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Pharming: Nutraceuticals and Pharmaceuticals, Opportunities And Challenges

Innovation is essential for sustaining and enhancing agricultural productivity. Agricultural innovation has always involved new, science-based products and processes that have contributed reliable methods for increasing productivity and environmental sustainability. The set of tools provided through biotechnology has introduced a new dimension to such innovation. Agricultural biotechnology offers efficient and cost-effective means to produce a diverse array of novel, value-added products and tools. It has the potential to increase food, feed and fiber production, reduce the dependency of agriculture on chemicals, alleviate biotic and abiotic stress and lower the cost of raw materials, all in an environmentally sustainable manner. Despite some vocal assertions to the contrary, in essence, commercialization of the first generation of products of recombinant DNA technology is just another facet in a long history of human intervention in nature for agricultural and food production purposes. And, as such, the same parameters of risk-based assessment should apply. Commercialization of products must be undertaken within a regulatory framework that insures adequate protection of the consumer and the environment while not stymieing innovation. We have over fifteen years of experience in using robust analysis in determining the optimum oversight of the first generation of biotechnology products. New products and new approaches on the horizon require a reassessment of appropriate criteria to manage risk while insuring that the development of innovative technologies and processes is encouraged to provide value-added commodities for the consumer.

The biotech industry will be a key player in this new market for value added traits and will be used to identify and isolate valuable genes and metabolites, with some of the later compounds being produced in mass quantities for niche market. This paper will focus on two value-added niche markets at the interface of agriculture and medicine; 1. The field broadly defined as nutraceuticals or “Functional Foods” 2. Plants as bioreactors for the production of valuable proteins and compounds. The latter system is broadly termed Plant Molecular Farming.

Possible molecular farming products include:	
1) Primary Products	2) Derived Products
Monoclonal Antibodies, Immunoglobulin (Ig) fragments-Fabs, scFv (Passive immunity)	Bio-plastics - PHAs (polyhydroxyalkanoates, chemically related to polyesters).
Antigens (vaccines) (Active immunity)	Nutraceuticals: Macro: Carbohydrates, Fats Micro: Vitamins, co-factors, minerals, Phytochemicals: carotenoids (beta-carotene, lycopene, lutein), flavonoids (quercetin, kaempferol, allicin), isoflavones (phytoestrogens - genistein and daidzein), isothiocyanates (glucosinolates, indoles, and sulforaphane), phenolics (reservatrol, catechin), tannins
Structural: proteins, peptides, hormones, (interleukins, interferons and colony stimulating factors)	
Enzymes: food, feed, industrial, therapeutic, diagnostic, cosmetic	

Anti-disease therapeutics: Factor VII,	Non-nutrient phytochemicals: fragrances, flavors
Enzyme inhibitors	Fibres: polymers, lignins

1. Nutraceuticals

Functional foods are defined as any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains (Thomas, 1994). The term, nutraceutical, was coined by the Foundation for Innovation in Medicine in 1991 and is defined as "any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease. Scientific evidence is accumulating to support the role of phytochemicals and functional foods in the prevention and treatment of disease. Epidemiological research has shown a positive association between dietary intake of food components. One estimate states that foods that are used for nutraceutical purposes made up 10 percent of the \$503 billion total U.S. retail food market.

Developing plants with improved quality traits involves overcoming a variety of technical challenges inherent to metabolic engineering programs. Both traditional plant breeding and biotechnology techniques are needed to produce plants carrying the desired quality traits. Continuing improvements in molecular and genomic technologies are contributing to the acceleration of product development.

Plants are remarkable in their capacity to synthesize a variety of organic substances, such as vitamins, sugars, starches and amino acids. As many as 80,000 to 100,000 different substances significant to human health are synthesized in plants. From a health perspective these can broadly be divided into three main categories macronutrients (proteins, carbohydrates, lipids [oils], and fiber), micronutrients (vitamins, minerals, phytochemicals) and anti-nutrients (allergens, toxins and substances such as phytate that limit bioavailability of nutrients). There is of course some overlap between these categories in terms of the balance of benefit to detriment of especially the latter two categories in the area of optimum intake of micronutrients for a given individuals needs and requirements. This is now being quantified by researchers in the field of personalized nutrition termed nutrigenomics.

Phytochemicals and functional food components have been associated with the prevention and/or treatment of at least four of the leading causes of death in the USA: cancer, diabetes, cardiovascular disease, and hypertension. The U.S. National Cancer Institute estimates that one in three cancer deaths are diet related and that eight of ten cancers have a nutrition/diet component (Steinmetz and Potter, 1996). Other nutrient-related correlations link dietary fat and fiber to prevention of colon cancer, folate to the prevention of neural tube defects, calcium to the prevention of osteoporosis, psyllium to the lowering of blood lipid levels, and antioxidant nutrients to scavenge reactive oxidant species and protect against oxidative damage of cells which may lead to chronic disease, to list just a few (Goldberg, 1994). One group of phytochemicals, the isothiocyanates (glucosinolates, indoles, and sulforaphane) are found in vegetables such as broccoli and target drug-metabolizing enzymes in humans, leading to decreased carcinogen-DNA interactions and increased carcinogen detoxification (Gerhauser et al., 1997).

