Transgenic Plants

Bio-Farming for the Future

by Leah J. Rosin

Plants have been improved for human uses since the advent of agriculture in ancient Sumeria. Before the introduction of hybridization and selective breeding, wild rice and maize produced fewer seeds, providing a smaller amount of food for human consumption. Plants have also been used for therapeutic purposes since ancient times, mostly providing medicinal compounds that have been extracted and used to treat illness. New therapeutic possibilities — combined with improvements in yield and other qualities — are found today in agricultural biotechnology.

Inoculation of *Nicotiana benthamiana* with a virus-derived transient expression vector, GENEWARE R, by pipetting inoculum onto the leaves and gently abrading the leaves manually. Within 10 to 14 days the recombinant protein will be expressed throughout the plant. (LARGE SCALE BIOLOGY CORPORATION; WWW.LBSC.COM)

HOW WE GOT HERE

In 1983, scientists at the Max-Planck-Institute for Breeding Research in Cologne, Germany, and Monsanto Inc. (www.monsanto.com; St Louis, MO) demonstrated the successful transfer of foreign genes into plant cells. The transfer involved the use of *Agrobacterium tumefaciens* (a common plant pathogen) as a vector for gene transfer using Ti-plasmids. This breakthrough opened the possibility of transferring genes into broadleaf plants. The technology of gene transfer now includes the direct physical insertion of DNA into plant cells. The transferred genes are expressed, and the cells synthesize the corresponding protein as they grow, making it available for use after harvest and purification. Thus, the plants become factories that manufacture therapeutic proteins. (For methods of gene insertion, see Making a Plant Transgenic below.)

Essentially, plant-made pharmaceuticals (PMPs) were investigated in an attempt to avoid the risks of pathogenic contamination associated with mammalian cell culture and transgenic animals, problems with inactive human proteins made by microbial systems, and the relatively small-scale production of proteins possible in some systems. Lower facility and production costs are associated with PMPs because plants are much simpler to grow and scale up. The biggest factor in reducing costs is the high yield of recombinant proteins extractable from transgenic plants. Production costs for corn systems are estimated to be between $10 and $100 per gram for proteins that, when produced in other systems, cost as much as $1000 per gram (1). Additionally, PMP growth is not limited to special manufacturing facilities and can easily be scaled up to meet increased and varied market demands. For development of new therapeutic proteins, the capital risk associated with a commercial facility can be greatly minimized not only by the reduced amount of capital

required but perhaps even more importantly, by the delayed timing of the capital spending decision. This is because conventional facilities require five to seven years to build and validate whereas PMP facilities, because of their relative simplicity, are expected to be built and validated in two to three years. Decreased downstream processing expense is also a potential benefit, especially when therapeutic proteins or vaccines could be consumed directly (if expressed in edible plants) or when the therapeutic protein is secreted into a simple liquid medium as is the case for some PMP systems. The potential lack of requirements for dedicated viral inactivation and clearance steps can further contribute to decreased downstream processing costs by eliminating expensive process steps and yield losses.

One class of therapeutic proteins that is becoming increasingly important is monoclonal antibodies (MAbs). Some 15 MAbs are already approved by the FDA, and they are the fastest growing segment of the pharmaceutical industry. Consumers could benefit from "Plantibody" (Epicyte Pharmaceutical Inc.; www.epicyte.com) production. With lower costs and larger scales than conventional methods, plant-produced antibodies could help patients who would not otherwise have access to them. A report published in 1998 demonstrated actual success in preventing disease through the use of plant-produced antibodies. The report detailed a human clinical trial in which a monoclonal antibody was produced in a transgenic plant and then topically applied to teeth. This treatment prevented colonization by *Streptococcus mutans,* the bacterium responsible for tooth decay (**2**). In addition, transgenic plants have been used to make antibodies directed against rheumatoid arthritis, cholera, *E. coli* diarrhea, malaria, certain cancers, Norwalk virus, HIV, rhinovirus, influenza, hepatitis B virus, and herpes simplex virus (**3**).

