Molecular farming in plants: An approach of agricultural biotechnology

Kunka Kamenarova[1]*, Nabil Abumhadi[1], Kostadin Gecheff[2] and Atanas Atanassov[1]

*[1]AgroBioInstitute, 8, Dragan Tzankov Blvd., 1164 Sofia, Bulgaria [2]Bulgarian Academy of Science, 1113 Sofia, Bulgaria (*author for correspondence)*

Abstract

Molecular farming is defined as the production of proteins or other metabolites valuable to medicine or industry in plants traditionally used in an agricultural setting. Crop plants can synthesize a wide variety of proteins that are free of mammalian toxins and pathogens. Crop plants produce large amounts of biomass at low cost and require limited facilities. Since plants have long been used as a source of medicinal compounds, molecular farming represents a novel source of molecular medicines, such as plasma proteins, enzymes, growth factors, vaccines and recombinant antibodies, whose medical applications are understood at a molecular level. Bio-pharming promises more plentiful and cheaper supplies of pharmaceutical drugs, including vaccines for infectious diseases and therapeutic proteins for treatment of such things as cancer and heart disease. "Plant-made pharmaceuticals" (PMPs) are produced by genetically engineering plants to produce specific compounds, generally proteins, which are extracted and purified after harvest. As used here, the terms molecular farming and PMP do not include naturally occurring plant products or nutritionally enhanced foods.

Bitkilerde moleküler tarım: Tarımsal biyoteknolojiye yaklaşım

Özet

Moleküler tarım, ilaç veya endüstri açısından değerli geleneksel olarak kullanılan proteinlerin veya diğer metabolitlerin üretimi olarak tanımlanır. Bitkiler memeli toksinleri ve patojenleri içermeyen çok çeşitli proteinleri sentez edebilir. Bitkiler düşük fiyata mal olan ve sınırlı olanaklara gerek duyan fazla miktarda biyomas üretebilir. Bitkiler çok uzun süredir tıbbi bileşenlerin kaynağı olarak kullanıldığından, moleküler tarım, tıbbi kullanımları moleküler düzeyde anlaşılmış olan plazma proteinleri, enzimler, büyüme faktörleri, aşılar ve rekombinant antikorlar gibi moleküler ilaçlar için yeni kaynaklardır. Biyo–tarım, enfeksiyon hastalıklar için gerekli aşılar, kanser ve kalp hastalıkları için kullanılan teröpatik proteinleri içeren bitkisel ilaçların daha bol ve daha ucuza elde edilmesi için umut vericidir. "Bitki yapımı farmasotikler" (PMP$_S$) genetik mühendisliği ile bitkilerden spesifik bileşikler, genellikle hasat sonrası ekstre edilip saflaştırılan proteinlerin üretilmesi için kullanılmaktadır. Burada da kullanıldığı gibi moleküler tarım ve PMP doğal meydana gelen bitkisel ürünleri veya besleyici değeri arttırılmış besinleri içermemektedir.

Introduction

Manufacturing pharmaceutical products in crops has been one of the promised benefits of plant genetic engineering for the past 20 years. The using of biotechnology, sometimes known as "pharming," "bio-pharming," or "molecular farming," has migrated from speculation to the testing phase in fields and greenhouses across the country.

While short peptide chains (containing fewer than 30 amino acids) can be synthesized chemically, larger proteins are best produced by living cells. The DNA that encodes the instructions for producing the desired protein is inserted into cells, and as the cells grow they synthesize the protein, which is subsequently harvested and purified. Plants have provided humans with useful molecules for many centuries, but only in the past 20 years it has became possible to use plants for the production of specific heterologous proteins. The use of transgenic higher plants to produce foreign proteins with economic value was being realized (Kusnadi et al., 1998). The first pharmaceutically relevant protein made in plants was human growth hormone, which was expressed in transgenic tobacco in 1986 (Barta, 1986). During the period 1986- 1999 many therapeutics produced in plants were reported for first time: human antibodies (During, 1988); secretory antibodies (Hiatt et al., 1989); egg proteins with important properties - avidin (Hood et al., 1997) and aprotinin, one of the first molecularly farmed pharmaceutical proteins produced in plants (Zhong et al., 1999).

