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Mini Review

Molecular Farming in Plants: A Current Perspective

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The low cost of production makes plants an ideal candidate for producing many high value compounds through genetic engineering. Expression of vaccines, therapeutic proteins, nutraceuticals, industrial enzymes, and other bio-polymers has been achieved in different plants. A few products for human health care that have been produced in plant systems are currently undergoing human clinical trials. Some recombinant molecules produced in plants for diagnostic use are currently available in the market and several other compounds are in the pipeline for commercialization. The involvement of several biotechnology companies and the successes achieved provide promise for the growth of this emerging field, "Molecular Farming".

Key words: molecular farming, edible vaccines, recombinant antibodies, therapeutic proteins.

The first two technological revolutions, the industrial and computer-based information technology revolutions, have already made a major impact on global economic growth. The third technological revolution will be based on biotechnology and genomics. These "third wave" technologies have great potential to revolutionize discovery in the treatment of several human disorders and in the provision of an alternative way for producing nutrition supplements, fuel, medicines, and other high value products from genetically modified crop plants. Since the first report of producing transgenic plants (1,2), several plant species have been modified to improve various agronomic traits and output traits, including their use as "biological factories". Transgenic plants with useful traits are already being cultivated in the USA. The global market for genetically engineered plants is estimated to be $3 billion in the current year with a trend to increase to $8 billion in 2005 and to $25 billion by 2010 (3). There is a great potential in the plant biotechnology sector for producing high value products in transgenic plants.

Traditionally, production and the supply of a desired protein were limited to the use of the original tissue wherein the proteins are synthesized in plants, animals or bacteria. This approach is not always cost effective and has many limitations, such as difficulties in obtaining proteins in sufficient quantities with acceptably high purity and biological activity. Advances in recombinant DNA technology have allowed the isolation of genes from diverse sources and their expression in heterologous host systems. Historically, bacterial systems were used as the host to overexpress recombinant proteins; however, in the last two decades other systems such as yeast, fungi, insect cells, mammalian cells, transgenic animals, and transgenic plants have been used as host to express the protein of interest.

It is now possible to genetically engineer / transform many agricultural crops and trees for useful traits by transferring genes from a heterologous source. Considerable success has been achieved through genetic engineering in improving agronomic traits for insect/pest resistance, herbicide tolerance, and other useful input traits that are available commercially. Plants are ideal host systems in that they are cost effective, can be rapidly scaled up, and have fewer ethical issues and a better public acceptance than transgenic animals. One of the emerging fields of plant biotechnology, referred to as "Molecular Farming", has been used in recent years to produce value-added products for nutraceuticals, pharmaceuticals, and other industrial applications. This mini-review focuses on the progress made and success achieved in using plants as factories to produce recombinant proteins and other bio-polymers for pharmaceuticals and industrial applications.

Choice of the Expression System for Expressing Recombinant Proteins

For a long time, bacterial systems were used as host organisms to produce recombinant proteins because of

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the ease of cloning and expressing useful genes and producing in high yields (4). The major disadvantages in bacterial systems are the lack of the ability to perform post-translational modifications and the high cost of production through fermentation systems. To overcome this problem, eukaryotic expression systems that are capable of performing post–translational modification were adopted. Insect cells, mammalian cell lines (5), and the milk of transgenic goats (6), rabbits (7), pigs and sheep (8) have been used for producing human therapeutic proteins. The main disadvantages of these expression systems are the high cost incurred in production, concerns about product safety, and public acceptance.

Plants are ideal candidates as host systems because of several features, such as ease of transformation, low cost of investment, dispersed capital requirements, rapid scale-up, high and controlled level of expression, and capability of performing post-translational modifications (9). Plants provide opportunities as a safe method of delivery of recombinant proteins for therapeutic use and easy storage and distribution. Further, there are less concerns about product safety and public acceptance, making plants the potentially best system as factories for producing recombinant proteins for a variety of uses, including but not limited to vaccines, therapeutics, diagnostics, industrial enzymes, and other bio-polymers. Several plants have been used in producing recombinant antibodies, enzymes, hormones, vaccines, and other useful bio-polymers.

Vaccines

Pathogenic organisms invade humans and animals and cause disease. It has been well established that inoculation of heat-killed pathogens or inactivated components of pathogens can induce an immune response in mammals without causing the disease, thus providing protection against the respective pathogen. This process, referred to as "vaccination", is a common method to protect humans and animals against several diseases (10). A successful example of mass vaccination is the eradication of small pox, a deadly disease, from the face of the Earth. By understanding the principles involved in the immune response, it was possible to ascertain that certain protein(s) or their subunits from pathogens could provide full protection against the disease. This offered a new trend in using small molecules for vaccination, a potentially safer procedure, compared to heat-killed whole pathogens.

The first step in developing effective vaccines is to identify the antigen (usually a protein or glycoprotein) that is involved in inducing the immune response. Once identified, the gene encoding the protein is isolated and expressed in another non-pathogenic system for large-scale production and its application in vaccination programmes. The first recombinant vaccine produced in a yeast system was the Hepatitis B surface antigen in the early 80's (HbsAg) (10). Considerable success has been achieved in producing recombinant vaccines; however, in many cases they are expensive due to the high cost of production and marketing. Further, injectable vaccines require the use of sterile disposable needles, syringes, and the availability of trained personnel. The poor people in developing nations neither can afford nor have access to a majority of the vaccines. Therefore, recent research has been focused on producing vaccines that can be administered orally in a cost-effective manner, preferably through ingestible food. Oral vaccination has the advantages of easy administration and of eliciting mucosal immunity, which offers a primary defense against the pathogens that enter the body through mucosal membranes. In the next step, a systemic immune response is triggered by the production of secretory antibodies to neutralize the pathogen. Plants (field and garden crops) are consumed by humans and animals as food, and therefore are a suitable choice of host system for the production of vaccines. Further, plants have the capacity of post-translational modification such as glycosylation, an important aspect in immunogenicity. The steps involved in the edible vaccine technology are identifying an antigen that provides the desired immune response, cloning and high-level expression of the antigen in a model plant system, analyzing the immune response in an animal model system, and extending it to human clinical trials.