Plant-derived vaccines are also possible. Vaccines have been produced in plants for *Vibrio cholerae*, enterotoxigenic *E. coli*, hepatitis B virus, Norwalk virus, rabies virus, human cytomegalovirus, rotavirus, and respiratory syncytial virus F (**3**). A therapeutic vaccine for protection against insulin-dependent autoimmune mellitus diabetes has been produced using insulin expression in plants. Large Scale Biology Corporation (Vacaville, CA; www.lsbc.com) has developed a personalized cancer vaccine produced in tobacco leaves for the treatment of lymphoma (**4**). Many plant-derived antigens have been purified and formulated for injectable delivery; however, oral delivery of some of these vaccines within food has also been successful. Edible vaccines offer potential benefits for people in developing countries where problems ensuring sterilization and adequate temperature control of traditional vaccine formulations exist. Edible vaccines are being tested in potatoes, tomatoes, bananas, and carrots (**4**). (See Production in Fruits and Vegetables below for more information about edible vaccines).

Since the early 1990s, the US Department of Agriculture (USDA) has allowed more than 200 field trials of pharmaceutical and industrial crops. In nearly three quarters of these tests, corn has been the crop of choice (**1**). Other crops that have seen USDA Animal and Plant Health Inspection Service (APHIS) regulatory field tests include tomato, rice, barley, alfalfa, sugarcane, soybean, potato, lettuce, lupine, tobacco, and rapeseed (canola) (**1**, **5**). Industry projects that the market for plant-produced pharmaceutical and industrial proteins could reach $200 billion by 2010.

Making a Plant Transgenic

Foreign genes can be inserted, or transformed, into plant cells using a variety of methods. Agrobacterium-mediated transformation and particle bombardment (biolistics) methods are the primary forms of stable insertion into the nuclear genome. Through these processes, the DNA coding for the protein of interest — and for a promoter to target its expression to a specific tissue or developmental stage — are integrated into the plant genome. When that plant is propagated, it will transmit those properties to its progeny, so large numbers of plants containing the transferred gene can be generated (**6**).

Another way to deliver foreign genes is by inserting them into the separate genome of plastids (chloroplasts and mitochondria) in plant cells. Plants have multiple copies of chloroplasts in each cell, which gives chloroplast transformation the potential for high expression levels and yields of recombinant proteins. Chloroplast transformation has been successful in tobacco, tomato, and potato plants, and investigational research is being conducted in many other species. Recombinant genes in chloroplast genomes are not usually transmitted through pollen, which makes it easier to contain them and prevent unwanted environmental contamination.

Transduction can be used to engineer plant protein expression. This method involves the use of a recombinant plant virus to deliver genes into plant cells. The DNA coding for the desired protein is incorporated into a plant virus, which then infects the host plant. A crop of host plants are cultivated and grown to the proper stage before being inoculated with the engineered virus. As the virus spreads and replicates, numerous copies of the target DNA are produced by the host plant, and high levels of protein expression can be achieved in a relatively short time. Viral particles are usually not transmitted by pollen, again controlling contamination of other plants to prevent the spread of genetic modifications.

Constitutive and developmentally regulated promoters have been used

to achieve high-level expression of therapeutic proteins in plants. Signal sequences can be used to target proteins for accumulation in specific areas of plant cells. Scientists believe this may be useful to ensure proper molecular folding and produce active proteins and enable or prevent posttranslational modifications (such as glycosylation) in the endoplasmic reticulum and Golgi apparatus. Recently, it has been shown that manipulation of specific glycosyl transferases in plant cells can yield a human-like glycosylation pattern on recombinant proteins expressed in plant systems. Greenovation Biotechnology GmbH (Freiburg, Germany; www.greenovation.com) and Dow Chemical (San Diego, CA; www.dowplantpharma.com) have active programs in this area. Compartmentalization can affect