Although transgenic animals, bacteria and fungi are also utilized for the production of proteins, highest economic benefit will likely be achieved with plants (Horn et al., 2004). Many protein-based drugs are currently produced in sterile fermentation facilities by genetically engineered microorganisms or mammalian cell cultures in stainless steel tanks. Another method for obtaining biopharmaceuticals is to extract them from animal and human tissues (e.g., insulin from pig and cow pancreas, or blood proteins from human blood (Freese, 2002). However, these are high-cost procedures that carry the risk of transmitting infectious diseases to humans. Due to advances in plant genetic engineering over the past two decades, plants can now be modified to produce a wide range of proteins. It is hoped this will result in therapeutic products at a price significantly cheaper than those obtained by the currently applied methods (Table 1).

The idea for using plants to produce human proteins was initially met with great skepticism. However, plants offer a unique combination of advantages over traditional microbial and animal expression systems. Molecular farming in plants began in earnest in 1989 with the remarkable demonstration that functional recombinant antibodies could be expressed in tobacco (Hiatt et al., 1989). Before this result was published, there was little support for the idea that plants could be used to produce therapeutic proteins. Since then, it has been shown that transgenic plants are extremely versatile and they have been used to produce a wide range of pharmaceutical proteins (Schillberg et al., 2003). According to Horn (Horn et al., 2004) the advantages of using higher plants for the purpose of protein production include: (1) significantly lower production costs than with transgenic animals, fermentation or

Table 1: Comparison of production systems for recombinant human pharmaceutical proteins (Ma et al., 2003).

System	Overall cost	Production timescale	Scale up capacity	Product quality	Glycosylation	Contamination risk	Storage cost
Bacteria	Low	Short	High	Low	None	Endotoxins	Moderate
Yeast	Medium	Medium	High	Medium	Incorrect	Low risk	Moderate
Transgenic animals	High	Very long	Low	Very high	Correct	Viruses, Oncogenic NA	Expensive
Plant cell cultures	Medium	Medium	Medium	High	Minor differences	Low risk	Moderate
Transgenic plants	Very Low	Long	Very High	High	Minor differences	Low risk	Inexpensive

bioreactors; (2) infrastructure and expertise already exists for the planting, harvesting and processing of plant material; (3) plants do not contain known human pathogens (such as 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.

Recombinant proteins expressed in plants

Until recently, pharmaceuticals used for the treatment of diseases have been based largely on the production of relatively small organic molecules, chemically or microbially synthesized. Presently, attention is focused on larger and more complex protein molecules as therapeutic agents. Examples of proteins that have been produced in plants are listed in table 2.

Horn (Horn et al., 2004) categorizes proteins currently being produced in plants for molecular farming purposes 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.

The group of parental therapeutics and pharmaceutical intermediates

Includes all proteins used directly as pharmaceuticals along with those proteins used in the making of pharmaceuticals. The list of such proteins is long, ever growing, and includes such products as thrombin and collagen (therapeutics), and trypsin and aprotinin (intermediates).

Industrial proteins

This group includes hydrolases, encompassing both glycosidases and proteases. Enzymes involved in biomass conversion for producing ethanol are candidates for molecular farming. All of these products are usually characterized by the fact that they are used in very large quantities and must therefore be produced very inexpensively (Hood et al., 1999).

Recombinant 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.

Plants are an alternative expression system to animals for the molecular farming of antibodies (Schillberg et al., 2003). The production of antibodies in plants represents a special challenge because the molecules must fold and assemble correctly to recognize their cognate antigens. Typical serum antibodies are tetramers of two identical heavy chains and two identical light chains; however, there are more complex forms, such as secretory antibodies, which are dimers of the typical serum antibody and include two extra polypeptide chains. Two different cell types are required to assemble such antibodies in mammals, but plants that express four different transgenes can assemble these antibodies in a single cell (Ma et al., 2003).

