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.

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.

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. 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
capabilities, 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
genome 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>
<thead>
<tr>
<th>Table 1: Pharmaceutical proteins that have been produced in plants</th>
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<tbody>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Human biopharmaceuticals</td>
</tr>
<tr>
<td>Human growth hormone</td>
</tr>
<tr>
<td>Human serum albumin</td>
</tr>
<tr>
<td>α-interferon</td>
</tr>
<tr>
<td>Erythropoietin</td>
</tr>
<tr>
<td>Human-secreted alkaline phosphatase</td>
</tr>
<tr>
<td>Aprotinin</td>
</tr>
<tr>
<td>Collagen</td>
</tr>
<tr>
<td>α1-antitrypsin</td>
</tr>
<tr>
<td>Recombinant antibodies</td>
</tr>
<tr>
<td>IgG1 (phosphonate ester)</td>
</tr>
<tr>
<td>IgM (neuropeptide hapten)</td>
</tr>
<tr>
<td>SlgA/V (Streptococcus mutans adhesin)</td>
</tr>
<tr>
<td>scFv-bryodin 1 immunotoxin (CD 40)</td>
</tr>
<tr>
<td>IgG (HSV)</td>
</tr>
<tr>
<td>LSC (HSV)</td>
</tr>
<tr>
<td>Recombinant subunit vaccines</td>
</tr>
<tr>
<td>Hepatitis B virus envelope protein</td>
</tr>
<tr>
<td>Rabies virus glycoprotein</td>
</tr>
<tr>
<td>Escherichia coli heat labile enterotoxin</td>
</tr>
<tr>
<td>Norwalk virus capsid protein</td>
</tr>
<tr>
<td>Diabetes autoantigen</td>
</tr>
<tr>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>Cholera toxin B and A2 subunits, rotavirus enterotoxin and enterotoxigenic E. coli fimbrial antigen fusion</td>
</tr>
<tr>
<td>Porcine transmissible gastroenteritis virus glycoprotein S</td>
</tr>
</tbody>
</table>

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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 f-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).
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 biomanufacturing 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.

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 (28). 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.

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