Table 1: Pharmaceutical proteins that have been produced in plants

Protein	Host Plant System	Comments
Human biopharmaceuticals		
Human growth hormone	Tobacco, sunflower	First human protein expressed in plants; initially expressed as fusion protein with *nos* gene in transgenic tobacco; later the first human protein expressed in chloroplasts, with expression levels ~7% of total leaf protein
Human serum albumin	Tobacco, potato	First full-sized native human protein expressed in plants; low expression levels in transgenics (0.1% of total soluble protein) but high levels (11% of total soluble protein) in transformed chloroplasts
α-interferon	Rice, turnip	First human pharmaceutical protein produced in rice
Erythropoietin	Tobacco	First human protein produced in tobacco suspension cells
Human-secreted alkaline phosphatase	Tobacco	Produced by secretion from roots and leaves
Aprotinin	Maize	Production of a human pharmaceutical protein in maize
Collagen	Tobacco	First production of human structural-protein polymer; correct modification achieved by co-transformation with modification enzyme
α1-antitrypsin	Rice	First use of rice suspension cells for molecular farming
Recombinant antibodies		
IgG1 (phophonate ester)	Tobacco	First antibody expressed in plants; full length serum IgG produced by crossing plants that expressed heavy and light chains
IgM (neuropeptide hapten)	Tobacco	First IgM expressed in plants and protein targeted to chloroplast for accumulation
SIgA/G (*Streptococcus mutans* adhesin)	Tobacco	First secretory antibody expressed in plants; achieved by sequential crossing of four lines carrying individual components; at present the most advanced plant-derived pharmaceutical protein
scFv-bryodin 1 immunotoxin (CD 40)	Tobacco	First pharmaceutical scFv produced in plants; first antibody produced in cell-suspension culture
IgG (HSV)	Soybean	First pharmaceutical produced in soybean
LSC (HSV)	*Chlamydomonas reinnhardti*	First example of farming in algae
Recombinant subunit vaccines		
Hepatitis B virus envelope protein	Tobacco	First vaccine candidate expressed in plants; third plant-derived vaccine to reach clinical trials
Rabies virus glycoprotein	Tomato	First example of an "edible vaccine" expressed in edible plant tissue
Escherichia coli heat labile enterotoxin	Tobacco, potato	First plant vaccine to reach clinical trials stage
Norwalk virus capsid protein	Potato	Second plant vaccine to reach clinical trials stage
Diabetes autoantigen	Tobacco, potato	First plant-derived vaccine for an autoimmune disease
Cholera toxin B subunit	Tobacco, potato	First vaccine candidate expressed in chloroplasts
Cholera toxin B and A2 subunits, rotavirus enterotoxin and enterotoxigenic *E. coli* fimbrial antigen fusion	Potato	First plant-derived multivalent recombinant antigen designed for protection against several enteric diseases
Porcine transmissable gastroenteritis virus glycoprotein S	Tobacco, maize	First example of oral feeding inducing protection in an animal

protein stability and purification procedures. If a therapeutic protein is secreted into the extracellular space or growth medium or localized in seed oil bodies, for example, it may be more easily purified (**4**).

Recombinant proteins can also be produced in plant cell culture. These cultures can be grown in conventional microbial fermentors with some minor technical modifications. Batch, fed-batch, and perfusion fermentation culture modes can all be used (**7**).

Production in Tobacco

At the research laboratory level, tobacco is the most widely used system for the production of pharmaceutical proteins. Its main advantages lie in an established technology for gene transfer and expression, high biomass yields, a potential for rapid scale-up because of high seed production, and the ready availability of large-scale infrastructure for processing (**7**). In addition, tobacco is not a food crop and therefore escapes some of the attention and controversy surrounding the potential contamination of food supplies.

For the most part, nuclear transgenic plants have been used for production of proteins occurring in the leaves. It is possible to target proteins to the secretory pathway, which can result in their exuding in roots or leaves (rhizosecretion or phyllosecretion) (**7**). This strategy requires no cropping or harvesting. Phytomedics Inc. (Dayton, New Jersey; www.phytomedics.com) is currently developing this technology for the production of human secreted alkaline phosphatase. Rhizosecretion offers easier extraction of proteins than from leaves, making it an attractive option (**7**).

Another method of protein production in tobacco is in transplastomic plants, which involves placing foreign DNA into the chloroplast genome within the tobacco plant. (See Making a Plant Transgenic above). Structurally active human growth hormone and serum albumin proteins have been produced in tobacco chloroplasts. A tetanus toxin fragment was expressed in tobacco chloroplasts, and tests revealed that it induced protective levels of antitetanus antibodies (**8**). Cholera toxin B subunit has also been expressed this way, demonstrating that plastids can fold and assemble oligomeric proteins correctly (**9**). However, plastids do not carry out glycosylation. Chloroplasts are therefore unlikely to be useful in the synthesis of human glycoproteins, at least in cases where the glycan chain structure is vital for protein activity (**7**).