Transgenic plants have been used for the production of antibodies directed against dental caries, rheumatoid arthritis, cholera, E. coli diarrhea, malaria, certain cancers, Norwalk virus, HIV, rhinovirus, influenza, hepatitis B virus, and herpes simplex virus (Thomas et al., 2002). Some of these have demonstrated preventative or therapeutic value and are currently in clinical trials.

Antigens for edible vaccines

Plant-derived vaccines have been produced against Vibrio cholerae, enterotoxigenic E. coli, hepatitis B virus, Norwalk virus, rabies virus, human cytomegalovirus, rotavirus and respiratory syncytial virus F (Thomas et al., 2002). Antigens specific to an individual patient's tumor are expressed in tobacco, harvested, purified, and administered to the patient. This entire process can take as little as 4 weeks, compared to 9 months for the same process using mammalian cell culture.

Many of these plant-derived antigens were purified and used as injectable vaccines, but oral delivery of these vaccines within foods has also been successful. In some cases, protection has actually been better with the edible vaccine than with the commercially available vaccine (Lamphear et al. 2004). In this way it could be overcome the need for injections and sterile needles and do not require refrigeration. Edible vaccines are being tested in potatoes, tomatoes, bananas, and carrots. Potatoes are usually cooked for consumption, which may inactivate the vaccine. Short

Table 2: Important pharmaceutical proteins that have been produced in plants (Thomas et al., 2002; Ma et al., 2003).

Protein	Host plant system	Comments/ Medical application
Human biopharmaceuticals		
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 size native human protein expressed in plants; low expression levels in transgenics (0.1% of total soluble protein) but high levels (11% of total leaf protein) in transformed chloroplasts/ Liver cirrhosis, burns, surgery
α-interferon	Rice, turnip	First human pharmaceutical protein produced in rice
Erythropoietin	Tobacco	First human protein produced in tobacco suspension cells/ Anemia
Human-secreted alkaline phosphatase	Tobacco	Produced by secretion from roots and leaves
Aprotinin	Maize	Human pharmaceutical protein produced in maize
Collagen	Tobacco	First human structural-protein polymer produced in plant; correct modification achieved by co-transformation with modification enzyme
α1-antitrypsin	Rice	First use of rice suspension cells for molecular farming
Lactoferrin	Rice, tomato	Antimicrobal activity
Protein C	Tobacco	Anticoagulant
Hirudin	Canola	Thrombin inhibitor
Granulocyte-macrophage colony-stimulating factor	Tobacco	Neutropenia
Enkephalins	*Arabidopsis*	Antihyperanalgesic by opiate activity
Epidermal growth	Tobacco	Wound repair and control of cell proliferation
Recombinant antibodies		
Immunoglobulin G1	Tobacco	First antibody expressed in plants; full length serum IgG produced by crossing plants that expressed heavy and light chains
Immunoglobulin M	Tobacco	First IgM expressed in plants and protein targeted to chloroplasts for accumulation
Secretory immunoglobulin A	Tobacco	First secretory antibody expressed in plants by sequential crossing of four lines carrying individual components; at present the most advanced plant-derived pharmaceutical protein
Immunoglobulin G (herpes simplex virus)	Soybean	First pharmaceutical protein produced in soybean
Hepatitis B virus envelope protein	Tobacco	First vaccine candidate expressed in plants; third plant-derived vaccine to reach clinical trials stage
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
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	Potato	First example of oral feeding inducing protection in an animal

storage life and length of production cycle may hinder vaccine production in tomatoes and bananas. Carrots have few storage problems and can be eaten raw, and carrots modified to produce the antigen used in hepatitis B vaccines are currently entering preclinical trials.

Other proteins of medical relevance

These include the milk proteins ß-casein, lactoferrin and lysozyme, which could be used to improve child health, and protein polymers that could be used in surgery and tissue replacement (Ma et al., 2003). Expression of thioredoxin in foods such as cereal grains would increase the digestibility of proteins and thereby reduce their allergenicity (Thomas et al., 2002). It has been shown that human collagen can be produced in transgenic tobacco plants and that the protein is spontaneously processed and assembled into its typical triple-helical conformation. The original plant-derived collagen had a low thermal stability owing to the lack of hydroxyproline residues, but this was remedied by co-expressing the enzyme proline-4-hydroxylase (Ma et al., 2003). Hood and colleagues (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 (Horn et al., 2004).