Intricate nutraceutical modifications will require more sophisticated plant manipulation than the simple (relatively speaking) addition or removal of one or two genes. In many instances complex trait modification will require engineering of metabolic pathways. Significant progress has been made in recent years in the molecular dissection of many plant pathways and in the use of cloned genes to engineer plant metabolism. Although there have been numerous success stories, there also has been a number of studies that have yielded unanticipated results. Trait modifications with the additions of one or two genes produce targeted, predictable outcome. For metabolic pathway manipulations, however, such data underscore our incomplete understanding of plant metabolism. Regulatory oversight of engineered products has been designed to detect such unexpected outcomes in biotech crops and, as more metabolic modifications are made, new methods of analysis are being developed to address these issues.

Some examples of successful outcomes to date are briefly outlined here. Most plants have a poor balance of essential amino acids relative to the needs of animals and humans. The cereals (maize, wheat, rice etc.) tend to be low in lysine, whereas legumes (soybean, peas) are often low in the sulfur-rich amino acids, methionine and cysteine. Successful examples to date of modifying amino acid content include high-lysine maize (O'Quinn et al., 2000) and high-lysine canola and soybeans (Falco et al., 1995). Consumption of foods made from these crops potentially can help to prevent malnutrition in developing countries, especially among children, as well as improving the quality of animal feed. These proteins can be from other natural sources, or be completely synthetic. A natural approach was achieved using an interesting novel method of increasing essential amino acids. Maize does produce a methionine-rich protein delta-zein but at a low level. Lai and Messing (2002) switched a bait site for a protein that degrades the mRNA and maximized the production of the methionine-rich protein. This modification could potentially save animal farmers \$1 billion per year in synthetic methionine supplements to corn-based feed. An example of the synthetic approach is an 11 kDa synthetic protein, MB1, which was created to contain the maximum number of the essential amino acids methionine, threonine, lysine, and leucine in a stable, helical conformation. The structure was also designed to resist proteases to prevent degradation in planta. It has been used to improve the protein content of soybean and sweet potato among other crops.

Plants make both polymeric carbohydrates (e.g., starches and fructans), and individual sugars (e.g, sucrose and fructose). The polymeric carbohydrates fructans are an important ingredient in functional foods as they promote a healthy colon and help reduce the incidence of colon cancer. Dutch researchers succeeded in modifying sugar beet to produce fructans without adversely affecting growth or phenotype. An starch metabolic enzyme ADP glucose pyrophosphorylase under the control of a seed-specific promoter has been used to increase grain weight on average by 23% in wheat. Taking the same gene and this time placing it under the tuber-specific patatin promoter in potatoes, starch content was increased by over 30%. This has an added bonus as the higher starch content lowers moisture resulting in less fat absorption on frying as moisture lost is replaced by oil uptake. However, there are still problems with granule distribution.

The techniques of biotechnology are providing powerful tools for modifying the composition of oilseeds to improve their nutritional value and provide the functional properties required for various food oil applications. The technology also has the potential to be used to produce industrial oils and chemicals in genetically engineered crops. While many lipids have important

health implications, the long-chain polyunsaturated fatty acids (PUFAs) especially the omega-3 fatty acids found in fish, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are present in the retina of the eye and cerebral cortex of the brain are some of the most well documented from a clinical perspective. DHA is also the predominant structural fatty acid in the gray matter of the brain. It is believed that EPA and DHA play an important role in the regulation of inflammatory immune reactions and blood pressure, brain development in utero, and, in early postnatal life, the development of cognitive function. They also possess anti-cancer properties. Omega-3 fatty acids also appear to be beneficial in certain neuropsychiatric illnesses such as bipolar disorder, schizophrenia and depression (Stoll et al.1999). Current Western diets tend to be relatively high in n-6 fatty acids and relatively low in n-3 fatty acids. This is due to our high intake of vegetable oils, which are rich in n-6 fatty acids, and our low intake of oils and foods rich in n-3 fatty acids, such as fatty fish. Ursin (2000) has introduced a gene from a fungus succeeding in producing omega-3 in canola. In a clinical study designed to determine the relative efficacy of various fatty acids, Ursin observed that the resultant precursor was superior in producing EPA and DHA by a factor of 3.6.

For select mineral targets (iron, calcium, selenium, and iodine) and a limited number of vitamins (folate, vitamins E, B6, and A), the clinical and epidemiological evidence is clear that they play a significant role in maintenance of optimal health and are limiting in diets worldwide. In addition, there is a growing knowledgebase indicating that elevated intakes of specific vitamins and minerals (e.g. vitamins E and C, carotenoids, and selenium) may reduce the risk of diseases such as certain cancers, cardiovascular diseases, and chronic degenerative diseases associated with aging. An important route to supplying such nutraceuticals is direct modification of levels in food crops. The advent of genomics during the past few years has provided new routes for such work. Using nutritional genomics, Della Penna (1999) isolated a gene, which converts the lower activity precursors to the highest activity vitamin E compound, alpha-tocopherol. With this technology, the vitamin E content of Arabidopsis seed oil has been increased nearly 10-fold and progress has been made to move the technology to agricultural crops such as soybean, maize, and canola. This has also been done for folates in rice. Chen et al (2003) took advantage of the fact that vitamin C can be regenerated by the enzyme dehydroascorbate reductase (DHAR) by introducing the gene encoding DHAR from wheat into maize and succeeded in increasing the amount of vitamin C by up to 100-fold. Similarly, a team led by Ingo Potrykus engineered rice to produce pro-Vitamin A which is an essential micronutrient and widespread dietary deficiency of this vitamin in rice-eating Asian countries has tragic consequences. Improved vitamin A nutrition would alleviate serious health problems and, according to UNICEF, could also prevent up to two million infant deaths because vitamin A deficiency predisposes them to diseases such as measles.