Another method of production in tobacco is cell culture. Expression of several recombinant proteins has been seen, including several antibody derivatives, in a suspension cell line derived from the tobacco strain BY-2 (**10**).

One disadvantage of producing therapeutic proteins in tobacco is the instability of recombinant protein in the leaves. To preserve any product, the leaves must be frozen, dried, or processed at the field site where they are grown before they begin to wilt and decay.

Production in Seeds

Some seeds have been found to retain stable antibodies after five months of storage at room temperature (**11**). Furthermore recombinant protein in rice grains remains stable and active after two years in storage (**12**). Seeds contain specialized storage compartments (protein bodies and storage vacuoles) that enhance their ability to accumulate proteins. "By including the appropriate signal peptide sequence or fusion responsible for directing expression and deposition, it is possible to target recombinant proteins to the lumen of the endoplasmic reticulum, vacuole, or other cellular compartments" (**13**).

Maize (corn) is the main commercial production crop for recombinant proteins. Its advantages include "high biomass yield, ease of transformation and in vitro manipulation, and ease of scale-up" (**7**). The first molecular farming venture by Prodigene (www.prodigene.com) used maize to produce the industrially valuable proteins avidin and ß-glucuronidase. Maize has also been used in the production of recombinant antibodies and enzymes such as lactase, trypsin, and aprotinin (**7**).

Other grains such as rice, wheat, and barley are also used to produce therapeutic proteins. These plants are self-pollinated, thus reducing the risk of gene flow. Ventria Bioscience (www.ventria.com) has developed a high-level protein expression system using the self-pollinating crops rice and barley (**14**). Expression of human lactoferrin and lysozyme in rice is reported to have "reached 1% of the rice grain weight or 40% of the total soluble protein" which is at least 25 to 40 times higher than the same molecules expressed in corn or tobacco plants (**14**).

Research conducted by SemBioSys (Calgary, AB, Canada; www.sembiosys.ca) recommends targeting seed oil bodies for protein expression. "Oleosines are highly expressed seed proteins, which comprise 2–10% of total seed protein and occur in all common oil seeds such as canola, sunflower, soybean, safflower, and peanuts. . . . Oleosin protein accumulates only on oilbodies and can be easily separated from other cellular contents by flotation centrifugation of aqueous seed extracts. Therefore, oilbody targeting can be used as an excellent carrier for recombinant proteins produced in oilseeds" (**13**).

A maize plantlet grown in ProdiGene's state-of-the-art greenhouse facility. Plants that contain the target gene are selected for further propagation.
PRODIGENE
(WWW.PRODIGENE.COM)

Soybeans and alfalfa are legumes — plants that fix atmospheric nitrogen — which have an advantage in their reduced need for chemical inputs. Researchers have reported the production of a functional, purified antihuman IgG through transgenic expression in perennial alfalfa (**15**).

The drawback of PMP production in grains is largely the risk of biocontamination, or genes spreading from PMP crops to food crops. This risk is also present in other edible crops, such as fruits and vegetables; but it can be avoided by the use of controlled and contained PMP systems (see Production in Aquatic Systems below).

Production in Fruits and Vegetables

Fruit and vegetables genetically modified to produce PMPs could be directly consumed by patients, bypassing the most costly part of protein production: purification.

Potatoes have been used to make edible vaccines and have been administered to humans in most of the clinical trials carried out using plant-derived vaccines so far (**7**). In 1999, volunteers who had been vaccinated against hepatitis B at a US national vaccine testing center were administered doses of raw potato containing hepatitis B vaccine. The immune response in those who received the vaccine-containing potatoes was comparable to people who had received a traditional booster vaccine (**16**). Potato tubers have been used for high-level production of a recombinant single-chain Fv (scFv) antibody and accumulated up to 2% of total soluble tuber protein. After they were stored at 4 °C for 1.5 years, half the amount of scFv present in fresh tubers was found (**17**).

Tomatoes offer the advantage of palatability and high biomass yields. Additionally, tomatoes grown in greenhouses can be contained more securely than field-grown plants (**7**). Transgenic tomatoes were used to produce the first plant-derived rabies vaccine. Some producing hepatitis B vaccine were grown in 2000. Drawbacks to tomatoes include their temperature sensitivity and the amount of heat required for prolific growth.