Protein expression systems

Plants are genetically enhanced to produce high-value proteins that are needed for the production of a wide range of therapeutics. The structure and functionality of a given protein is determined by its sequence of amino acids, which, in turn, determines its three-dimensional conformation, or structure. Internal bonds (sulfur and hydrogen bonds) among the amino acids give the protein its final shape and form. Complex proteins undergo further processing such as the addition of phosphate groups (phosphorylation) or carbohydrate molecules (glycosylation), which modify the proteins' functions. Information stored in DNA directs the protein-synthesizing machinery of the cell to produce the specific proteins required for its structure and metabolism.

Genetic aspect of producing of PMPs

To achieve specific protein production in plants, the DNA that encodes the desired protein must be inserted into the plant cells. This can be done as a stable transformation when foreign DNA is incorporated into the genome of the plant. A promoter associated with the inserted DNA then directs the cells to produce the desired protein, often targeting it to accumulate only in specific tissues such as the seed. Alternatively, a plant virus can be used to direct expression of a specific protein without genetically modifying the host plant. The transformation and expression systems used to engineer these proteins in plants affect the stability,

Table 3: Examples of recombinant proteins targeted to subcellular compartments in transgenic plants.

Proteins	Host plants	Tissue expression	Subcellular targets	References
α-Amylase	Tobacco	Leaves	Apoplast	Seon et al., 2002
Avidin	Corn	Seeds	Apoplast	Hood et al., 1997
Secretory antibodies	Tobacco	Leaves	Apoplast	Hiatt et al., 1989
ß-Glucuronidase	*Brassica*	Seeds	Oil bodies	Seon et al., 2002
Anti-oxazolone	Tobacco	Leaves	ER	Seon et al., 2002
Xylanase	*Brassica*	Seeds	Oil bodies	Seon et al., 2002
Anti-phytochrome	Tobacco	Leaves	Cytosol	Seon et al., 2002
Anti- ß-1,4-endoglucanase	Potato	Roots	Cytosol	Seon et al., 2002
Hirudin	*Brassica*	Seeds	Oil bodies	Seon et al., 2002
Vicilin	Tobaco, alfalfa	Leaves	ER	Wandelt et al., 1992

yield, cost of purification, and quality of the proteins produced (Thomas et al., 2002). In addition, the methods used affect the procedures needed to prevent the spread of the engineered traits to other plants during their growth in the field.

Foreign genes may be inserted, or transformed, into plants via a number of methods. Stable transformation into the nuclear genome is done primarily using *Agrobacterium* mediate transformation or particle bombardment methods (Suslow et al., 2002). In each case, the DNA coding for the protein of interest and an associated promoter to target its expression to a particular tissue or developmental stage is integrated into the genome of the plant. Thus, when the plant is propagated, each plant will transmit this property to its progeny and large numbers of plants containing the transferred gene are readily generated. It is also possible to deliver genes into the separate genome of plastids (chloroplasts and mitochondria) in plant cells. Chloroplast transformation has been successful in tobacco and potato, and research is being done to expand to other crops. Because genes in chloroplast genomes are not transmitted through pollen, recombinant genes are easier to contain, thereby avoiding unwanted escape into the environment. A second method of engineering plant protein expression is transduction, the use of a recombinant plant virus to deliver genes into plant cells. The DNA coding for the desired protein is engineered into the genome of a plant virus that will infect a host plant. A crop of the host plants is grown to the proper stage and is then inoculated with the engineered virus. As the virus replicates and spreads within the plant, many copies of the desired DNA are produced and high levels of protein production are achieved in a short time. A limitation with this system is that the green plant matter must be processed immediately after harvest and cannot be stored (Thomas et al., 2002).