Unlike vitamins and minerals, the primary evidence for the health-promoting roles of phytochemicals comes from epidemiological studies, and the exact chemical identity of many active compounds has yet to be determined. However, for select groups of phytochemicals, such as non-provitamin A carotenoids, glucosinolates, and phytoestrogens, the active compound or compounds have been identified and rigorously studied. Other targets include improved iron content, through the production of iron-rich storage protein ferritin, bio-available phosphorus released from phytate, and isoflavonoids. Some work being done in the area includes increase of the carotenoid lycopene in tomatoes by modifying enzymes involved in the ripening process.

Lycopene lowers oxidized low-density lipoprotein (LDL), mitigates epithelial cancers, and captures twice as many oxygen ions as beta-carotene. The polyphenolics resveratrol and catechin, which are found in a wide range of plant species, including grapevine and rhubarb, are powerful antioxidants, cancer chemopreventatives and platelet aggregation inhibitors responsible for the French wine paradox. They are being modified in a number of plant species (Hipskind and Paiva, 2000, Ebler, 2002). Genistein and daidzein are naturally occurring plant isoflavones that are being studied for their putative health benefits. They are found almost exclusively in soybeans and other leguminous plants. The reported health benefits include estrogenic and anticancer activity, assistance in the prevention of arterogenic oxidation of LDLs, and increasing bone mass. Jung et al (2000) have cloned and expressed the isoflavone synthase (IFS) gene in both monocot and dicot demonstrating that introduction of IFS alone could result in the novel production of isoflavones in non-legume plants. However, studies by Bao and Williamson (2001) demonstrate that maximized dietary intake is not always correlated with optimized dietary benefit. Quercetin is a flavonoid that has been demonstrated to work optimally at very low concentrations in protecting against cancerous cell proliferation and the actions of the carcinogen PhIP found in cooked meat. As the concentration was increased the effect was attenuated. It is possible that similar effects may be found for other phytochemicals. This also illustrates the importance of taking a cautious approach to any research to increase phytochemicals with putative beneficial effects under the premise of “more is better”.

For many other health-promoting phytochemicals, decisions will need to be made regarding the precise compound or compounds to target and which crops to modify so that the greatest nutritional impact and health benefits are achieved. However, a large body of credible scientific research is still needed to confirm the benefits of any particular food or component. For functional foods to deliver their potential public health benefits, consumers must have a clear understanding of, and a strong confidence level in, the scientific criteria that are used to document health effects and claims. Because these decisions will require an understanding of plant biochemistry, mammalian physiology, and food chemistry, strong interdisciplinary collaborations will be needed among plant scientists, nutritionists, and food scientists to ensure a safe and healthful food supply for this new century.

2. Pharmaceuticals

In addition to being a source of nutrition, plants have been a valuable wellspring of therapeutics for centuries. During the past decade, however, intensive research has focused on expanding this source through rDNA biotechnology and essentially using plants and animals as living factories for the commercial production of vaccines, therapeutics and other valuable products such as industrial enzymes and biosynthetic feedstocks.

Possibilities in the medical field include a wide variety of compounds, ranging from edible vaccine antigens against hepatitis B and Norwalk viruses (Arntzen, 1997; Dixon and Arntzen, 1997; Mason et al., 1996, 1998, Richter, 2000) and *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Brennan et al., 1999) to vaccines against cancer and diabetes (McCormick, 1999) enzymes (Hogue et al., 1990; Verwoerd et al., 1995), hormones (Leite et al., 2000; Staub et al., 2000), cytokines, interleukins (Magnuson et al., 1998), plasma proteins (Sijmons et al., 1990),

and human alpha-1-antitrypsin (Terashima et al., 1999). Thus, plant cells are capable of expressing a large variety of recombinant proteins and protein complexes. Therapeutics produced in this way are termed plant made pharmaceuticals (PMPs).

Two of the seminal papers that gave proof of the concept of the use of transgenic plants as pharmaceutical production systems appeared in 1995 (Haq et al, 1995, Ma et al, 1995, 1997, 1998). In 1998 the same two groups reported the results of successful human clinical trials with their transgenic plant-derived pharmaceuticals: an edible vaccine against *E. coli*-induced diarrhea and a secretory monoclonal antibody directed against *Streptococcus mutans*, for preventative immunotherapy to reduce incidence of dental caries. Haq et al. (1995) reported the expression in potato plants of a vaccine against *E. coli* enterotoxin (ETEC) that provided an immune response against the toxin in mice. Human clinical trials suggest that oral vaccination against either of the closely related enterotoxins of *Vibrio cholerae* and *E. coli* induces production of antibodies that can neutralize the respective toxins by preventing them from binding to gut cells. In the clinical trial, two groups of volunteers consumed either 50 g or 100 g of raw potato tubers expressing LT-B (equivalent to 0.5 mg or 1 mg LT-B per dose, respectively) and were compared with a third group that ate untransformed potato tubers. The first two groups developed specific anti-LT-B mucosal and systemic immune responses while the control group did not. These responses are comparable to those observed when humans are challenged with ETEC bacteria. The human clinical trials demonstrate that edible plant vaccines are immunogenic in humans, as was previously shown in mice (Tacket, 1998). Similar results were found for Norwalk virus oral vaccines in potatoes (Tacket, 2000). For developing countries, the intention is to deliver them in bananas or tomatoes (Arntzen, 2002).

