Bioindustrial and biopharmaceutical products from plants

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Abstract

Plant production of biopharmaceuticals and bioindustrial proteins is a potentially viable industry with tremendous upside potential. Plants allow large amounts of biomass and can be easily and inexpensively produced, with seed based systems being the most economical. Significant technologies, including promoters with tissue-specific activity, and sub-cellular targeting sites that offer protein stability, have been successfully developed to address protein accumulation issues. Crop choices for production, whether for domestic or wild species, are based on many criteria including type of product, cost of production, and safety of the product. Production steps are numerous—from growing and harvesting the crop to transportation, storage, processing, extraction and in some cases protein purification. Examples of products and crop choices will be presented. Legal and regulatory issues also will enter into the decision-making process. The industry players must work together to solve a number of problems that are currently at issue, such as regulatory requirements and public acceptance. However, with attention to these details and good stewardship, the success of the industry is possible.

Media Summary

Plant production of biopharmaceuticals and bioindustrial proteins is a potentially viable industry with tremendous potential. Plants allow large amounts of biomass and can be easily and inexpensively produced.

Key Words

Bioindustrial proteins, biopharmaceuticals, human health, industrial enzymes, transgenics.

Introduction

Plant biotechnology was developed to improve agricultural products, but is now being used to manufacture heterologous proteins for application in industry and human health. The number of proteins successfully expressed in plants is large and is expected to grow rapidly in the future. The types of proteins targeted for this technology include antibodies, food and feed additives, products for human and animal health and industrial enzymes. The use of transgenic plants for large-scale production of proteins (molecular farming) has been shown to be commercially feasible and can be economically advantageous (Hood and Jilka, 1999; Nikolov and Hammes, 2002; Hood and Howard, 2002). In this chapter, the focus is on the production of human health products and industrial enyzmes in transgenic plants.

Recombinant protein production systems

Many production systems have been used to generate proteins for industrial and pharmaceutical applications. One option is to isolate the protein from its natural source, since proteins thus acquired are usually in their native form and, therefore, functional. However, this approach can be problematic where the source material is expensive, in limited supply or where access is restricted by geography, climate or social issues. If the target protein is a small peptide, chemical synthesis may be a viable alternative to extraction. However, this option is limited to proteins shorter than approximately 50 amino acid residues due both to the cost of synthesis and the difficulty in maintaining structural accuracy in longer molecules. Foreign proteins have been produced in animal cell cultures and transgenic animals, methods that are very expensive and time intensive, particularly in the scale-up of cultures or herds large enough even for high-value pharmaceutical products (current use), making them highly impractical for industrial enzyme

production. Bacteria and fungi are relatively simple fermentative systems but require a large initial investment for capital equipment, and scaling the production systems involves further capital investment.

Why are plants the production system of choice?

Plants represent an attractive alternative to other expression systems because large amounts of biomass can be produced easily and inexpensively. Transgenic plants require the lowest capital investment (mainly for dedicated harvesting equipment and storage) of all production systems, and they are easily scalable simply by increasing production acreage. The cost of producing crude recombinant protein in plants could be three orders of magnitude lower than that of the mammalian cell system, and 10-fold less than microbial fermentation (Hood and Woodard, 2002).

Several additional advantages to plant-based products can be noted, particularly when used for direct delivery. Direct delivery refers to applications where the plant material can be used directly in food, feed or as an industrial feedstock without purification. In some cases, the plant material itself may have value in the application independently of the recombinant proteins contained in it. Furthermore, plant systems allow for proteins free from human pathogens.

Technology Options

The choice of plant to be utilized depends on a number of factors including its cultivation, transformability, growing cost, production and processing of the target tissue, existence of wild relatives, and degree of out-crossing. Current systems include corn, soybean, canola, alfalfa, *Lemna*, tobacco, tomatoes, potatoes and safflower. These crops are examples of wild plants, e.g., *Lemna*, domesticated non-food crops, e.g., tobacco and alfalfa, and food crops—rice, corn, soybeans, tomatoes, potatoes, canola, and safflower.

The type of tissue that is utilized for protein accumulation is often chosen because of the type of plant used, or vice versa. For example, *Lemna*, alfalfa and tobacco are leafy crops, and the seed or fruit would not be appropriate as production vehicles. Potatoes are a root crop and thus the tubers are used. For fleshy fruits, tomatoes can be used for production. However, grain crops such as corn, rice, soybeans, canola and safflower have the most stable production vehicle, the seed. While multiple factors affecting protein accumulation and crop choice are interrelated, only generating the transgenic material and protein expression will be discussed.