Use of secretion pathways for subcellular targeting

To understand the factors controlling stability and accumulation of heterologous proteins, it is important to know where the protein of interest is located within specific plant cells or tissues and how this localization changes during development and as a result of environmental conditions. Isolation and purification of the desired protein may be greatly facilitated by

sequestering the protein into a particular cellular compartment. The secretion pathway in plants regulates and determines the passage of polypeptides to tonoplast-derived protein bodies, endoplasmic reticulum (ER)-derived protein bodies or secretion into the apoplastic space (Table 3). They may undergo specialized folding and post-translational modification that requires components of the ER. 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 ER, vacuole or other cellular compartments. As an advantage of this pathway it may be indicated that the secretion into one of the cellular compartments may separate the desired protein from proteases likely to catalyze its breakdown. Secretion has also been found to enhance protein stability by facilitating proper folding. Targeting signals can be used to intentionally retain recombinant proteins within distinct compartments of the cell to protect them from proteolytic degradation, preserve their integrity and to increase their accumulation levels (Seon et al., 2002). In this direction it is now possible to design gene constructs which contain ER-targeting signal peptide, KDEL, and to increase the level of accumulation of foreign proteins in transgenic plants. The presence of the ER-targeting signal led to a greatly enhanced accumulation of the heterologous protein. For example, the gene for the pea seed protein vicilin was modified by the addition of a sequence coding for this tetrapeptide. In lucerne and tobacco leaves, the level of vicilin-KDEL protein was 20 and 100 times higher than that of the unmodified vicilin, respectively (Wandelt et al., 1992). In the case of recombinant antibodies, it is very interesting that the recombinant full-size antibodies do not accumulate in the cytosol, due to incorrect/incomplete assembly and folding of heavy and light chain and consequent protein degradation. Cytosolic accumulation of recombinant antibodies has only been successful for single polypeptide chains, such as antibody heavy or light chains or scFvs (Schillberg et al., 2003).

The protein-synthesis pathway is highly conserved between plants and animals, so human transgenes that are expressed in plants yield proteins with identical amino-acid sequences to their native counterparts. However, there are some important differences in post-translational modification. The main difference between proteins that are produced in animals and

plants, however, concerns the synthesis of glycan side chains. All eukaryotes add glycan chains to proteins as they pass through the secretory pathway, but owing to differences in the levels of different modification enzymes, the glycan-chain structures vary widely across different taxa (Ma et al., 2003). Plant-derived recombinant proteins tend to lack the terminal galactose and sialic acid residues that are normally found in mammals, but have the carbohydrate group α(1,3)-fucose, which has a (1,6) linkage in animal cells, and ß(1,2)-xylose, which is absent in mammals although present in invertebrates (Ma et al., 2003).

These minor differences in glycan structure could potentially change the activity, biodistribution and longevity of recombinant proteins compared with the native forms. The possibility of plant-specific glycans inducing allergic responses in humans has been considered (Ma et al., 2003) and the finding that human serum contains antibodies that are reactive against these residues has been interpreted as evidence that the α(1,3)-fucose and ß(1,2)-xylose residues might lead to adverse reactions (Ma et al., 2003). However, carbohydrates are rarely allergenic. Moreover, the presence of antibodies in serum is not indicative of an adverse reaction. Finally, these glycan residues are also associated with every normal plant glycoprotein that is found in our diet. So, it is highly unlikely that they will be associated with adverse reactions. Indeed, studies in which mice were administered a recombinant antibody that contained plant-specific glycans showed no evidence of an antiglycan immune reaction (Ma et al., 2003). Nevertheless, the perceived negative effect of 'foreign' glycan structures is one of the most important issues that affect the use and acceptance of plant-derived recombinant proteins. Therefore, recent attention has focused on the development of strategies to 'humanize' the glycosylation patterns of recombinant proteins. Strategies that have been attempted in transgenic plants include the use of purified human ß(1,4)-galactosyltransferase and sialyltransferase enzymes to modify plant-derived recombinant proteins in vitro (Ma et al., 2003), and the expression of human ß(1,4)-galactosyltransferase in transgenic tobacco plants to produce recombinant antibodies with galactose-extended glycans. In the latter case, ~30% of the recovered antibody was galactosylated (Ma et al., 2003). *In vivo* sialylation is unlikely to be achieved in the near future because

plants seem to lack the metabolic pathway for the precursors of sialic acid, so several new enzymes would need to be introduced and coordinately expressed.