Ma et al. (1995, 1998) showed that tobacco plants could express secretory antibodies or “plantibodies” against the cell surface adhesion protein of *S. mutans*. Used as a bactericidal mouthwash, the antibodies prevented bacterial colonization by the microorganism and development of dental caries for four months. A similar approach showed that soybean produced antibodies protected mice against infection by genital herpes (Zeitlin et al., 1998). Compared to antibodies produced in mammalian cell culture, the plantibodies had similar physical properties, remained stable in human reproductive fluids, and exhibited no differences in their affinity for binding and neutralizing herpes simplex virus. Hence, the difference in the glycosylation processes of plants and animals does not appear to affect the immune functions of the plant-derived antibodies which is of real interest to companies such as Centocor, Abginex and Immunex (now Amgen) who are looking to more effective, efficient and economic ways to produce humanized monoclonals.

The most effective method to rapidly produce large quantities of foreign protein in a plant is to use a viral vector in a manner similar to using a floppy disk to run a program in a computer. This is termed transient expression and optimizes the production capabilities of the plant while not imposing a genetic load that incorporating the gene in the plant’s genome may do. A system using tobacco mosaic virus (TMV) was developed to produce in tobacco plants a therapeutic vaccine against non-Hodgkin’s B-cell lymphoma in a mouse model (McCormick et al., 1999). Because of variability, effective therapy for non-Hodgkins B-cell lymphoma requires “personalized medicine” tailored to the genetic makeup of each patient’s tumor. Production of customized antibodies in sufficient quantities is difficult and those in conventional treatment also

tend to be expensive and unreliable, and have solubility and conformation problems. TMV DNA was modified with a tumor-specific sequence from the gene coding for the immunoglobulin cell surface marker in malignant B-cells of mice. Plants were then infected with the modified virus, resulting in expression of cancer-specific antibodies. Extracted B-cell proteins were used for vaccination of the mice. Eighty percent of the mice receiving the plant-derived vaccine survived the lymphoma, while all untreated mice died within three weeks of contracting the disease.

This approach has also been applied to diabetes. Blanas et al. (1996) describes the development of a potato-based insulin vaccine that is almost 100 times more powerful than the existing vaccine in preventing insulin-dependent diabetes mellitus (IDDM), an autoimmune disease in which insulin-producing cells of the pancreas are destroyed by the cytotoxic T lymphocytes, in a mouse model. Centocor is currently researching the production of and characteristics of two antibodies working with Monsanto and with Epicyte/Dow.

Apart from those specific applications where the plant system was optimum there are many other advantages to using plant production. Many new pharmaceuticals based on recombinant proteins will receive regulatory approval from the United States Food and Drug Administration (FDA) in the next few years. As these therapeutics make their way through clinical trials and evaluation, the pharmaceutical industry faces a production capacity challenge. Pharmaceutical discovery companies are exploring plant-based production to overcome capacity limitations, enable production of complex therapeutic proteins, and fully realize the commercial potential of their biopharmaceuticals. Bacterial protein synthesis machinery is incapable of reproducing the complexity of eukaryotic proteins, which often require extensive folding, post-translational modification and assembly. Many mammalian proteins expressed in bacteria are produced as insoluble misfolded aggregates or lack the features required for their activity. Plant cells carry out many of the post-translational modifications required for optimal biological activity of mammalian proteins. Four therapeutic molecules in the area of one of the systems described above, that is the production of monoclonal antibodies (Mabs), or fragments of immunoglobulins termed Fabs (fragments of antibodies), consume 75 percent of existing biologics capacity today and comprises fourteen percent of treatments in clinical trials. Producing 1000 kilograms of human antibodies in hamster cells costs about \$105 to \$175 per gram. Transgenic plants might be able to produce the same amount for \$15 to \$190 per gram. There are 100 Mabs in those trials covering cancer, infectious disease, cardiovascular disease, central nervous system disease, metabolic disorders, and immune/inflammatory disease, 20 to 30 approvals are expected by 2010 (Dry, 2002). Another “inert” protein of therapeutic and especially cosmetic interest is collagen. The company Fibrogen is establishing partnerships with seed companies for production systems.

As with any manufacturing process, production space is an issue in the area of pharmaceutical production. It takes five to seven years to build a biologics plant compared to one to three years for a conventional pharmaceutical plant. As a result, biotech firms contract out 90 percent of biologics production compared to 55 percent of conventional pharmaceuticals. Plants are among the most efficient bioreactors. The use of plants as bioreactors is of special interest as they allow production of recombinant proteins in large quantities and at relatively low costs. With sunlight and soil-based nutrients as inputs, plants produce large quantities of material. This production step replaces the traditional production step of fermentation. The benefits of plant-based production include: The ability to increase production at low cost by planting more acres, rather

than building fermentation capacity; lower capital and operating costs compared to traditional production facilities; simplified downstream processing compared to traditional technology. From a technology perspective plants have an advantage over microbes in that they can accommodate the production of a wide range of protein types, including some not possible by traditional production technology; in most instances they undertake correct post-translational modification; there is no propagation of human pathogens or other mammalian contaminants; no other mammalian contaminants are created de novo thus reducing screening costs for viruses and bacterial toxins; asepsis can begin at purification, not inoculation so there is less opportunities for contamination and scale-up utilizes the same technology used in agriculture to-day.