Surface protein antigen A (spaA) was the first antigen produced in transgenic tobacco plants (11). However, the first proof of concept that recombinant antigens expressed in plants could stimulate the immune system was demonstrated by expressing the heat labile enterotoxin B subunit (LT-B) toxin in transgenic potato (12). The LT-B toxin is produced by enterotoxigenic *Escherichia coli* that cause diarrhea commonly called 'Travellers Diarrhea". LT-B is closely related to cholera toxin (CT-B) produced by *Vibrio cholera*. The initial attempts resulted in an expression level of 0.01% of total soluble protein; however the level of expression was significantly increased by including endoplasmic reticulum (ER) retention signal sequences and by optimizing the gene with plant preferred codons (12-14). CT-B has also been expressed in transgenic potato (15). Due to similar properties, CT-B and the LT-B could be used as an oral adjuvant to enhance the immune response (16). When CT-B insulin fusion protein was

Table 1. Vaccines produced in different plant host systems

Antigen	Causal organism/ Disease	Host system	Reference
Rabies virus glycoprotein	Rabies	Tomato, Tobacco, Spinach	18, 19
Capsid protein epitope	Mink enteritis virus	Cowpea	20
Spike protein	Piglet diarrhea	Tobacco	21
CT-B toxin	Cholera	Potato	22, 15
LT-B toxin	Travellers diarrhea	Potato	13, 14
Hepatitis B surface antigen	Hepatitis B	Tobacco, Potato	23, 24
Human cytomegalovirus glycoprotein B	Human cytomegalovirus	Tobacco	25
Norwalk virus antigen	Gasterointestinal distress	Tobacco, Potato	26
Foot and mouth disease antigen	Foot and Mouth disease	Cowpea	27
Malarial antigens	Malaria	Tobacco	28
Gp41 peptide	HIV-1	Cowpea	29, 30
Hemagglutinin	Influenza	Tobacco	31
c-Myc	Cancer	Tobacco	31

expressed in transgenic potato and tested on non-obese diabetic mice, the results showed a substantial reduction in pancreatic islet inflammation and a delay in progression of clinical diabetes (15,17). Table 1 gives a list of antigens that have been expressed in plants for the production of edible vaccines.

Two major approaches that are used to produce edible vaccines is the use of an *Agrobacterium*-based vector for production of transgenic plants and a viral coat protein-based vector, where the antigen is expressed on the surface of a virus particle. Three antigens (LT-B, HbsAg, NVCP) have been successfully expressed in transgenic potato and are currently undergoing human clinical trials. The results of the first human clinical trial suggest that edible LT-B can induce mucosal and systemic immune responses (32). Hepatitis B virus surface antigen (HbsAg) is currently undergoing Phase I clinical trials (33), and Norwalk virus capsid protein (NVCP) has been shown to elicit an immune response (34).

In the last few years, several investigators and biotechnology companies have been focusing on producing "autoantigens" in plants to induce autoimmune tolerance. Insulin-dependent diabetes mellitus (IDDM), also known as type I diabetes, is a common autoimmune disease that affects 1 in 300 individuals in the United States. The development of diabetes may be associated with a T lymphocytes-mediated destruction of beta cells in the pancreas resulting in the shortage of insulin, a hormone that controls the blood sugar level. Two major proteins, insulin and glutamic acid decarboxylase (GAD), have been found to play a major role in eliciting autoimmunity for IDDM. Both these proteins (insulin and GAD) have been expressed in transgenic potato (35,15). Feeding experiments of potato tubers expressing insulin or GAD in non-obese diabetic mice indicates a delay in progression of clinical diabetes. The preliminary results are promising and warrant further exploration of this approach. This novel approach is exciting because it offers a new strategy in providing autoantigens as supplements in food to combat autoimmune disorders.

Antibodies

Antibodies are used both in diagnostics and as therapeutic agents in biomedical health care. Monoclonal antibodies produced through hybridoma technology have been extensively used for this purpose (36). Several attempts have been made to produce recombinant antibodies in plants, since they are an excellent host system for expressing foreign proteins (Table 2). The first reported attempt at producing antibodies in plants was in tobacco (45). Several functional antibodies (46,47), fragment antigen-binding (Fab) (48), and single chain antibody fragments (scFv) (49-51) can be expressed in the leaves and seeds of plants without the loss of binding specificity. Expression of antibodies varies between different plant species, and a high level expression of scFvs was achieved in tobacco leaves, 7% of total soluble protein (52). The first clinical trial using antibodies produced in plants was to prevent human tooth decay caused by *Streptococcus mutans* (52). Expression of an anticancer monoclonal antibody in tobacco plants using a viral vector has been reported, and trials are underway to test tumor-suppressive activity (38). Monoclonal antibodies against genital herpes (HSV-2), and sperm as a contraceptive, are currently being

Table 2. Antibodies produced in different plant host systems

Potential use	Antigen used	Host	Reference
HSV-2	Glycoprotein B of HSV	Soybean	37
Colon cancer	Colon cancer antigen	Tobacco	38
Dental care (tooth decay)	*S. mutans* antigen	Tobacco	39
Hodgkin's lymphoma	ScFv of IgG from mouse B-cell lymphoma	Tobacco	40
Tumor associated marker antigen	ScFvT84.66 against carcinoembryogenic antigen	Cereals	41
Research	Human creatine kinase	*Arabidopsis*	42
Phytoremediation	Atrazine	Tobacco	43
Plant protection	Nematode antigen	Tobacco	44

developed in transgenic corn (53). The current success provides hope to produce antibodies in plants to treat various mucosal infections in humans.

Recombinant Proteins

Recombinant proteins have been used for treating metabolic and genetic disorders. Unlike the edible vaccines, where purification is not critical, recombinant proteins for therapeutic use require purification. In order to commercially produce recombinant proteins in plants the following criteria are important: the choice of the host system, cost of production, capability of post-translational modifications, stability and shelf life of the product, choice of expression system, purification strategies involved, and market potential (54). The choice of crop plant as host depends on the recombinant proteins that are to be produced. The cost of producing a recombinant protein varies among different crops due to differences in expression levels and processing, and ease of use (55); *eg.*, the expression of recombinant proteins in seeds and tubers provides for better storage and longer shelf life. Tissue-specific promoters have been employed in expressing recombinant proteins in potato tubers and in seeds of corn, *Arabidopsis*, tobacco and canola (56-60). Hirudin, an anti-coagulant protein, produced in transgenic canola retained the specific activity to inhibit thrombin (60). Gastric lipase produced in transgenic corn has successfully completed European Phase I trials in treating cystic fibrosis (61). The number of other recombinant proteins that have been expressed in plants for therapeutic purposes is promising (Table 3).