Lettuce has been used in a series of clinical trials for a vaccine against hepatitis B virus. Human volunteers were found to develop a specific serum-IgG response to the plant-produced protein (**18**). Carrots have also been investigated as potential producers of hepatitis B vaccine. They have the same palatability advantage as tomatoes but are less affected by heat.

Bananas have also been considered as hosts for the production of recombinant vaccines. The founder of this idea — and the lead researcher on many of the edible vaccine projects conducted to date — is Dr. Charles Arntzen of Arizona State University. He first came up with the idea after watching a mother in Thailand soothe a fussy infant with bits of banana. He was struck by the question: "What if, in addition to quieting her child, the mother could also administer a life-saving vaccine — in the banana?" (**19**). But bananas posed weighty technical challenges, so Arntzen redirected his work to potatoes and tomatoes. Both have shorter growing seasons than bananas, are easier to manipulate in an experimental setting, and can be freeze-dried to control doses (**19**).

Potato Tubers As Biopharmaceutical Factories

The Industrial Tuber technology developed by former MPB Cologne provides a unique concept for fully contained and controlled potato production in growth rooms. It meets the precepts of pharmaceutical production directors and regulatory agencies: biomass production in closed rooms under fully controlled conditions, a continuous production process linked to downstream processing, a stable genetic background by vegetative propagation of potato, economical efficiency through use of simple standard production tools, and strong optimization potential for molecular biological, biotechnological, and technical parameters. This suggests an alternative biomanufacturing process to be realized at significantly lower investment and cost of operations, requiring reasonable space only in production halls.

Potato tubers have been used for expression and purification of several single chain antibodies (SCA) as model products as well as for other proteins. Yields of 150–200 mg of extractable SCA and 75–100 mg of purified SCA per kilogram of tubers were reached with a standard expression system. The novel biomaufacturing process was developed to pilot scale productions aiming at some 50 g of purified SCA. All biomass production and downstream processing steps were established at technical scale. Final purification of the SCAs unfortunately could not be realized due to the insolvency of MPB Cologne. The technology is now owned by MPB´s founder Dr. Klaus Düring and marketed by my firm, Axara Consulting (www.axara-consulting.com).

A key technology building block is the proprietary post-harvest production technology that can significantly increase efficiency in containment production but also allows strictly increased biosafety over other systems if the potatoes are grown in field. The target protein will not be present in the growing plant but is expressed only after harvest by induction through nitrogen flooding.

Dr. Klaus Düring
President
Axara Consulting

Production in Aquatic Systems

Some aquatic plants have also been used for PMP production. Among the most prominent is the use of Lemna (duckweed) by Biolex (Pittsboro, NC, www.biolex.com).

Lemna is propagated clonally, without the need for pollen or seeds, making it environmentally safer than corn. Clonal propagation also has the advantage of a very high level of genetic stability, which is often not achieved with seed systems until after several generations. Lemna is the fastest growing higher plant, doubling its biomass every 36 hours. The generation of stable transgenic Lemna requires only two months from the start of transformation compared to about a year with many other plant systems.

Biolex grows Lemna in a controlled and contained chamber and has shown very high levels of recombinant protein accumulation (**20**). Some of the therapeutic proteins made in Lemna are α-interferon, β-interferon, GM-CSF, human growth hormone, plasminogen, human serum albumin, therapeutic peptides, and certain MAbs (**20**). Biolex is advancing its lead product, α-interferon, toward an IND in 2004.

Aseptically banked Lemna plants are transformed, selected, and screened using proprietary automation technology at Biolex.

BIOLEX (WWW.BIOLEX.COM)

Regulations in the United States

APHIS regulates the production of PMPs in the United States when the plants are grown in an open field environment. The Food and Drug Administration (FDA) Center for Biologics Evaluation and Research (CBER) regulates many biologics from clinical research through commercialization. The Center for Drug Evaluation and Research (CDER) regulates the PMPs that are drug related. Open-field grown PMPs are unlike other transgenic crops in that they are always grown under APHIS permit and thus regulated concurrently by the FDA and USDA.