Plants are faster, cheaper, more convenient and more efficient than the principal eukaryotic production system namely Chinese Hamster Ovary (CHO) cells. Hundreds of acres of protein-containing seeds could inexpensively double the production of a CHO bioreactor factory. In addition, proteins can be expressed at the highest levels in the harvestable seed and plant-made proteins and enzymes formulated in seeds have been found to be extremely stable, reducing storage and shipping costs. Seeds are easier and more economical than whole plants to transport to a processing factory where proteins can be extracted and purified in preparation for processing or possible oral delivery of valuable proteins. Purification is easier because of the limited number of soluble proteins present in the maize seed. There is a low microbial load, which reduces contamination and low proteolytic activity, which enhances final target protein levels. Pharming may also enable research on drugs that cannot currently be produced. CropTech in Blacksburg, Va., is investigating a protein that seems to be a very effective anticancer agent. The problem is that this protein is difficult to produce in mammalian cell culture systems as it inhibits cell growth. This should not be a problem in plants.

Furthermore, production size is flexible and easily adjustable to the needs of changing markets. Making pharmaceuticals from plants is also a sustainable process, because the plants and crops used as raw materials are renewable. The system also has the potential to address problems associated with provision of vaccines to people in developing countries. Products from these alternative sources do not require a so-called “cold chain” for refrigerated transport and storage and those being developed for oral delivery also obviates the need for needles and aseptic conditions often a problem in those areas.

But nothing is ever without a downside. Molecular farming may fall short of its potential if there are unanticipated purification problems, or if therapeutic substances made in plants are not as effective as those made in mammalian cells, such as for example if the glycan in the plant-produced antibody looks different from what would naturally occur in a human, then there is a possibility the body might recognize it as foreign and eliminate it from the system, thereby influencing the efficacy of the drug. Where such differences in processing do represent a problem, it may be possible to engineer plants with altered protein maturation pathways (Cabanes-Macheteau, 1999). Even with more palatable alternatives to raw potatoes (e.g. bananas or tomatoes), accumulation levels may limit the practicality of edible vaccines. Two solutions to overcome this limitation are being explored. First, techniques to enhance antigen accumulation in plant tissues are being explored. These include, optimization of the coding sequence of bacterial or viral genes for expression as plant nuclear genes, and defining the subcellular compartment in which to accumulate the product for optimal quantity and quality. Several laboratories are also

developing alternative expression systems to improve accumulation. For example the expression in plastids is advocated by some (Daniell, et al., 2001; Ruf, et al., 2001) a concomitant advantage of which maybe containment as discussed below. Other systems involve plant viruses for expression of foreign genes (e.g. Nemchinov, et al., 2000) or coat-protein fusions (e.g. Modelska, et al., 1998) and even viral assisted expression in transgenic plants (Mor, et al., 2002).

In addition to technical pitfalls, the development of pharma plants could be hampered if cost savings are not realized as the bottom line on this is that other “omics”, economics. In addition it could be hampered by legal barriers or by marketing issues related to negative public perceptions that have surrounded the entire field of genetic engineering. There are, indeed, new challenges to oversight, risk assessment, and public perception. The most pressing concern is to insure adequate segregation from conventional foods to prevent unintentional commingling.

While such products will be controlled as medicines, however, unlike contained bioreactors there is potential for environmental and food safety mishaps. Especially in the case of active pharmaceutical ingredients (API), there is genuine concern of gene escape and the possibility of failure to segregate from the food supply. No matter how comprehensive the containment facilities and measures in place or how vigilant the oversight and monitoring procedures, the potential exists for unexpected events. Such as happened with field tests by the company ProdiGene. ProdiGene produces animal vaccines in maize. Federal officials are penalizing ProdiGene for two similar incidents involving its test plots of GM maize being raised under contract by local growers, one farm in Nebraska and another in Iowa. In the Nebraska case, officials realized that some 500,000 bushels of harvested soybeans were contaminated (adulterated in the parlance of the Food Drug and Cosmetics Act) with small amounts of GM maize, which had been grown during 2001 on the same plot, because the farmer did not weed "volunteer" tasseled plants from the field in which the soy was grown. That failure to remove the volunteer maize before it tasseled, and to remove the tasseled maize before the soybeans were harvested, are violations of the regulations of such test plots under the Plant Protection Act. In Iowa, federal officials required a local producer to destroy some 155 acres of maize because it could have been cross-pollinated by ProdiGene's engineered maize being raised in a nearby field. Without admitting to those violations, ProdiGene agreed to post a \$1 million bond and also to reimburse the USDA for the costs, which could be several million dollars, involved in disposing of the contaminated crops. These incidences underscores the fact that, while manufacture in maize permits an attractive production, storage and delivery system it raises the specter of skewed risk to benefit of growing such pharmaceutical maize in the midst of a region where the majority of farmland is used to produce commodity food and feed crops.

The current regulatory authority responsibility has been legislatively placed in the hands of four Federal agencies: USDA's Animal and Plant Health Inspection Service (APHIS), the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and the Occupational Safety and Health Administration (OSHA). APHIS is responsible for permits for growing PMPs during the R&D and production phase. Molecular farming crops will need perpetual permitting from APHIS. Permits for growing small acreages of these crops for development purposes are already being issued, and APHIS has published its mandates for ensuring maximum environmental protection (USDA-APHIS, 2002, 2003).