Expression of transgenes could be controlled by using different promoter sequences that are constitutive, tissue-specific, developmental-specific, and inducible through chemicals or wounding. Constitutive expression of recombinant proteins is not always a desired feature, for it can affect the stability and yield of the protein. Tissue-specific expression in seeds and tubers has resulted in better stability of the recombinant protein. Inducing the expression of recombinant proteins by the use of chemicals or wounding could be ideal in the sense that the proteins are synthesized in large amount within a short period of time. This allows the flexibility of expressing the proteins when required for purification and processing. A post-harvest expression strategy has been adopted to express recombinant proteins using promoter sequences that induce the expression after harvesting the leaves (76). Researchers at Crop Tech Corporation, VA, USA, have modified promoter sequence elements from a defense-related gene that activate gene expression in response to mechanical wounding and defense elicitors. This strategy has several advantages in producing recombinant proteins, such as growth of the plant is not affected and uncontrolled environmental factors impinging on the growing plant do not affect the protein yield and quality. Further, based on the stability of the particular gene product, the timing of protein extraction can be adjusted to optimize the yield of active recombinant proteins. One of the major concerns in plant-based production of recombinant proteins is the purification system, which depends on the choice of the recombinant protein. For example, edible vaccines and some industrial enzymes do not require high purification and could be used either directly or after partial purification. However, recombinant proteins for therapeutics require high purification and different strategies are employed. A few strategies are listed below.

Use of affinity tags to purify the target protein could be used, in which the expressed fusion protein is

Table 3. Therapeutic proteins produced in different plant host systems

Therapeutic protein	Potential use	Host	Reference
α-and β Haemoglobin	Blood substitute	Tobacco	62
Human serum albumin (HAS)	Blood substitute	Potato	63
Protein C	Anticoagulant	Tobacco	64
Glucocerebrosidase	Gaucher disease	Tobacco	65
α-interferon	Viral protection anticancer	Rice	66
Epidermal growth factor	Mitogen	Tobacco	67
Erythropoietin	Mitogen	Tobacco	59
Trout growth factor	Mitogen	Tobacco	68
Hirudin	Anticoagulant	Canola	60
Glutamate decarboxylase	Diabetes	Tobacco	35
Human somatotropin	Hypopituitary dwarfism	Tobacco	69
Calcitonin	Paget disease, osteoporosis, parathyroid gland carcinomas	Potato	70
β-interferon	Protection	Tobacco	71
Human granulocyte-macrophage colony- stimulating factor	Neutropenia	Tobacco	72
Enkephalins	Antihyperanalgesic by opiate activity	Oil seed	72
Human homotrimeric collagen I	Collagen	Tobacco	73
α- tricosanthin	HIV Therapy	Tobacco	74
Angiotensis-1-converting enzyme	Hypertension	Tobacco/ Tomato	75

recovered by binding to a ligand immobilized onto a matrix support. Different ligand pairs such as maltose binding protein-amylose, histidine residues-metal ion, and protein A-IgG used in bacterial systems could be adopted in plant systems. The fusion protein, after affinity purification, could be proteolytically cleaved and with another purification step yield the pure target protein. An example of the affinity tag is the purification of human glucocerebrosidase-FLAG epitope fusion produced in tobacco, where the fusion protein was purified using anti-FLAG antibody affinity matrix (65). It is also possible to express the recombinant protein as a fusion protein to the virus capsid protein. Incorporation of a protease-cleaving site between the capsid protein and the target protein allows cleavage from the capsid protein; however, the disadvantage of this system is that proteins of large size will impair the assembly of the viral coat.

Oil bodies primarily store triacylglycerides and are present in seeds of many plant species. In nature a unique protein, "olesin", is targeted to, and found to be associated with the oil bodies. Targeting recombinant proteins into oil bodies is a new and exciting strategy to purify recombinant proteins. Expression of olesin-Gus fusion constructs in transgenic plants produces foreign protein fusions with olesin resulting in targeting to oil bodies (77). This approach offers an easy step of purification involving grinding of the seeds in aqueous buffer, separation of the oil phase, proteolytic cleavage of olesin and the target foreign protein, yielding the protein in the aqueous phase. Hirudin is an anticoagulant protein that inhibits thrombin and has high therapeutic value. A synthetic hirudin gene fused to the 3' end of the gene encoding *Arabidopsis* olesin with the incorporation of a cleavage site for endoprotease Factor Xa between the two was transformed to *Brassica napus* (60). The olesin-hirudin fusion was highly stable in seeds stored at room temperature for about 30 months and retained the specific activity in the extracts (78). Since olesins are not exposed to endoplasmic reticulum or to other organelles for post-translational modification, this approach is limited to proteins that do not require post-translational modifications.

Secretion of recombinant proteins through roots, referred to as "rhizosecretion", is a new alternate strategy for easy purification. Roots of different plants secrete proteins into their growth environment. Three heterologous genes (green fluorescent protein, secreted alkaline phosphatase, and xylanase) under the control of root-specific promoter and secretory signals have been found to be secreted through the roots of transgenic tobacco plants (79). Another method by which

recombinant proteins could be secreted is through the guttation fluid — a phenomenon referred to as "Phyllosecretion". In nature, guttation fluid is observed during high water absorption and reduced transpiration especially during the dawn and cool conditions. Recently, using endoplasmic reticulum signal peptides to recombinant proteins, it was demonstrated that the recombinant proteins could be secreted through the intercellular spaces into the guttation fluid in transgenic tobacco plants. (80). Although this method provides continuous supply of recombinant proteins, with easy downstream processing, efforts are required for optimization for production in large scale in an economically feasible manner. The use of an affinity tag, targeting of proteins to specific bodies, phyllosecretion and rhizosecretion are a few examples of innovations pertaining to downstream processing for recombinant protein purification.