Plant-expression hosts

The range of plant species amenable to transformation is growing at a phenomenal rate and it is unclear at present which species are optimal for molecular farming. Many factors need to be taken into consideration (Schillberg et al., 2003). The yield of functional protein in a given species needs to be evaluated carefully, since this factor has to be weighed against the total biomass yield over a given planted area and any associated overhead costs. The storage and distribution of the product is also a consideration. The costs of grain storage and distribution are minimal compared with those of freshly-harvested tobacco leaves or tomato fruits, but the costs of extraction and purification are lower for watery plant material than desiccated seed. The compromise between production costs and profit is likely to be a key factor in selecting the crops used, because most pharmaceuticals will be produced by industry.

Ma end colleagues (Ma et al., 2003) have arranged the most spread plant production systems in three groups: 1) tobacco production system; 2) cereals and legumes and 3) fruit and vegetables.

Tobacco

Tobacco has an established history as a routine system for molecular farming. The main advantages of tobacco include the mature technology for gene transfer and expression, the high biomass yield, the potential for rapid scale-up owing to prolific seed production, and the availability of large-scale infrastructure for processing. Although many tobacco cultivars produce high levels of toxic alkaloids, there are low-alkaloid varieties that can be used for the production of pharmaceutical proteins (Ma et al., 2003).

As an alternative to nuclear transgenics, transplastomic plants are produced by introducing DNA into the chloroplast genome rather than the nuclear genome, a process that is generally achieved by particle bombardment. Human growth hormone, serum albumin, a tetanus toxin fragment and the

cholera toxin B subunit have been produced at high levels in tobacco chloroplasts, and found to be structurally authentic and biologically active. These data show that plastids can fold and assemble oligomeric proteins correctly (Ma et al., 2003). One disadvantage of the chloroplast transgenic system is that plastids do not carry out glycosylation. It is therefore unlikely that chloroplasts could be used to synthesize human glycoproteins in cases in which the glycan-chain structure is crucial for protein activity. One of the disadvantages of recombinant-protein production in tobacco is the instability of the product, which means that the leaf tissue must be frozen or dried for transport, or processed at the farm.

Cereals and legumes

The accumulation of recombinant antibodies in seeds allows long-term storage at ambient temperatures because the proteins amass in a stable form. Seeds have the appropriate biochemical environment for protein accumulation, and achieve this through the creation of specialized storage compartments, such as protein bodies and storage vacuoles, which are derived from the secretory pathway. Seeds are also desiccated, which reduces the exposure of stored proteins to non-enzymatic hydrolysis and protease degradation. Cereal seeds also lack the phenolic substances that are present in tobacco leaves, so increasing the efficiency of downstream processing (Ma et al., 2003).

Maize is now the main commercial production crop for recombinant proteins, which reflects advantages such as high biomass yield, ease of transformation and in vitro manipulation, and ease of scale-up. Maize is also being used for the production of recombinant antibodies (Hodd et al., 2002a) and further technical/pharmaceutical enzymes, such as laccase, trypsin and aprotinin (Hood, 2002b).

The use of barley grains as bioreactors for highly active and thermo-tolerant hybrid cellulase (1,4-ß-glucanase) was investigated (Xue et al., 2003). Of crescent interest are the production of marker-free transgenic plants and the use of cultivars without herbicide or antibiotic resistance. Toward this, transgenic barley plants whose genome contains genes for production of human antithrombin III, α1-antitrypsin, lysozyme, serum albumin and lactoferrin were generated (Stahl et al., 2002). Successful expression of human lactoferrin was achieved in rice

by Anzai and colleagues (Anzai et al., 2000). Recombinant antibody of a single-chain Fv against carcinoembryonic antigen was produced in rice and wheat. It was confirmed that this antibody can be stored for at least five months at room temperature, without significant loss of the amount or the activity (Stöger et al., 2000).