Currently, test plots for crops engineered to grow pharmaceuticals are regulated by the U.S. Department of Agriculture's (USDA) permit system. USDA regulations require developers to have clearly written procedures, to handle wastes properly, and to maintain production and control records. Plant made pharmaceuticals also come under the U.S. Food and Drug Administration's (FDA) purview. The FDA will monitor the manufacturing process as well as the purity and consistency of the products under its "good manufacturing practices" guidelines. Nevertheless, both USDA and FDA recognize that this is a new application of the technology and are in the process of coming up with new guidance specifically for pharmaceutical plants. FDA has domain over any products produced by PMP farming. This authority ensures integrity (purity, correct dosages), and safety of the product. They will have already ruled on safety and efficacy of the PMP product. Risk assessment testing will have to be conducted under Good Laboratory Practice (GLP) standards, similar to EPA pest regulations. FDA covers the entire manufacture of the pharmaceutical. FDA GMPs (Good Manufacturing Practices) ensure consistent manufacturing processes, product safety, purity, and potency. GMPs will essentially spread out from its historical application within the walls of the factory to the field. EPA would be initially involved if the plants contained pest-protection characters or herbicide-tolerance characters which would be deemed a new use pattern for a herbicide and thus would require a pesticide product label change.

In September 2002 a draft guidance document termed Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals was developed by a number of agencies including the U.S. Department of Health and Human Services, Food and Drug Administration (FDA), Center for Biologics Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDER), Center for Food Safety and Applied Nutrition (CFSAN), Center for Devices and Radiological Health (CDRH), Center for Veterinary Medicine (CVM, U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS), Center for Veterinary Biologics (CVB) and the Biotechnology Regulatory Services (BRS). This document is the result of a combined effort by the FDA and the USDA to provide guidance with regard to the use of bioengineered plants or plant materials to produce biological products, including intermediates, protein drugs, medical devices, new animal drugs, and veterinary biologics regulated by FDA or USDA (FDA/USDA, 2002). The document is directed at the issues unique to the use of bioengineered pharmaceutical plants as source material for the production of FDA and/or USDA regulated products.

The guidelines address, the host plant, gene source, containment, environmental impact harvesting etc. Consideration is taken of the plant species, the source of the desired regulated product and the potential for the plant to express an allergen or toxin. It also covers the method of plant propagation and the measures to ensure confinement. For a food crop species engineered to produce non-food material, it addresses the methods in place for the prevention of commingling with food or feed. As noted, such contamination would constitute food adulteration under the Food, Drug & Cosmetic Act.

When determining oversight review of host plants consideration is given to the growth habitat of the plant such as to whether the plant is an annual, perennial, or biennial; the timing of sexual maturity and duration of flowering; seed production and harvesting; the recognized practices for maintaining seed stock purity for the cultivar; the conditions of growth; timing of harvest;

method of harvesting and transporting, storage and sorting of harvested materials; levels of any toxins, anti-nutrients, and allergens known to be produced by the plant species and whether it is known to accumulate heavy metals.

The modifying gene may be transiently added to the plant or it may be stably inserted. General considerations for bioengineered source plants include characterization of the recombinant DNA constructs or viral vectors used to transfer genes, the origin and function of all component parts of the construct, including coding regions, antibiotic- or herbicide-resistance genes, origins of replication, promoters, and enhancers; any changes in codons in order to optimize codon usage in plants. For stable transformation systems with a gene from a pathogenic organism or nucleic acid sequences from a pathogen, the pathogen, the strain, and the gene(s) involved must be described. For transient transfection systems the virus-mediated transient transfection systems must be described. If the bioengineered pharmaceutical plant lines are derived through stable transformation both the phenotype and genotype must be demonstrated as stable. For plants that are infertile or for which it is difficult to produce seed (such as vegetatively propagated male-sterile potatoes), data must be provided to demonstrate that the trait is stably maintained and expressed during vegetative propagation over a number of cycles that is appropriate to the crop.

The tissue distribution of expression products is important. For all inserted coding regions, it must be demonstrated whether the protein is or is not produced as intended in the expected tissues consistent with the associated regulatory sequences driving its expression (e.g., if the gene is inducible, is the gene expressed in the expected tissues conditions). Quantitative data must be provided characterizing the distribution of the product in the major plant tissues (e.g., leaves, roots, stalks, seeds).

The growing and harvesting of the crop itself must comply with principles of confinement which essentially means keeping the crop and its products on the land where it was grown until removed for processing, with no inadvertent exposure to the public and minimal exposure of products to workers and the environment. Adequate analytical methods for detection of expression (i.e., protein) products must be demonstrated and all confinement systems and procedures must be based on sound scientific principles. Identity preservation within a closed loop system to prevent co-mingling of pharm crops with food crops will be a prime directive for industry as well as regulatory agencies. The misadventures over the co-mingling with food maize of the non-food Bt-maize hybrid known as StarLink, which was only registered for animal feed, will not be repeated. Precaution demands that Standard Operating Practices (SOPs) be implemented for a functional identity preservation system. Such a system ensures that the pharm crop is completely segregated from all other crops and that protocols are in place for production and handling of the crop. Achieving this goal is possible with implementation of chain-of-custody procedures that track the product through every stage of production and processing. A closed loop identity preservation system not only protects the quality and purity of the final protein product, but it complements confinement to ensure maximal environmental, worker, and food supply protection. Remedial plans must be in place that will trigger procedures to mitigate any potential effects if the confinement system does not achieve its desired results. Furthermore, confinement systems need to be modified as needed to improve performance and ensure adherence to the overriding confinement and identity preservation-closed loop principles.

Candidate plants for the production of PMPs include familiar crops like alfalfa, canola, maize, potato, rice, safflower, soybean, and tobacco. These food and non-food crops will be treated altogether differently than the biotechnology-derived crops designated for food purposes. Issues of safety and efficacy are evaluated when considering candidacy plants. Candidate pharmaceutical producing plants have been studied with respect to pollination, genetics, seed dormancy, and weediness potential. This information can be useful for addressing several concerns, including pollen movement and subsequent gene flow between conventionally bred and biotechnology-derived crops. A long history of cultivation shows that the candidate crops are the least likely to be invasive of “natural” ecosystems. This information will be used to ensure maximal isolation of the plants from food producing crops.