Generation of transgenic material

Recovery of stably transformed plants exhibiting traits of interest requires the combination of several technologies: 1) appropriate selectable marker genes and selection conditions, 2) an efficient culture system that allows recovery of plants from target tissues, 3) a DNA delivery system, and 4) joining of these technologies so that transformed, fertile adult plants can be recovered. Variety is the driver for these technologies because of the plethora of plant species available as targets for plant production of foreign proteins (Hood, 2003). For maximum utility and efficiency, however, DNA delivery systems should be simple, efficient and preferably inexpensive.

Molecular farming requires that DNA for encoding the protein of choice be introduced at will into the plant of choice. To this end, multiple methods of foreign DNA transfer into plant nuclei have been developed over the last 20 years. These methods include *Agrobacterium*-mediated transformation and several direct gene transfer methods, e.g., microprojectile bombardment, electroporation, silicon carbide fibers, electrophoresis and microinjection (Songstad et al., 1995; Hood, 1999). Microprojectile bombardment has also been used to transform chloroplasts of solanaceous plants (Maliga, 2002: Daniell et al., 2002). Of these numerous methods, the *Agrobacterium*-mediated nuclear transformation and microprojectile bombardment-mediated nuclear and chloroplast transformation methods are most often used today. Viral vectors are being used for commercial development of transiently expressed therapeutic proteins in tobacco (Lindbo et al., 2001: Grill et al., 2002). The operative word here is variety. Crop plants span many species in many genera, families and classes, and no single method is appropriate for gene transfer.

Selection of transformed tissues requires inclusion of genes that allow identification of the transformed cells. Selectable marker genes come in a variety of types with quite varied substrates (Hood, 2003). Again, the operative word is variety, because a large array of methods and markers are critical to the success of plant biotechnology. Selectable markers can provide selection pressure on plant tissues resulting in death of non-transformed cells, or through the starvation of unwanted cells because selective growth of transformed cells is supported.

Selectable markers have recently been deemed as undesirable traits because of their perceived danger to public health. Their perceived danger is because of the potential for allergenicity of the protein or the potential of resistance gene transfer to gut microorganisms (antibiotic resistance). Maintenance of selectable marker genes in the plant after establishment of the desired trait is assumed to be unnecessary. The assumption is that these genes have no utility after transformed plant recovery. However, herbicide resistance genes have utility because they confer downstream advantages for selection of transformed plants in the field. Herbicide resistance gene products allow growth and recovery of plants prior to establishment of homozygous lines so that early field performance of the transgenic plants can be assessed. Moreover, the pflp gene (Chen et al., 2000; You et al., 2003), a selective trait, confers resistance to plant pathogens, also giving it value beyond selection. Thus, a discriminating assessment of marker gene value versus risk should be undertaken before wholesale removal of the trait is endorsed.

Gene expression and protein accumulation

Gene expression with its resulting protein accumulation is the single most important factor for expressing most recombinant proteins. The economics of the product as well as regulatory issues will be dictated by gene expression patterns and amounts. Expression of the gene depends on many factors including transcription, translation, targeting and the ability of the plant to accumulate the protein.

In the case of foreign protein production of pharmaceuticals and industrial enzymes, it is desirable to sequester the protein as much as possible into specific target tissues. This is accomplished with the use of tissue specific promoters that are active only in limited tissue types. An example is the globulin-1 promoter from maize (Belanger and Kriz, 1991) that is primarily limited to embryo specific expression (Hood et al., 2003; Woodard et al., 2003).

In addition to promoter specificity, the subcellular compartments that are noteworthy within that tissue present the array of potential subcellular locations that are likely to maximize that promoter's work. An example of the effect of alternative targeting on protein accumulation is shown in Table 1. Clearly, cell wall targeting effected the highest accumulation of protein 1, whereas the vacuole was the best subcellular location for accumulation of protein 2.

The cellular machinery responsible for targeting proteins is under intense study and has been elegantly reviewed (Kermode, 1996; Pyke, 1999; Sanderfoot and Raikel, 1999). The rough endoplasmic reticulum (RER) contributes protein to the several compartments that receive their member proteins from the membrane/secretion pathway, e.g., the vacuole, plasma membrane, the endoplasmic reticulum (ER), the Golgi and the cell wall. Secretion to the exterior of the plasma membrane is the default pathway, and a signal sequence that begins this process is generally necessary and sufficient to have a protein arrive on the cell surface (Vitale and Denecke, 1999). Other destinations in this pathway require additional information.