APHIS, under 7 CFR part 340, regulates the interstate movement and environmental release of plants engineered for the production of PMPs (**21**). CBER/CDER also regulate the manufacturing of PMPs and consider fields of pharmaceutical crops to be "factories" (**3**). APHIS requires that manufacturers "document the required crop management practices to maintain containment of seed, pollen, or any plant product, and ensure that Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) are followed" (**3**). Under 7 CFR part 340 (c)(5), manufacturers are required to specify their procedures for maintaining control of the crop during planting, harvesting, and disposal of crop residues, as well as crop volunteers in following seasons to ensure that these products do not enter the food supply.

GMP procedures are required to ensure the safety, consistency, and potency of PMPs. They include "standards for quality management, personnel training, buildings and facilities, process equipment, documentation and records, materials management, production and in-process controls, packaging and labeling for transport, storage and distribution, laboratory controls, and process validation" (**3**).

In addition, APHIS regulations require that PMPs be isolated from other fields of same-species crops at greater distances than are required for Foundation and Certified seed-production operations. Self-pollinating crops have relatively short minimal isolation distances compared with corn. For example, the minimum isolation distance for rice is 100 ft and for corn is 1 mile (1.6 km) (**3**). Further methods of containment for corn include the removal of tassels (which produce the pollen) or using male-sterile varieties that do not produce viable pollen (**3**). APHIS recommends that plants that are pollinated by bees, that can produce dormant seeds, or that can cross-pollinate with wild crops not be used for PMP production. Additionally, PMP crops could be planted at different times than food crops to prevent overlap of pollination times. Leaving fallow border rows around transgenic crops can be useful in monitoring the possible growth of volunteer transgenics that may spread. Plants that have been genetically modified using plastid transformation provide an extra level of safety because proteins expressed in plastid genomes will have little or no transmission via pollen. A number of these environmental and trade concerns can be overcome by growing the PMP plants in a contained and controlled facility.

Regulations in Europe

Regulations in Europe are overseen by the European Agency for the Evaluation of Medicinal Products (EMEA). These regulations are similar to those found in the United States but are subject to individual member country regulations (or restrictions) as well. Additionally, to market any medicinal product containing biological active substances that have been manufactured using transgenic plants in the European Union, companies must use the centralized application procedure described in Part A of the Annex to Council Regulation (EEC) 2309/93 (**22**).

The EMEA requires a vast amount of information to be presented in applications to produce PMPs. This includes justification for the choice of host plant; characterization of the heterologous gene; a description of the expression

construct and a characterization of the final construct map; description of materials, procedures, and methods used in plant transformations; a rigorously characterized master cell bank; a global strategy description that includes relative parameters characterizing the expression construct, the plant, and the genetic stability of the production system; and detailed characterizations of expression constructs and final purified proteins, including nucleic acid and expressed protein analysis and validated methods for analysis.

European regulations concerning the manufacture of PMPs are similar to FDA and APHIS requirements (see Regulations in the United States). GMP and GAP practices are required and are meant to ensure containment of the transgenic crop and prevent contamination of wild or domestic plants in addition to providing consumers with a safe product.

What Does the Future Hold?

A world of possibility exists for the use of transgenic plants in the production of biopharmaceutical proteins and antibodies. However, optimism is balanced by the unknown risks of changing food crops into biopharmaceutical factories. Use of nonfood crops grown in contained and controlled environments may be a near-term answer to the regulatory and public perception challenges of PMPs. It may be ironic that the first research in the area of transgenic plants occurred in the laboratory of Lucien Ledoux in Mol, Belgium, in the late 1950s (**23**). Ledoux's laboratory was housed in Belgium's Nuclear Study Center, another technology that scientists viewed as a major breakthrough and positive innovation that has since become a source of many problems and controversy. In Chapter 6, the ethics and political impact of transgenic plants and animals is discussed. If the science is going to be successful, the risks must be weighed and the public must be informed about them along with the potential benefits of PMPs.

Acknowledgments

I would like to thank Bipin Dalmia of Biolex and Gina Melville of Large Scale Biotechnology Corporation for their contributions to this chapter.

References

1 Union of Concerned Scientists. *Pharm and Industrial Crops: The Next Wave of Agricultural Biotechnology.* www.ucsusa.org/pharm/pharm_why.html.