The infrastructure of regulation has been in place for nearly a decade, and it continues to evolve as experience with biotechnology-derived food crops grows. Risk management must necessarily focus on providing protection for human health (worker and consumer) and ecosystems. The Biotech Industry Organization (BIO) a biotech trade group in October 2002 adopted a policy including geographic restrictions which its members will be expected to follow. The policy will prevent the PMP maize from being grown in an area stretching from eastern Nebraska to western Ohio and from southern Minnesota to Missouri. All of Iowa, Illinois and Indiana are part of the region. The principle concerns are for gene flow and co-mingling. Gene flow from PMP farms is likely to be the most contentious issue during the crop production phase of PMP technology. Concerns are already being addressed by industry, APHIS, and the Canadian Food Inspection Service (BIO 2002, USDA APHIS 2002, CFIS 2001); the main ones revolve around pollination of food crops and subsequent inadvertent setting of seed containing the active pharmaceutical ingredient (API).

According to Ellstrand (2002) in assessing risks posed by various pharma crops, the worst possible plant with regards to confining its genes would be one that: routinely breeds with related crop varieties; produces large amounts of pollen and seed (and the seeds are particularly small); serves as an important food and feed crop; spontaneously mates with wild relatives and is widely planted throughout the world. Stevens (2002) tested the parameters for confinement of an outcrossing food crop that meets many of these criteria in this instance maize. He tested pollen spread from a yellow-kernel inbred (dominant) to a white-kernel hybrid using simple, reliable markers. The yellow-kernel pollen source was planted in a 10-acre block in middle of a 160-acre cotton and bean field, various distances from the block four-row strips of white-kernel hybrid were sown at a number of planting dates. The yellow kernel inbreds were tagged with molecular markers and detasseled to various extents - 0%, 80%, 90% and 100% tassels were removed. He found that pollen dispersal dropped as distance and detasseling increased. At distances less than prescribed by APHIS (900 ft), he found only a 0.0013% incidence of contaminant from 90% detasseled pollen, and 0.0% incidence from the 100% detasseled source. Given that an average maize ear contains 500 seeds, the significance of cross-pollination at the 900-foot is that there will occur one seed containing a hypothetical pharm protein for every 150 ears. No contamination was observed in comparable studies in Missouri, California and in Washington State (Halsey et al., 2002).

In essence, the data generated so far on gene flow potential support the APHIS regulatory requirements for separation distances between PMP crops and food crops. Pertinently, the

minimum separation distances required by APHIS are significantly longer than the distances shown to have almost no gene flow in the Missouri, California, and Washington State experiments. However, a major caveat is that one kernel among tens of thousands can still pose a worry, so the question to be posed is what the consequence to people or the environment would be of such a low exposure to an active pharmaceutical ingredient (API).

Following this and other feedback, APHIS has modified its regulations. It now requires an increase in the number of field site inspections to assure compliance with inspections corresponding to critical times in production. Permit conditions specifically for maize designed to produce PMP or industrial compounds have as a requirement that no maize be grown less than one mile of a field test site that involves open-pollinated maize. There is now a restriction on the production of food and feed crops at the field-test site and perimeter fallow zone (which is increased from 25 to 50 feet) in the following season. Dedicated mechanized equipment must be used for planting and harvesting as well as dedicated facilities for the storage of equipment. Equipment cleaning procedures and seed cleaning and drying procedures must be submitted and approved by APHIS to minimize the risk of seed movement. Training programs are required to ensure that personnel are prepared to successfully implement and comply with permit conditions.

There are several methods to control gene flow with relative levels of effectiveness. Some strategies to reduce the risk of gene flow from transgenic crops, such as the use of male sterile plants, work well but are limited to a few species. For the many crops in which chloroplasts are strictly maternally inherited, which is to say not transmitted through pollen, transformation of the chloroplast genome should provide an effective way to contain foreign genes. But it is not fool proof as demonstrated by recent papers indicating some illegitimate recombination with genomic DNA. In the past few years, patents have been issued for techniques linking "suicide" genes to DNA "switches" that can be tripped inside pollen cells, impeding their development, and also issued for techniques based on genes that kill off hybrid seeds as they attempt to germinate. These Genetic Use Restriction Technologies, or GURTs (pejoratively termed "terminator technology"), of which there are several, basically render crop fertility into a trait that can be switched on or off with messengers.

As noted, tests to date show even when cross-pollination in maize has occurred; the probability of finding one seed in thousands of seeds is pretty low. It is reasonable to conclude that under proper confinement measures, any inadvertent exposures attributable to gene flow are very low, and thus the risk of adverse effects are correspondingly low if any one is inadvertently exposed through their food to an API. Fortunately, the APIs under development are proteins (not active vaccines). Bioavailability and fate of proteins in the environment and in organisms is well studied. Some therapeutic Igs are actually coded for using human gene constructs. As with the stability test for allergens, all proteins can be tested for digestibility in the stomach or intestine. For example, Igs are already known to be rapidly digestible, for that reason, therapeutic doses are given by injection or intravenously rather than orally. However, in this business perception is reality and the discovery, or indeed deliberate adulteration, of the food supply despite low or no true risks could potentially scupper the whole promising industry.