The plastids, mitochondria, nucleus and cytoplasm receive their proteins from cytoplasmically translated messages, and the proteins have specific transit peptide sequences that allow their import into each organelle (Kermode, 1996). The plastids are an interesting group of organelles that are derived from a single pre-organelle, the proplastid (Esau 1977), and include chloroplasts, chromoplasts, amyloplasts and elaioplasts. The import process for chloroplast proteins encoded by nuclear genes has been reviewed recently (Keegstra and Cline, 1999). The underlying principle is that a transit peptide is necessary for recognition by a receptor on the surface of the plastid envelope. Some features of this transit peptide are common to all plastids (de Boer et al., 1988; Lawrence et al., 1997). However, each member of the

plastid group most likely has features of its import apparatus that limit uptake of proteins to those specifically required for the unique functions of the various plastids (Wan et al., 1996).Transit peptides and signal sequences have been utilized by all players in the field of molecular farming. In most cases, these signals are a part of the strategy to achieve maximal accumulation of the target protein, which is the measured parameter. In very few cases has subcellular *in situ* localization of the target protein been performed. In one case, the bacterial endotoxin from *E. coli* (Lt B) was found in an unpredicted compartment (Chikwamba et al., 2003).

In order for proteins to accumulate, the protein must be stable to the particular environment in which it is found. Carbohydrate, protein and lipid content, as well as pH and salt may influence the stability of the protein. Since these will differ in different plants, tissues and subcellular locations, protein accumulation will also vary considerably. While this must be explored empirically today there is hope that the future may bring predictability to the fate of proteins based on their specific characteristics in plants and tissues to achieve the highest overall accumulation.

Table 1. Effect of promoter and targeting signal on protein accumulation in maize seed

Protein location	Protein example	Targeting sequence	Highest T1 seed
Embryo [a] Cell wall	1	BAASS	0.8% TSP
Constitutive [a] Cell wall	1	BAASS	0.19% TSP
Constitutive [a] ER	1	BAASS KDEL	0.12% TSP
Constitutive [a] Cytoplasm	1	None	0.07% TSP
Constitutive [b] Vacuole	2	SS Vacuole	10% TSP
Constitutive [b] Cell wall	2	BAASS	2% TSP
Constitutive [b] ER	2	BAASS KDEL	0.08% TSP
Constitutive [b] Cytoplasm	2	None	0.0008% TSP

[a] Data taken from Hood et al., 2003
[b] Data taken from Streatfield et al., 2003

Production Options

Production practices for recombinant proteins refers to the growing, harvesting, transport, storage and tissue processing of the crop, as well as the extraction and purification of the protein (Table 2). With thousands of species to select from and the wide variety of products that are possible, it is highly unlikely that any one system will work best for each of these steps. Moreover, different product categories will require different amounts of purification and different formulations. Each plant production system has distinct characteristics that may prove advantageous depending on the product. The key features of the best production system include a potential for low cost of goods, maintenance of protein integrity, flexibility with regard to time and temperature for harvest and maintenance of product as well as environmental safety (Delaney, 2002; Nikolov and Hammes, 2002).

In selecting a crop for production one must decide if working with a domesticated species would be preferred over use of a wild species (Figure 1). Native plants only grow well in specific environments because of light, temperature and soil conditions. In contrast, the major commercial crops have been adapted for a wide range of conditions, which extends their geographic boundaries. In many cases only one crop per year can be grown in a geographic area. However, additional production of this same crop can be done in other geographic locations at different times of the year, extending the seasonal growing of the crop to year-round production. Wild species have an advantage in that they will be unlikely to be mistaken for food crops and therefore unlikely to be inadvertently mixed with the food supply. Unfortunately, they would be more likely than food crops to outcross with native species. One consideration for foreign protein production is whether to use an open-pollinated or a self-pollinating plant, an issue for either domesticated or wild species. For most domesticated crops, self-pollination usually means that the seed planted by the grower can be saved every year and replanted the following season. This is in contrast to open-pollinated crops that are produced as hybrids. For these, subsequent production is not from plants in the production fields but from parent seed stocks.

