova S, Brazdova M, Vacek J, Bailey M, Jelen F, Howard JA (2003) Application of avidin-biotin technology and adsorptive transfer stripping square-wave voltammetry for detection of DNA hybridization and avidin in transgenic avidin maize. *Anal Chem* 75:2663–2669
- Mitra A, Zhang Z (1994) Expression of a human lactoferrin cDNA in tobacco cells produces antibacterial protein(s). *Plant Physiol* 106:997–981
- Nandi S, Suzuki YA, Huang J, Yalda D, Pham P, Wu L, Bartley G, Huang N, Lönnerdal B (2002) Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Sci* 163:713–722
- Nikolov Z, Hammes D (2002) Production of recombinant proteins from transgenic crops. In: Hood EE, Howard JA (eds) Plants as factories for protein production, Kluwer, Dordrecht, pp 159–174
- Raskin I (2000) Methods for recovering polypeptides from plants and portions thereof. US Patent 6 096 546
- Ruggiero F, Exposito J-Y, Bournat P, Gruber V, Perret S, Comte J, Olganier B, Garrone R, Thiesen M (2000) Triple helix assembly and processing of human collagen produced in transgenic tobacco plants. *FEBS Lett* 469:132–136
- Samyn-Petit B, Gruber V, Flahaut C, Wajda-Dubos J-P, Farrer S, Pone A, Desmaizieres G, Slomianny M-C, Theisen M, Delannoy P (2001) N-Glycosylation potential of maize: the human lactoferrin used as a model. *Glycoconjugate J* 18:519–527
- Schillberg S, Emans N, Fischer R (2002) Antibody molecular farming in plants and plant cells. *Phytochem Rev* 1:45–54
- Stoger E, Sack M, Perrin Y, Vaquero C, Torres E, Twyman RM, Christou P, Fischer R (2002a) Practical considerations for pharmaceutical antibody production in different crop systems. *Mol Breed* 9:149–158
- Stoger E, Sack M, Fischer R, Christou P (2002b) Plantibodies: applications, advantages and bottlenecks. *Curr Opin Biotechnol* 13:161–166
- Streatfield SJ (2002) The greening of vaccine technology. *Curr Drug Discovery* Nov:15–18
- Streatfield SJ, Howard JA (2003) Plant-based vaccines. *Int J Parasitol* 33:479–493
- Streatfield SJ, Lane JR, Brooks CA, Barker DK, Poage ML, Mayor JM, Lamphear BJ, Drees CF, Jilka JM, Hood EE, Howard JA (2003) Corn as a production system for human and animal vaccines. *Vaccine* 21:812–815
- Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc Natl Acad Sci USA* 90:913–917
- Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. *Proc Natl Acad Sci USA* 87:8526–8530

- Tackett CO, Clements JD, Wasserman SS, Streatfield S (2003) Immunogenicity of a recombinant bacterial antigen delivered in transgenic corn. Abstract S4, 6th Annual Vaccine Conference, 5–7 May 2003, Arlington, Va.
- Verch T, Yusibov V, Koprowski H (1998) Expression and assembly of a full-length monoclonal antibody in plants using a plant virus vector. *J Immunol Methods* 220:69–75
- Witcher DR, Hood EE, Petersen D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehig R, Kappel W, Register J, Howard JA (1998) Commercial production of  $\beta$ -glucuronidase (GUS): a model system for the production of proteins in plants. *Mol Breed* 4:301–312
- Woodard SL, Mayor JM, Bailey MR, Barker DK, Love RT, Lane JR, Delaney DE, McComas-Wagner JM, Mallubhotla HD, Hood EE, Dangott LJ, Tichy SE, Howard JA (2003) Maize-derived bovine trypsin: characterization of the first large-scale, commercial protein product from transgenic plants. *Biotechnol Appl Biochem* 38:123–130
- Yu J, Langridge WHR (2001) A plant-based multicomponent vaccine protects mice from enteric diseases. *Nat Biotechnol* 19:548–552
- Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA, Whaley KJ (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat Biotechnol* 16:1361–1364
- Zhong G-Y, Peterson D, Delaney DE, Bailey M, Witcher DR, Register JC III, Bond D, Li C-P, Marshall L, Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA (1999) Commercial production of aprotinin in transgenic maize seeds. *Mol Breed* 5:345–356