What about persistence of therapeutic proteins in the environment? All organisms, including soil bacteria and fungi, contain proteases, enzymes that degrade protein. Once in the moist soil

virtually all proteins, including the pharmaceuticals, will break down into their basic constituents, amino acids. Stotzky (2000) suggested in a paper in 2000 that some proteins such as crystalline proteins of the Bt endotoxin may stick to clay particles under certain conditions, but they are not biologically available nor are they significantly mobile, especially under natural soil moisture conditions (Carpenter et al. 2002).

In addition to the points raised above it must also be remembered that in most instances one highly defined product will be produced in very limited acreage. For example, about 1000 acres may be required to produce enough recombinant immunoglobulins of any kind. Under optimum conditions according to Felsot (2002), that acreage will not be placed in one area. Although hotly countered by the states adversely affected, it is to be expected that the sites chosen will be in a number of locations in states, or areas thereof, where little of the food crop counterpart of the PMP crop is in production. Thus, not only will the acreage be more manageable owing to a limitation in size in any one location, but the specific location itself will be comparatively devoid of the food crops subject to cross pollination.

As to the protein or protein product itself, those for use as therapeutics will already have undergone rigorous review and, in most instances, extensive clinical trials under the FDA's comprehensive and thorough safety and efficacy review and trial process given to all pharmaceuticals whether they be synthetic or biological. As noted at the beginning obtaining medicine from plants is as old a concept, almost, as medicine itself and modern techniques for growing and extracting "natural" plant therapeutics ensure the highest level of integrity and safety. The therapeutic proteins themselves are not "novel" per se. Most of these proteins have already been produced as medicines using CHO cells in large stainless steel bioreactors and, as above, have been thoroughly characterized and analyzed from an in vitro and in vivo perspective and undergone up to 13 years of clinical trials at a cost of between \$400 to \$800 million. The manufacturing process is the real novelty, but it will be regulated stringently as if the protein was being manufactured in a factory.

While other specialty crop production practices, such as for foundation seed, already have a well tested and verified oversight, for molecular farming crops, especially PMPs, standard operating practices will be as thorough as that for stainless steel bioreactors. Oversight will be required from even more regulatory agencies than the latter system and the level of scrutiny at every step of the process, pre- and post-production, will be unprecedented for either agriculture or pharmaceutical production. In addition, confinement systems will be optimized to ensure adherence to the overriding confinement and identity preservation-closed loop principles. No doubt there will be mishaps as occurred with the Prodigene trial but adequate remediation systems will be in place to mitigate any potential effects if the confinement system does not achieve its desired results.

A number of companies such as Dow, Monsanto, and partnerships with smaller biotech companies such as Centocor and Epicyte have applied for and received permits from regulatory agencies for field trials of biopharmaceutical-containing crops. These permits have been granted based on review of detailed, credible, and practical plans for producing plant-based pharmaceuticals. In addition, only a few select growers were identified, trained and supervised to grow these crops and the seeds will be available only from the manufacturer. As part of this

commitment, and corporate policy, these companies apply a comprehensive risk-focused process at key stages throughout the lifecycle of every product, from conceptualization and development through to production and post marketing. While these systems hold enormous promise for all stakeholders from the company through the producer to the patient, analytical-deliberative processes along with proactive continuous dialogue will be necessary between all the relevant stakeholders and partners including companies, the government, agro-biotech experts, the food industry, public policy groups and the health care system to insure optimum benefits for all with minimum impact on the environment and none on the food supply.

Examples of Plant made Pharmaceuticals

Alfalfa	Plasma Proteins Foot-and-mouth disease {livestock}
Maize	Anti- HIV and Anti herpes Simplex Antibodies Microbiocides for pulmonary infection Mabs for cancer, autoimmune disease (rheumatoid arthritis, Crohns disease) Vaccines for hepatitis B, Norwalk virus (Travelers disease), Vaccines and Mabs for animal disease prevention Transmissible Gastroenteritis Vaccine for Pigs Aprotinin for blood loss and heart surgery
Lemma	Human tissue plasminogen activator for peripheral arterial occlusion, Alpha Interferon
Lettuce	Vaccines for Hepatitis B
Moss (bryophyte)	Factor IX for haemophilia B
Pigeonpea plant	Rinderpest
Rice	Alternatives to antibiotics in poultry diets, Lysozyme for Gastrointestinal health, Topical infections and inflammations, B-cell lymphoma idiotype vaccine
Safflower	Therapeutics and oil-body-based products for oral and dermal delivery
Spinach	Protective antigen for vaccine against Bacillus anthracis
Soybean	Herpes simplex virus 2 - Tobacco extensin signal peptide - Anti-HSV-2 (IgG)
Tobacco	Non-Hodgkins B-cell lymphoma surface antigen scFv TGF-b glucocerebrosidase for Gauchers Syndrome Alpha galactosidase for enzyme replacement therapy IgGs for prevention of dental decay, common cold, neutralization of chemotherapeutic drug toxicity GAD 7 cytokines for type 1 Diabetes, IL-10 for inflammatory bowel Glycoprotein B from human cytomegalovirus (hCMV) Colon cancer surface antigen - Murine IgG signal peptide Fabrazyme fat-storage disorder called Fabry disease
Tobacco and Corn	Gastric lipase for cystic fibrosis Lactoferrin for gastrointestinal infections, Sjogran's (eye) syndrome
Tomato Potato	Edible vaccines against Enterotoxigenic E. coli, Norwalk virus, Hepatitis B, Vibrio cholera, Rabies virus-intact Glycoprotein

Potato Tuber Banana (someday!)	Antimicrobial peptides, Rabbit hemorrhagic disease virus
Wheat	Carcinoembryonic antigen - Murine IgG signal peptide

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