Table 2: Steps in Production with Key Considerations

Production step	Consideration	Applicable products
Growing	Requirements Geographic Limitations Seasonal Limitations Recombinant Protein Yield Confinement	
Harvesting	Mechanical Issues Time Sensitivity	
Transportation	Temperature sensitivity Cost	
Storage	Temperature sensitivity Protein stability in tissue	
Tissue Processing	Stability in tissue Potential for Enrichment	First critical step for specific product type[a]
Extraction	Protein stability	Not necessary for direct delivery

	Biomass Quantity	
Purification	Interfering agents CGMP	Primarily for pharmaceuticals

[a] Vaccines, industrial enzymes, purified pharmaceuticals

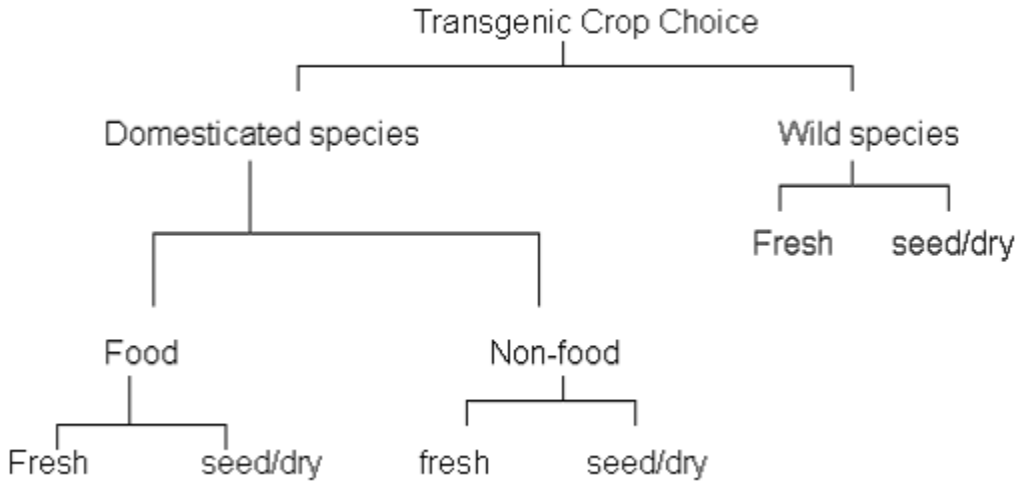

Figure 1. Characteristics of Plant Systems

With the exception of some specialty crops, most of the crops today are harvested mechanically. After the plant tissue is harvested and transported to its designated location, it must be stored for some amount of time before it is processed. Leaves and fruits contain water, making storage problematic. In contrast, if a plant storage organ is used, e.g., seeds or tubers, the plant part is in a dormant state with little metabolic activity, and storage is easy.

Tissue processing after the crop is harvested is necessary whether for direct delivery or for highly purified products. One of the most critical aspects is the amount of total protein present in the harvested tissue. The amount of protein as a percentage of total biomass can range from less than 1% to over 40% depending on the plant and tissue source. This feature is critical because in addition to obtaining relatively high percentages of total soluble protein for ease in purification, the overall cost of tissue processing and extraction is directly related to the amount of necessary biomass to obtain the required product amount.

Tissue can also be processed and separated into fractions enriched with recombinant proteins by mechanical means. As an example, it is possible to generate a germ or endosperm fraction from grains by using standard procedures common in the industry today (Watson, 1988). These fractions can have much higher protein content than the whole grain because the expression technology used may sequester the protein in a specific sink. Furthermore, the remaining part of the grain can be used for other industrial applications.

Extraction is relatively easy in most cases by simply adding an aqueous buffer to ground tissue. The pH and salt content can be optimized on a case by case basis to reduce extraction of endogenous proteins and preferentially solubilize the recombinant protein (Evangelista et al., 1998; Bai and Nikolov, 2001). Fresh tissue will most likely require refrigeration to prevent protein degradation. Seeds, however, usually have the advantage of endogenous protease inhibitors, which allows greater flexibility in extraction times and temperatures.

Protein can be purified from the aqueous extracts in a manner similar to extracts from other production systems. The advantages and disadvantages for a variety of plant types when considering production characteristics are summarized in Table 3. No one type of plant rises to the top as the clear choice. This suggests the possibility that one could modify an existing crop specifically for recombinant protein production. As an example, a cultivated crop could be altered to have a higher protein content that could translate into higher amounts of recombinant protein. For industrial feedstocks, selecting a major crop that already is used in industrial applications would have benefit. For orally delivered therapeutic proteins or vaccines, a food crop that has GRAS (Generally Recognized as Safe) status would be best. For protein stability and ease of transport a grain would be a good choice.