Bio-polymers

Protein-based polymers have immense use in medical and non-medical applications. Bioelastic protein-based polymers are used in wound coverings for post surgical adhesions and scars. Non-medical applications of bio-polymers include transducers and biodegradable plastics. The protein-based polymer gene $(GVGVP)_{121mer}$ was synthesized and transferred to tobacco through chloroplast transformation (81). In spite of the presence of a high level of transcripts, the expression of protein was low. Since $(GVGVP)_{20mer}$ has been successfully used in post surgical adhesion, expressing the smaller protein polymer (20 mer *vs* 121 mer) may be more suitable (81).

Polyhydroxyalkanoates (PHAs) are a class of polymers found in many bacteria that have properties similar to that of plastics. Many microorganisms can use PHA as source of energy. Therefore, these molecules are biodegradable. A range of commercial products using the biodegradable plastic polymer, poly-hydroxybutarate-co-valerate (PHBV) obtained from the bacteria *Ralstonia eutropha*, are available (82). Three genes (*phbA, phbB, and phbC*) of *R. eutropha* code for enzymes that are involved in the production of polybydroxybutyrate (PHB) pathway using acetyl-CoA as substrate (83). When these three gene products were targeted to the chloroplasts by fusing with the chloroplast transit peptide in *Arabidopsis*, PHB accumulation was found to be 14% of dry mass (84). Another co-polymer polyhydroxybutyrate-co-hydroxyvalerate (PHBV), has better properties than PHB and has a similar biosynthetic pathway except for the initial step. The initial step involved in the biosynthetic pathway of PHBV is the condensation between the two substrates, acetyl-CoA and propionyl-CoA. In plants propionyl-CoA could be obtained from threonine by introducing a gene encoding threonine deaminase which converts threonine to 2-ketobutyrate, and further to propionyl-CoA by the pyruvate dehydrogenase complex present in the plastids. Introduction of four distinct genes, *ilvA* (*E. coli*), *Bktb, PhbB, PhbC* (*R. eutropha*), with chloroplast targeting sequence in *Arabidopsis* and *Brassica* resulted in the formation of PHBV (85). Although the level of expression of PHBV was too low to be commercially exploited, there is scope for improving the expression levels by adopting different strategies. Another excellent example is the production of medium chain length polyhydroxyalkanoates (MCL-PHA) in *Arabidopsis* by introducing *PhaC1* synthase from *Pseudomonas aeruginosa* (86). These are examples of another emerging field of biotechnology referred to as "Metanomics" and "Metabolic Engineering" in which the metabolic pathways of plants are directed for producing useful compounds.

Industrial Proteins and Enzymes

Production of recombinant proteins and enzymes for industrial purposes generally do not require high levels of purification. Therefore, plants are an ideal host system for producing important industrial proteins and enzymes economically, compared with fermentation systems. Several proteins and industrial enzymes have been produced in plants (Table 4). Avidin and β-glucouronidase (GUS) were the first successful commercial recombinant products produced in corn and are marketed by Sigma as research reagents (Avidin Cat No: A8706; GUS Cat No. G2035). The level of expression in the seeds was found to be 2-3% for avidin and 0.4-0.7% for GUS of total soluble protein (97). Avidin from transgenic plants was at least fifty times more cost effective than that extracted from the eggs that are the traditional source of avidin (98). Commercialization of these two recombinant proteins provides evidence that other proteins and macromolecules can be effectively produced for industrial application. Expression of a thermostable 1,4-β-D-glucanase enzyme that has high demand for several industrial applications such as ethanol production, textiles and detergents (97) was achieved in transgenic *Arabidopsis* (98). In addition to industrial application, an excellent example of producing a nutritional supplement is the use of phytase in poultry feed. Currently phytase as a supplement to animal feed is produced through a relatively expensive fermentation system. Production of phytase in transgenic alfalfa (89),

Table 4. Industrial enzymes and proteins produced in different plant host systems

Industrial enzymes	Potential use	Host	Reference
α-amylase	Industry	Tobacco	87, 88
Phytase	Industry	Alfalfa, Tobacco	89-91
Cellulase	Industry	Alfalfa, Potato, Tobacco	92
Manganese peroxidase	Industry	Alfalfa, Tobacco	88
β(1,4) xylanase	Industry	Tobacco, Canola	93, 94
β(1,3-1.4)glucanase	Industry	Tobacco, Barley	93, 95
Avidin	Research Reagent	Maize	57
Glucuronidase	Research Reagent	Maize	96

tobacco (91) and soybean seeds has demonstrated the potential of plant-produced phytase for use in poultry and animal feed (99). The enzyme phytase converts phytate (stored phosphorus) to myo-inositol and inorganic phosphate. Many monogastric animals cannot use phytate and thus require inorganic phosphorus as a supplement in animal feed. As an alternative to phytase from fermentation systems, the milled seeds of transgenic crops expressing phytase could compensate for low levels of dietary phosphorus (91). Manganese-dependent lignin peroxidase, a potentially important industrial enzyme for the paper industry and other industrial sectors, has been expressed in transgenic tobacco and alfalfa plants (88). Another innovative approach is to produce industrial enzymes in non-conventional plants, such as poplar, a fast growing forest tree adapted to various agro-climatic conditions. This could offer poplars as a high-value alternative crop that would benefit farmers and various industries. The above and other examples indicate that plants can serve to be a good source in the production of industrial enzymes on an economically commercial scale.

Future Prospects

Current progress in the use of transgenic plants for producing recombinant proteins for therapeutics, vaccines, and industrial applications indicates that molecular farming is an exciting and commercially promising area. However, expression levels sometimes limit economic feasibility. Expression levels can be optimized through the use of the correct crop species, the use of strong tissue-specific promoter sequences, changing the codon sequence to that of plant-preferred codons, targeting to organelles such as chloroplasts, or expressing the genes in the chloroplasts. Changing the gene sequences to plant-preferred codons has increased the level of expression of transgenes (100). Chloroplast transformation with transgenes has resulted in a high level of expression due to the multiple copies of chloroplast within each cell (101, 102, 69).