Alfalfa and soybean produce lower amounts of leaf biomass than tobacco, but have the advantage of using atmospheric nitrogen through nitrogen fixation, thereby reducing the need for chemical inputs. Both species have been used to produce recombinant antibodies (Ma et al., 2003). Pea is being developed as a production system, although at present the yields that are possible with this species are low (Ma et al., 2003).

Fruit and vegetables

The main benefit of fruit, vegetable and leafy salad crops is that they can be consumed raw or partially processed, which makes them particularly suitable for the production of recombinant subunit vaccines, food additives and antibodies for topical passive immunotherapy (Ma et al., 2003). Potatoes have been widely used for the production of plant-derived vaccines and have been administered to humans in most of the clinical trials. The potential of potato tubers for antibody production was first shown by Artsaenko and colleagues (Artsaenko, 1998; Ma et al., 2003), and recently this crop has been investigated as a possible bulk-production system for antibodies (Ma et al., 2003). Potatoes have also been used for the production of diagnostic antibody-fusion proteins and human milk proteins (Ma et al., 2003).Tomatoes, which were used to produce the first plant-derived rabies vaccine (Ma et al., 2003), are more palatable than potatoes and offer other advantages including high biomass yields (~68,000 kg per hectare) and the increased containment that is offered by growth in greenhouses. Lettuce is also being investigated as a production host for edible recombinant vaccines, and has been used in one series of clinical trials for a vaccine against HBV (Ma et al., 2003). Bananas have been considered as hosts for the production of recombinant vaccines, as they are widely grown in the countries in which vaccines are most needed and can be consumed raw or as a puree by both adults and children (Ma et al., 2003).

Discussions and conclusions

Like many other aspects of crop biotechnology, supporters and critics of PMP crops differ strongly over the benefits and risks of this new application. Proponents stress the societal benefits of a cheaper and more plentiful source of pharmaceuticals, while opponents emphasize the risks of contamination of the food supply and unknown effects on ecosystems. Given the uncertainties surrounding bio-pharm crops, it is difficult to predict whether and to what extent this technology will become part of our future agricultural and health care systems. Several questions remain to be answered, including: (1) Are PMPs safe and effective medicines for humans and animals? (2) Will production costs of PMPs, especially for the purification process, be reduced sufficiently to bring the promised economic benefits? (3) What will be the appropriate combinations of crop species, plant parts, growing environments, and production safeguards that will provide acceptable levels of gene containment and environmental protection? (4) Are our regulatory structures adequate to the task of regulating and monitoring bio-pharm crops, and, if not, what changes will be necessary? (5) To what extent will crop-based pharmaceuticals provide new economic opportunities for farmers and rural communities?

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. The fear is that the non-food products may inadvertently enter the food chain and present an unintentional risk (Horn et al., 2004). The use of plants to produce non-food products is not unlike the current use of other food products, such as eggs or yeast, which 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 started with the arising of 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 including plants it is not possible to eliminate all possibilities of unintended exposure due to unforeseen circumstances such as an accident, a natural disaster, etc.

One of the keys to success in the future will undoubtedly be the level of expression of the recombinant protein in plants. This is one of the most important aspects with regard to economics. Expression is also a major regulatory concern (Horn et al., 2004). 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 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 (Horn et al., 2004). 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.

Reference

Anzai H, Takaiwa F and Katsumata K. Production of human lactoferrin in transgenic plants. *Elsevier Science* B.V. 265-271, 2000.

Artsaenko O, Kettig B, Fiedler U, Conrad U. and Düring K. Potato tubers as a biofactory for recombinant antibodies. *Mol. Breeding*. 4: 313-319, 1998.

Barta A. The expression of nopaline synthase human growth

hormone chimaeric gene in transformed tobacco and sunflower callus tissue. *Plant Mol. Biol.* 6: 347-357, 1986.