While a general discussion of different plant types provides a framework, it is also possible to compare actual systems used today. Characteristics of a single representative of several different plant families/types for their ability to be a host production system are summarized in Table 3. A number of assumptions must be made to even begin to compare them, and this should only be used as a general guide.

Table 3. Ratings for selected crop types as suitable protein production systems

Crop Type	Product Safety	Environmental safety	Lab ease	Growing Ease	Harvest Transport Storage	Process Purification	By-Product Credits
Leafy crops							
Tobacco	C	A	A	B	C	B	C
Fruits							
Tomato	A	B	A	B	C	C	C
Cereals							
Maize	A	B	B	A	A	A	A
Mustards							
Canola	B	C	A	A	A	B	B
Legume seeds							
Soybeans	B	B	C	A	A	A	B
Modified industrial	A	A	A	A	A	A	A

grain

A score: best performance
B score: moderate performance
C score: least desirable performance

Products

High purity human health products made in plants have the distinct advantage of being free from pathogens that are inherent in animal production systems and from the contaminants of microbial systems. As stated above, the actual purification procedures and costs after extraction of the plant material are similar to these systems. Trypsin and aprotinin manufactured in transgenic maize are examples of high purity products from a plant system (Table 4; Woodard et al., 2003; Delaney et al., 2002). Each of these products had special requirements to achieve high expression. Because trypsin is an aggressive protease, the zymogen form was expressed in the seed and these parameters were shown to be essential for success (USP#6,087,558). Aprotinin expressed from a seed-preferred promoter with the protein accumulating in the cell wall space generated extremely high-expressing lines. Each protein can be produced from a small acreage to fill the required market amounts.

Table 4: Production characteristics of different product types

Product type	Acreage	Overall production cost	Protein example	Expression level for production[a]	Formulation	Market
Highly purified human health	Low	High	Trypsin Aprotinin	0.01% dw	Pure protein	Cell culture Open heart surgery
Orally delivered human health	Low	Medium	TGEV	0.01% dw	Ground corn meal	Pig vaccine
Industrial enzyme	High	Low	Manganese peroxidase Laccase	0.1% -0.5% dw	Concentrated extract Corn meal	Pulp bleaching Bioglues

[a]Using cost models for maize production, the minimum expression level necessary for cost-effective production.

Protection against infection by transmissible gastroenteritis virus (TGEV) has been demonstrated following oral delivery of a subunit vaccine produced in transgenic maize (Streatfield et al., 2001). This was the first demonstration of disease protection following this oral delivery methodology. Again, the major advantage is freedom from human and animal pathogens in the production system. However, the other advantages include low overall cost of production and utilization of a feedstuff directly from a crop (Table 4).

Industrial enzymes are ideal for use as catalysts in industrial processes because they can function at ambient temperatures in aqueous solutions at neutral or physiologic pH levels. Furthermore, enzymes have a high specificity, low toxicity, and catalyze reactions relatively quickly at low concentrations.

However, temperature and pH extremes can limit enzymatic activity, and because these extremes are present in large-scale industrial processes, significant changes are required when moving from chemical to enzymatic catalysts. Nevertheless, the large market opportunity for industrial enzymes and the environmental benefits they provide offer an incentive to invest in the development of efficient production systems and to make changes in industrial processes to accommodate enzymatic catalysts.

Cost models indicate that industrial enzymes can be produced cost-effectively at 0.1% of grain dry weight. With today's technology it is possible to obtain 1% of grain dry weight as a single recombinant protein (Figure 2). Because plant biotechnology is young, improvements will be achieved over the next several years that will enable substantial increases in recombinant protein expression.