Plants expressing uniform expression levels of the desired antigen have to be identified in order to administer the correct dosage of vaccine. The choice of plant has to be critically evaluated for producing edible vaccines. As potatoes are consumed after cooking, this may result in the denaturation of the vaccine and probably will not trigger the immune response when compared to the native form. Therefore, plants such as banana, tomato and other plant fruits that are eaten raw will be ideal candidates for producing edible vaccines. Use of ethylene-inducible genes linked to fruit-ripening would allow inducible expression of the antigen, and since ripening affects the color of many fruits it may be possible to develop a correlation between the color of the fruit and the level of antigen, to ensure an adequate dosage of the vaccine. Due to the adjuvant property of LT-B and CT-B to enhance the immune response, a strategy to co-express LT-B or CT-B antigens along with target antigens will further reduce the amount of antigen needed for successful immunization. Production for edible vaccines would allow mass vaccination at a reduced cost more effectively and in a safe manner, producing tremendous benefit in the developing world.

Initial attempts at producing several therapeutic proteins indicate the requirements of a high level of expression, better storage and shelf life of the product, and a quick and easy method of purification of the recombinant proteins. Targeting the recombinant proteins to seeds is a promising feature to increase the expression levels in a controlled manner and provides better shelf life. Use of chemical-inducible promoter sequences may allow the expression of the target protein in high amounts within a short span of time without affecting the growth

Table 5. Some of the major industry players in producing high-value products in plants through Molecular Farming

Company	Web Address	Product / Area of Research
Astra Zeneca	www.astrazeneca.com	Enzymes
Aventis	www.aventis.com	Biopharmaceuticals
BASF AG	www.basf.de	Phytaseed Canola
Crop Tech Corp	www.croptech.com	Biopharmaceuticals
Dow AgroSciences	www.dowagro.com	Vaccines, Biopharmaceuticals
Dupont	www.dupont.com	Bio-polymers, Enzymes
FibroGen	www.fibrogen.com	Biopharmaceuticals
EPIcyte Pharmaceuticals	www.epicyte.com	Biopharmaceuticals
Genencor International.	www.genencor.com	Gene discovery, Functional genomics, Enzymes
Gist Brocades	www.gist-brocades.nl	Enzymes
Meristem Therapeutics	www.meristem-therapeutics.com	Biopharmaceuticals
Monsanto	www.monsanto.com	Biopharmaceuticals, Bio-polymers
NeoRx Corporation	www.neorx.com	Biopharmaceutical
Novartis	www.novartis.com	Enzymes
Pioneer Hy-Bred International Inc.	www.pioneer.com	Biopolymers, Enzymes
Planet Biotechnology Inc.	www.pano.com	Antibodies
Prodigene	www.prodigene.com	Vaccines, Biopharmaceuticals, Enzymes
SemBioSys Genetics	www.sembiosys.ca	Olesin technology
Syngenta	www.syngenta.com	Enzymes

of the plants. Targeting the proteins to oil bodies offers an easy and efficient method of downstream processing in purification of recombinant proteins. Secretion of recombinant proteins from roots and leaves will have to be evaluated for cost effectiveness of production and product stability.

Choice of the host plant has to be evaluated for production of each recombinant protein. As stated above, expression levels and site of production of the recombinant product will have a major influence on commercial potential and vary with plant species. To reduce product degradation, production of recombinant proteins in leaves requires processing soon after harvest (103), whereas targeting to seeds will provide better stability and long shelf life. Due to the requirement of many recombinant proteins for various applications, it is possible to choose several crops including forest trees for producing certain recombinant proteins and make them into a profitable crop. Production of industrial enzymes in tree species, where high biomass is available, should be evaluated for commercial exploitation. *Populus* could be an ideal host for producing high-value recombinant proteins for industrial applications. A program was recently initiated at the University of Minnesota-Duluth to research the role of transgenic *Populus* in producing valuable proteins for industrial commercial application.

The commercial success of molecular farming will depend on several other factors, including market demand, cost of cultivation of crops, purification costs, and public acceptance. These factors have to be considered in order for plant production to compete with existing and alternative methods. The relationship of molecular farming with industries, products, technologies and business factors has been schematically represented in Fig. 1. The involvement of several biotechnology companies in molecular farming is promising for the potential of this technology in meeting the demands for various applications in vaccines, therapeutic proteins, industrial enzymes, and bio-polymers. Some of the leading companies actively involved in producing recombinant proteins in plants for industrial and health care applications are listed in Table 5. Several startup biotechnology companies are also focusing on this area. The agricultural biotechnology industry is undergoing tremendous change in the past few years, primarily through acquisition, mergers, buy-outs and joint venture. These actions will result in a few significant vertically and horizontally integrated conglomerates that will eventually survive and control the leadership in the area of biotechnology and will have a major effect on molecular farming. These companies will hold a large number of patents on technologies that will be required for delivering

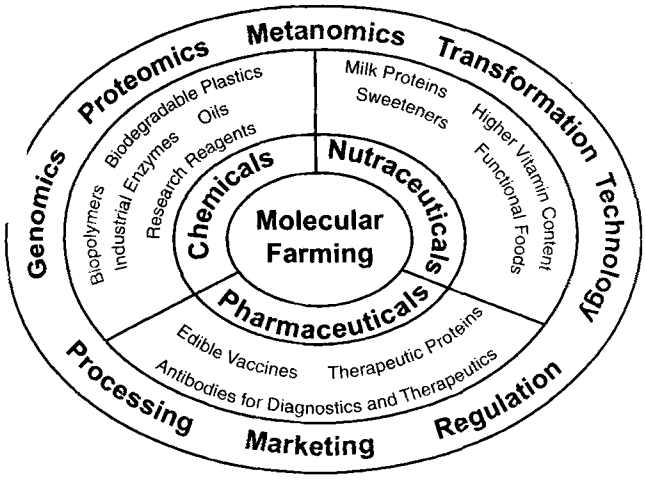

Fig. 1. The relationship of molecular farming with industries, products, technologies, and business factors. The inner circle represents major areas of molecular farming by industry; the middle circle represents potential products through molecular farming and the outer circle represents technologies and business factors that could enable or improve molecular farming.

any successful product. A few examples of such nucleus companies are shown in Fig. 2. It is yet to be determined how the developing nations will honour such nucleated companies and their intellectual property.