During K. Wound-inducible expression and secretion of T4 lysozyme and monoclonal antibodies in *Nicotiana tabacum. Ph. D Thesis.* Mathematisch-Naturwissenschaftlichen Fakultat der Universität zu Köln, 1988.

Freese B. Manufacturing drugs and chemical crops: Biopharming poses new threats to consumers, farmers, food companies and the environment. Available from GE Food Alert, www.gefoodalert.org, 2002.

Hiatt A, Cafferkey R and Bowdish K. Production of antibodies in transgenic plants, *Nature.* 342 (6245): 76-78, 1989.

Hood EE, Kusnadi A, Nikolov Z, Howard JA. Molecular farming of industrial proteins from transgenic maize. In: *Chemicals via higher plant bioengineering. Kluwer/Plenum.* Shahidi F, Kolodziejczyk P, Whitaker JR, Munguia AL, Fuller G, New York, 127–147, 1999.

Hood EE, Witcher DR, Maddock S, Meyer T, Baszczynski C, Bailey M, Flynn P, Register J, Marshall L, Bond D, Kulisek E, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh RJ, Hernan R, Kappel WK, Ritland D, Li CP and Howard JA. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Mol Breed* 3: 291–306, 1997.

Hood EE. From green plants to industrial enzymes. *Enzyme Microbial Technol.* 30: 279–283, 2002a.

Hood EE, Woodard SL and Horn ME. Monoclonal antibody manufacturing in transgenic plants - myths and realities. *Curr. Opin. Biotechnol.* 13: 630–635, 2002b.

Horn ME, Woodard SL and Howard JA. Plant molecular farming: systems and products. *Plant Cell Rep.* 22: 711–720, 2004.

Kusnadi AR, Hood EE, Witcher DR, Howard JA and Nikolov ZL. Production and purification of two recombinant proteins from transgenic corn. *Biotechnol Prog.* 14: 149–155, 1998.

Lamphear BJ, Jilka JM, Kesl L, Welter M, Howard JA and Streatfield SJ. A corn-based delivery system for animal vaccines: an oral transmissable gastroenteritis virus vaccine boosts lactogenic immunity in swine. *Vaccine* (in press), 2004.

Ma JK-C, Drake PMW and Christou P. The production of recombinant pharmaceutical proteins in plants. *Genetics.* 4: 794-805, 2003.

Schillberg S, Fischer R and Emans N. Molecular farming of antibodies in plants. *Naturwissenschften.* 90: 145-155, 2003.

Seon J-H, Szarka JS and Moloney MM. A unique strategy for recovering recombinant proteins from molecular farming: affinity capture on engineered oilbodies. *J. Plant Biotechnology.* 4 (3): 95-101, 2002.

Stahl R, Horvath H, Van Fleet J, Voetz M, Von Wettstein D and Wolf N. T-DNA integration into the barley genome from single and double cassette vectors. *Proc. Natl. Acad. Sci.* 99 (4): 2146-2151, 2002.

Stöger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P and Fischer R. Cereal crops as viable production and storage systems for pharmaceutical scFV antibodies. *Plant Mol.Biol.* 42: 583-590, 2000.

Suslow TV, Thomas BR and Bradford KJ. Biotechnology provides new tools for planting. *Oakland: University of California Division of Agriculture and Natural Resources*, Publication 8043, 2002.

Thomas BR, Van Deynze A and Bradford KJ. Production of Therapeutic proteins in plants. *Agricultural Biotechnology in California Series*, Publication 8078, 2002.

Wandelt CI, Khan MR, Craig S, Schroeder HE, Spencer D anf Higgins TJ. Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *The Plant Journal.* 2: 181, 1992.

Xue GP, Patel M, Johnson JC and Smith DJ. Selectable marker-free transgenic barley producing a high-level of cellulase (1,4-ß-glucanase) in developing grains. *Plant Cell Rep.* 21: 1088-1094, 2003.

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 and Howard JA. Commercial production of aprotinin in transgenic maize seeds. *Mol Breed.* 5: 345–356, 1999.