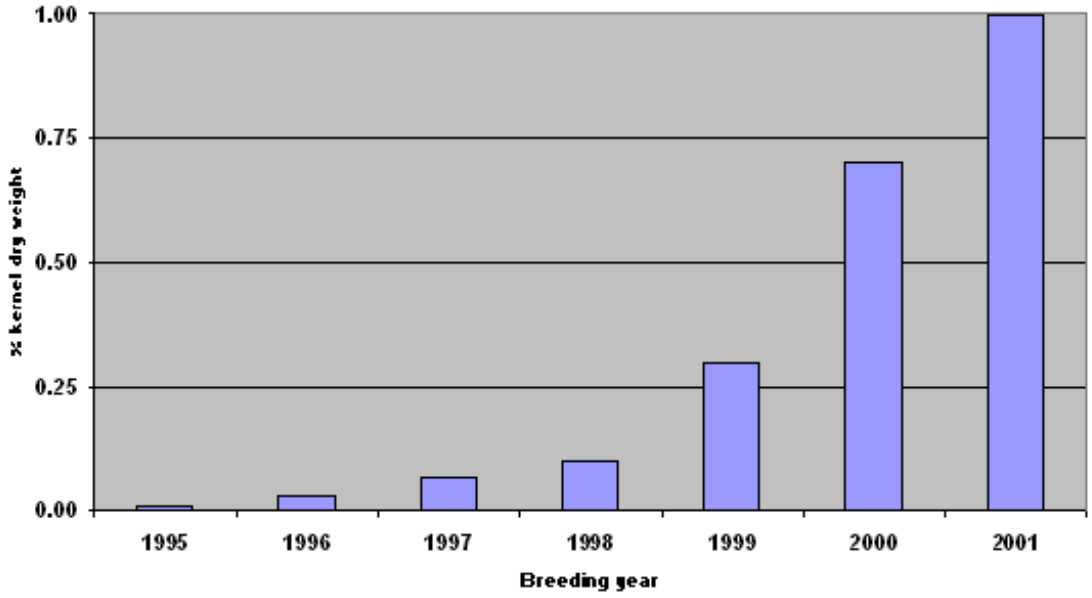

Figure 2. Increase in avidin expression in maize kernels by breeding and selection.

Laccase and manganese peroxidase represent two examples of industrial enzymes produced in maize (Table 4; Hood et al., 2003; Hood et al., unpublished). These two oxidation/reduction enzymes have many potential applications in industries including wood products and textiles because of their activities on lignin. Laccase is actually more active as a ground corn fraction than in an extract (Bailey et al., 2003), whereas manganese peroxidase is easily extracted and concentrated (unpublished). Application of these enzymes in industry has been hampered by the ability to produce them economically in large quantities. Their high expression in transgenic maize should enable this process.

Other production issues

Production is composed of the mechanics of product development, manufacturing and formulation. However, in order to bring a product to market, several other considerations are necessary. This discussion is not meant to be a treatise on these considerations, but to highlight the areas that require attention.

Legal issues. A consideration of legal issues surrounding any new industry is important. In the case of plant molecular farming, legal considerations include such things as patent protection of intellectual property and licensing of patented technology to provide freedom to operate (Sweeney, 2002). Consideration should also be given to a strategy for invention versus licensing. In many cases, it may be more expensive to invent new technology than to license existing technology. The issues are complex,

and expert legal advice is key to success in this field. A more complete consideration of this topic is given by Sweeney (2002).

Regulatory issues. Regulatory issues are becoming more complex. Regulations that are based on scientific principles rather than reaction to public fears are the goal of scientists as well as the regulatory agencies. However, in order for the agencies to accomplish that, the data must be collected. Never have agricultural products undergone such scrutiny, whether food products or crops containing pharmaceuticals or industrial enzymes. We are beginning to understand the dynamics of crop gene flow and insect and weed resistances in ways not even thought of in the past, whether the crops were developed through conventional breeding or genetic engineering. Whatever regulations are set forth in response to the collected data, compliance by players in the industry is critical to everyone's success.

Public acceptance. The public must see a positive benefit from genetically modified crops in order for acceptance to be a reality. Product safety is also at issue here, whether the crop is a foodstuff with herbicide or insect resistance genes, or a crop containing a pharmaceutical or industrial product. The safety of the crop can be assessed quantitatively using models for assessing risk/benefit ratios (Howard and Donnelly unpublished). Briefly, risk is proportional to the hazard of the product in question, times the exposure to that hazard. The hazard of the product is based on the inherent properties of the protein, for example the Bt protein or an orally delivered vaccine, a pharmaceutical or an industrial enzyme. The exposure is equal to the dose per exposure multiplied by the number of exposures. Therefore, the risk factor is product-specific and confinement procedures should be tailored to the hazard of the product.

Summary

In conclusion, molecular farming for biopharmaceuticals and bioindustrial protein products is a potentially viable industry with tremendous upside potential. Many products are in development by many laboratories and companies, and sales could begin quite soon. The industry must work together to solve a number of problems that are currently at issue, such as regulatory requirements and public acceptance. However, with attention to these details and good stewardship, the success of the industry is possible.

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