The emerging fields of genomics, proteomics, and metanomics will allow us to understand the function of the several genes that are involved in metabolic disorders

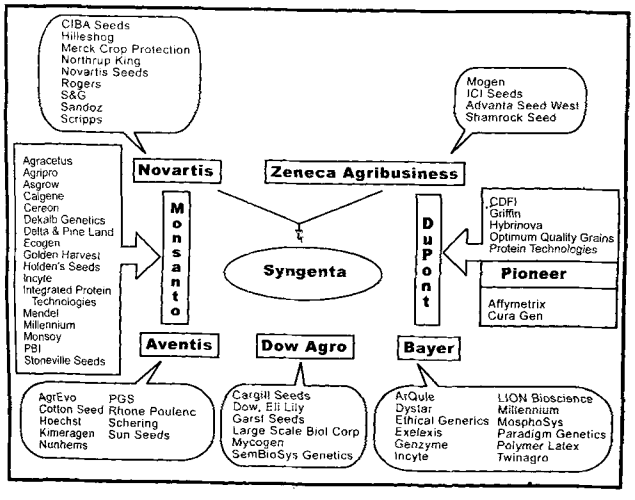

Fig. 2. Growth of biotechnology companies through acquisition, buyouts, mergers, and joint ventures resulting in gain of intellectual property rights and technology capability leading to success in R & D and commercialization.

and pathways and will provide insight to provide tools, including molecular farming, to cure several disorders. Further, technological advances in instrumentation have been focused on high-throughput analysis that will help in identifying the functions of genes very rapidly. Use of microarray technology, high-throughput analysis and bioinformatics have great potential in identifying high-value molecules. Molecular farming is a young and highly competitive field that is growing rapidly to fulfill the high demand for many recombinant proteins in a safe and cost effective manner.

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References

1 **Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL & Woo SC,** *Proc Natl Acad Sci,* USA, **80** (1983) 4803.

2 **Zambryski P, Joos H, Genetello C, Leemans J, Van Montagu M & Schell J,** *EMBO J,* **2** (1983) 2143.

3 **Persidis A,** *Nat Biotechnol,* **17** (1999) 612.

4 **Olins PO & Lee SC,** *Curr Opin Biotechnol,* **4** (1993) 520.

5 **Benatti L, Scacheri E, Bishop DH & Sarmientos P,** *Gene,* **30** (1991) 255.

6 **Denman J, Hayes M, O'Day C, Edmunds T, Bartlett C, Hirani S, Ebert KM, Gordon K & McPherson JM,** *Biotechnology,* **9** (1991) 839.

7 **Buhler TA, Bruyere T, Went DF, Stranzinger G & Burki K,** *Biotechnology,* **8** (1990) 140.

8 **Wall RJ, Pursel VG, Shamay A, McKnight RA, Pittius CW & Hennighausen L,** *Proc Natl Acad Sci,* USA, **88** (1991) 1696.

9 **Sharma AK, Mohanty A, Singh Y & Tyagi AK,** *Curr Sci,* **77** (1999) 524.

10 **Richter L & Kipp PB,** *Curr Top Microbiol Immunol,* **240** (1999) 159.

11 **Curtiss R & Cardineau G,** *European patent application,* (1989)

12 **Haq TA, Mason HS, Clements JD & Arntzen CJ,** *Science,* **268 [5211]** (1995) 714.

13 **Mason HS, Haq TA, Tichter L & Arntzen CJ,** *Plant Physiol,* **111** (1996) 40.

14 **Mason HS, Haq TA, Clements JD & Arntzen CJ,** *Vaccine,* **16** (1998) 1336.

15 **Arakawa T, Yu J, Chong DK, Hough J, Engen PC & Langridge WH**, *Nat Biotechnol,* **16** (1998) 934.

16 **Dickinson BL & Clements JD,** In *Mucosal vaccines* (H Kiyono, PL Ogra, JR Mcghee, Editors) Academic Press, San Diego (1996) p 73.

17 **Arakawa T, Yu J & Langridge WHR**, *Adv Exp Med Biol,* **464** (1999) 161.

18 **McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, Koprowski H & Michaels FH,** *Biotechnology,* **13** (1995) 1484.

19 **Modelska A, Dietzschold B, Sleysh N, Fu ZF, Steplewski K, Hooper DC, Koprowski H & Yusibov V,** *Proc Natl Acad Sci,* USA. **95** (1998) 2481.

20 **Dalsgaard K, Uttenthal A, Jones TD, Xu F, Merryweather A, Hamilton WD, Langeveld JP, Boshuizen RS, Kamstrup S, Lomonossoff GP, Porta C, Vela C, Casal JI, Meloen RH & Rodgers PB,** *Nat Biotechnol,* **15** (1997) 248.

21 **Tuboly T, Yu W, Bailey A, Degrandis S, Du S, Erickson L & Nagy E,** *Vaccine,* **18** (2000) 2023.

22 **Arakawa T, Chong DK, Merritt JL & Langridge WH,** *Transgenic Res,* **6** (1997) 403.

23 **Mason HS, Lam DM & Arntzen CJ,** *Proc Natl Acad Sci,* USA, **89** (1992) 11745.

24 **Ehsani P, Khabiri A & Domansky NN,** *Gene,* **190** (1997) 107

25 **Tackaberry ES, Dudani AK, Prior F, Tocchi M, Sardana R, Altosaar I & Ganz PR,** *Vaccine,* **17** (1999) 3020.

26 **Mason HS, Ball JM, Shi JJ, Jiang X, Estes MK & Arntzen CJ,** *Proc Natl Acad Sci,* USA, **93** (1996) 5335.

27 **Usha R, Rohll JB, Spall VE, Shanks M, Maule AJ, Johnson JE & Lomonossoff GP,** Virology, **197** (1993) 366.

28 **Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V & Grill LK,** *Biotechnology,* **13** (1995) 53.