2 Pew Initiative on Food and Biotechnology. *Harvest on the Horizon: Future Uses of Agricultural Biotechnology.* http://pewagbiotech.org/research/harvest/harvest.pdf. September 2001.

3 Thomas, BR.; Van Deynze, A; Bradford, KJ. *Production of Therapeutic Proteins in Plants.* Agricultural Biotechnology in California Series, Publication 8078. Seed Biotechnology Center, University of California, Davis. 2002.

4 Gillis, J. Tobacco Plants May Grow Cancer Drugs. *Washington Post.* July 23, 2002.

5 Biotechnology Industry Organization. *Plant Made Pharmaceuticals: Frequently Asked Questions,* www.bio.org/pmp/factsheet2.asp. 2004.

6 Suslow, TR; Thomas, BR; Bradford, KJ. *Biotechnology Provides New Tools for Plant Breeding.* Agriculture Biotechnology in California Series, Publication 8043. Seed Biotechnology Center, University of California, Davis. 2002.

7 Ma, JK-C; Drake, PMW; Christou, P. The Production of Recombinant Pharmaceutical Proteins in Plants. *Nature Reviews Genetics.* 4: 794–805. October 2003.

8 Tregoning, J; et al. Expression of Tetanus Toxin Fragment C in Tobacco Chloroplasts. *Nucleic Acids Res.* 2003, 31: 1174–1179.

9 Daniell, H; et al. Expression of the Native Cholera B Toxin Subunit Gene and Assembly as Functional Oligomers in Transgenic Tobacco Chloroplasts. *J. Mol. Biol.* 311(5): 1001–1009. 31 August 2001.

10 Fischer, R; et al. Towards Molecular Farming in the Future: Using Plant-Cell-Suspension Cultures as Bioreactors. *Biotechnol. Appl. Biochem.* 30: 109–112. 1999.

11 Stoger, E; et al. Cereal Crops as Viable Production and Storage Systems for Pharmaceutical scFv Antibodies. *Plant Mol. Biol.* 42(4): 583–590. March 2000.

12 Huang J, et al. Expression of Natural Antimicrobial Human Lysozyme in Rice Grains. *Mol. Breeding.* 10: 83–94. 2002.

13 Seon, J-H; Szarka, JS; Moloney, MM. A Unique Strategy for Recovering Recombinant Proteins from Molecular Farming: Affinity Capture on Engineered Oilbodies. *J. Plant Biotech.* 4(3): 95–101. 2002.

14 Huang, N. High-Level Protein Expression System Uses Self-Pollinating Crops As Hosts. *BioProcess Int'l.* 2(1): 54–59. January 2004.

15 Khoudi, H; et al. Production of a Diagnostic Monoclonal Antibody in Perennial Alfalfa Plants. *Biotechnol Bioeng.* 64(2): 135–143. 20 July 1999.

16 Arntzen, CJ. Future Pharming, presented to the Philadelphia Society for Promoting Agriculture. www.pspaonline.com/arntzensum.html. 1 March 2001.

17 Artsaenko, O; et al. Potato Tubers as a Biofactory for Recombinant Antibodies. *Mol. Breeding.* 4(4): 313–319. 1998.

18 Kaputsa, J; et al. A Plant-Derived Edible Vaccine Against Hepatitis B Virus. *FASEB J.* 13(15): 1796–1799. October 1999.

19 Redig, M. Banana Vaccines: A Conversation With Dr. Charles Arntzen. *J. of Young Investigators.* Vol. 7. 1 March 2003.

20 Spencer, D. Center for Business Intelligence Conference on Plant Made Pharmaceuticals, Washington DC, 8–9 December 2003.

21 United States Department of Agriculture, Animal and Plant Health Inspection Service. 7 CFR 340. www.aphis.usda.gov/brs/7cfr340.html.

22 European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products. Draft: *Points to Consider on Quality Aspects of Medicinal Products Containing Active Substances Produced by Stable Transgene Expression in Higher Plants.* CPMP/BWP/764/02. 13 March 2002.

23 Lurquin, PF. *The Green Pheonix: A History of Genetically Modified Plants.* Columbia University Press. New York, NY. 2001.