29 **McLain L, Durrani Z, Wisniewski LA, Porta C, Lomonossoff GP & Dimmock NJ,** *Vaccine,* **14** (1996) 799.

30 **Yusibov V, Modelska A, Steplewski K, Agadjanyan M, Weiner D, Hooper DC & Koprowski H** *Proc Natl Acad Sci,* USA, **94** (1997) 5784.

31 **Beachy RN, Fitchen JH & Hein MB,** In *Engineering plants for commercial products and applications* (GB Collins, RJ Sheperd, Editors) New York Acad of Sci, NY (1996) p 43.

32 **Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM & Arntzen CJ,** *Nat Med,* **4** (1998) 607.

33 **Hood EE & Jilka JM,** *Curr Opin Biotechnol,* **10** (1999) 382.

34 **Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM & Arntzen CJ,** *J Infect Dis,* **182** (2000) 302.

35 **Ma SW, Zhao DL, Yin ZQ, Mukherjee R, Singh B, Qin HY, Stiller CR & Jevnikar AM,** *Nat Med,* **3** (1997) 793.

36 **Cramer CL, Boothe JG & Oishi KK,** *Curr Top Microbiol Immunol,* **240** (1999) 95.

37 **Zeitlin L, Olmsted SS, Moench TR, Co MS, Martinell BJ, Paradkar VM, Russell DR, Queen C, Cone RA & Whaley KJ,** *Nat Biotechnol,* **16** (1998) 1361.

38 **Verch T, Yusibov V & Koprowski H,** *J Immunol Methods,* **220** (1998) 69.

39 **Ma JK, Hikmat BY, Wycoff K, Vine ND, Chargelegue D, Yu L, Hein MB & Lehner T,** *Nat Med,* **4** (1998) 601.

40 **McCormick AA, Kumagai MH, Hanley K, Turpen TH, Hakim I, Grill LK, Tuse D, Levy S & Levy R,** *Proc Natl Acad Sci,* USA, **96** (1999) 703.

41 **Stoger E, Vaquero C, Torres E, Sack M, Nicholson L, Drossard J, Williams S, Keen D, Perrin Y, Christou P & Fischer R,** *Plant Mol Biol,* **42** (2000) 583.

42 **De Neve M, De Loose M, Jacobs A, Van Houdt H, Kaluza B, Weidle U, Van Montagu M & Depicker A,** *Transgenic Res,* **2** (1993) 227.

43 **Longstaff M, Newell CA, Boonstra B, Strachan G, Learmonth D, Harris WJ, PorterAJ & Hamilton WD,** *Biochim Biophys Acta,* **1381** (1998) 147.

44 **Baum TJ, Hiatt A, Parrott WA, Pratt LH & Hussey RS,** *Plant Microbe Interact,* **9** (1996) 382.

45 **Hiatt A, Cafferkey R & Bowdish K,** *Nature,* **342** (1989) 76.

46 **Voss A, Niersbach M, Hain R, HirschHJ, Liao YC, Kreuzaler F & Fischer R,** *Mol Breed,* **1** (1995) 39.

47 **De Wilde C, De Neve M, De Rycke R, Bruyns AM, De Jaeger G, Van Montagu M, Depicker A & Engler G,** *Plant Sci,* **114** (1996) 231.

48 **De Neve M, De Loose M, Jacobs A, Van Houdt H, Kaluza B, Weidle U, Van Montagu M & Depicker A,** *Transgenic Res,* **2** (1993) 227.

49 **Tavladoraki P, Benvenuto E, Trinca S, De Martins D & Galeffi P,** *Nature,* **366** (1993) 469.

50 **Owen M, Gandecha A, Cockburn B & Whitelam G,** *Biotechnology,* **10** (1992) 790.

51 **Artsaenko O, Peisker M, Zur Nieden U, Fiedler U, Weiler EW, Muntz K & Conrad U,** *Plant J,* **8** (1995) 745.

52 **Ma JK, Hiatt M, Vine ND, Wang F, Stabila P, Van Dolleweerd C, Mostov K & Lehner T,** *Science,* **268** (1995) 716.

53 **Potera C,** *Gen Eng News,* Feb (1999) 20.

54 **Cramer CL, Boothe JG & Oishi KK,** *Curr Top Microbiol Immunol,* **240** (1999) 95.

55 **Kusnadi AR, Nikolov, ZL & Howard JA,** *Biotech & Bioeng,* **56** (1997) 473.

56 **Oakes JV, Shewmaker CK & Stalker DM,** *Biotechnology,* **9** (1991) 982.

57 **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 & Howard JA,** *Mol Breed,* **3** (1997) 291.

58 **Vandekerckhove J, Damme JV, Lijsebettens MV, Botterman J, Block MD, Vandewiele M, Clercq AD, Leemans J, Montagu MV & Krebbers E,** *Biotechnology,* **13** (1989) 72.

59 **Matsumoto S, Ikura K, Ueda M, & Sasaki R,** *Plant Mol Biol,* **27** (1995) 1163.

60 **Paramenter DL, Boothe JG, Van Rooijen GJH, Yeung EC & Moloney MM,** *Plant Mol Biol,* **29** (1995) 1167.

61 **Fox S,** *Gen Eng News,* **20** (2000) 38.

62 **Dieryck W, Pagnier J, Poyart C, Marden MC, Gruber V, Bournat P, Baudino S & Merot B,** *Nature,* **386 (6620)** (1997) 29.

63 **Sijmons PC, Dekker BM, Schrammeijer B, Verwoerd TC, van den Elzen PJ & Hoekema A,** *Biotechnology,* **8** (1990) 217.

64 **Cramer CL, Weissenborn DL, Oishi KK, Grabau EA, Bennett S, Ponce E, Grabowski GA, & Radin DN,** In *Engineering plants for commercial products and applications* (GB Collins, RJ Shepherd, Editors,) New York Acad of Sci, New York (1996) p 62.

65 **Cramer CL, Weissenborn DL, Oishi KK & Radin DN,** In *Transgenic plants: A production system for industrial and pharmaceutical proteins* (MRL Owen, J Pen, Editors) Wiley, Chichester (1996) p 299.

66 **Zhu Z, Hughes KW, Huang L, Sun B, Lui C, Li Y, Hou Y & Li X,** *Plant Cell Tiss Org Cult,* **36** (1994) 197.

67 **Higo K, Saito Y & Higo H,** *Biosci Biotechnol Biochem,* **57** (1993) 1477.

68 **Bosch D, Smal J & Krebber E,** *Transgenic Res,* **3** (1994) 304.

69 **Staub JM, Garcia B, Graves J, Hajdukiewicz PT, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye G & Russell DA,** *Nat Biotechnol,* **18** (2000) 333

70 **Ofoghi H, Moazami N, Domonsky NN & Ivanov I,** *Biotech Letters* **22** (2000) 611.

71 **Eldelbaum O, Stein D, Holland N, Gafni Y, Livneh O, Novick D, Rubinstein M & Sela I,** *J Interferon Res,* **12** (1992) 449.

72 **Goddiijn OJM & Pen J,** *Trends Biotechnol,* **13** (1995) 379.

73 **Halling-Sørensen B, Nielsen SN, Lanzky PF, Ingerslev F, Holten Lützhøft HC & Jørgensen SE,** *Chemoshphere,* **36** (1998) 357.

74 **Kumagai MH, Turpen TH, Weinzettl N, Della-Cioppa G, Turpen AM, Donson J, Hilf ME, Grantham GL, Dawson WO, Chow TP, Piatak Jr M, & Grill LK,** *Proc Natl Acad Sci,* USA, **90** (1993) 427.

75 **Hamamoto H, Sugiyama Y, Nakagawa N, Hashida E, Matsunaga Y, Takemoto S, Watanabe Y & Okada Y,** *Biotechnology,* **11** (1993) 930.

76 **Cramer CL & Weissenborn DL,** *US Patent 5,670,349* (1997)

77 **Van Rooijen GJ & Moloney MM,** *Biotechnology,* **13** (1995) 72.

78 **Boothe JG, Saponha JA & Parmenter DL,** *Drug Dev Res,* **42** (1997) 172.

79 **Gleba D, Borisjuk NV, Borisjuk LG, Kneer R, Poulev A, Skarzhinskaya M, Dushenkov S, Logendra S, Gleba YY & Raskin I,** *Proc Natl Acad Sci,* USA, **96** (1999) 5973.

80 **Komarnytsky S, Borisjuk NV, Borisjuk LG, Alam MZ & Raskin I,** *Plant Physiol,* **124** (2000) 927.

81 **Guda C, Lee SB & Daniell,** *Plant Cell Rep,* **19** (2000) 257.

82 **Hanley Z, Siabas T & Elborough KM,** *Trends Plant Sci,* **5** (2000) 45.

83 **Poirier Y, Dennis DE, Klomparens K, & Somerville C,** *Science,* **256** (1992) 520.

84 **Nawrath C, Poirier Y & Somerville C,** *Proc Natl Acad Sci,* USA, **91**(1994) 12760.

85 **Slater S, Mitsky TA, Houmiel KL, Hao M, Reiser SE, Taylor NB, Tran M, Valentin HE, Rodriguez DJ, Stone DA, Padgette SR, Kishore G & Gruys KJ,** *Nat Biotechnol,* **17** (1999) 1011.

86 **Mittendorf V, Robertson EJ, Leech RM, Kruger N, Steinbuchel A & Poirier Y,** *Proc Natl Acad Sci,* USA, **95** (1998) 13397.

87 **Pen J, Molendijk L, Quax WJ, Sijmons PC, van Ooyen AJ, van den Elzen PJ, Rietveld K & Hoekema A,** *Biotechnology,* **10** (1992) 292.

88 **Austin-Phillips S, Bingham ET, Mathews DE, Shahan MN, Will J & Burgess RR,** *Euphytica,* **85** (1995) 381.

89 **Austin-Phillips S , Koegel RG, Straub RJ & Cook M,** *US Patent No: US5900525* (1999).

90 **Verwoerd TC, van Paridon PA, van Ooyen AJ, van Lent JW, Hoekema A & Pen J,** *Plant Physiol,* **109** (1995) 1199.

91 **Pen J, Verwoerd TC, van Paridon PA, Beudeker RF, van den Elzen PJM, Geerse K, van der Klis JD, Versteegh HAJ, van Ooyer AJJ, & Hoekema A,** *Biotechnology,* **11** (1993) 811.

92 **Ziegelhoffer T, Will J, & Austin-Phillips S,** *Mol Breed,* **5** (1999) 309.

93 **Herbers K, Flint HJ & Sonnewald U,** *Mol Breed,* **2** (1996) 81.

94 **Liu J, Selinger B, Cheng D, Merrit J & Langridge W,** *Mol Breed,* **3** (1997) 463.

95 **Jensen LG, Olsen O, Kops O, Wolf N, Thomsen KK & von Wettstein D,** *Proc Natl Acad Sci,* USA, **93** (1996) 3487.

96 **Witcher DR, Hood EE, Peterson D, Bailey M, Bond D, Kusnadi A, Evangelista R, Nikolov Z, Wooge C, Mehigh R, Kappel W, Register J & Howard JA,** *Mol Breed,* **4** (1998) 301.

97 **Hood EE, Kusnadi A, Nikolov Z & Howard J,** *Adv Exp Med Biol,* **464** (1999) 127.

98 **Ziegler MT, Thomas SR & Danna KJ,** *Mol Breed,* **6** (2000) 37.

99 **Denbow DM, Grabau EA, Lacy GH, Kornegay ET, Russel DR & Umbeck PF,** *Poultry Sci,* **77** (1998) 878.

100 **Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, & Sen SK,** *Proc Natl Acad Sci,* USA, **94** (1997) 2111.

101 **McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, & Maliga P,** *Biotechnology,* **13** (1995) 362.

102 **Daniell H, Datta R, Varma S, Gray S, & Lee SB,** *Nat Biotechnol,* **16** (1998) 345.

103 **Austin-Phillips S, Bingham ET, Koegel RG, Mathews DE, Shahan MN, Straub RJ & Burgess RR,** *Ann NY Acad Sci,* **721** (1994) 235.