

BIOINDUSTRIAL AND BIOPHARMACEUTICAL PRODUCTS PRODUCED IN PLANTS

John A. Howard¹ and Elizabeth Hood²

¹Applied Biotechnology Institute, College Station, Texas 77845, USA

²Arkansas State University, Jonesboro, Arkansas 72403, USA

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Over the past several years there have been many advances in plant biotechnology that have led to the successful commercialization of agricultural products for crop improvement. Plant biotechnology is now being considered as a tool to produce non-food products such as biopharmaceuticals and bioindustrial products. This chapter reviews the status of the field with particular emphasis on different plant systems. Key factors such as transformation, expression, growth, harvest, transport, storage, processing, and purification of the plant material are included. The chapter also evaluates the characteristics of different systems and their utility for different types of products. While no one system stands out as the ideal platform, this chapter does point to systems that have broader appeal and speculates as to future platforms and utilities.

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I. INTRODUCTION

Investigators have been exploring the use of plants as an alternative production system for biologics over the past several years (Daniell *et al.*, 2001; Fischer and Emans, 2000; Fischer *et al.*, 1999; Giddings, 2001; Hood, 2002; Hood and Howard, 1999, 2002; Hood and Jilka, 1999; Hood *et al.*, 2002, 2003a; Kusnadi *et al.*, 1997; Ma *et al.*, 2003). While it seems unlikely that no one production system could meet all potential needs for the diversity of products, plants do offer some clear theoretical advantages over other systems. One obvious advantage is that the biologics can be produced free of animal source tissue, thereby eliminating the fear of transmitting animal pathogens, including prions responsible for such conditions as “mad cow disease.”

Plants also offer the potential for a reduced cost of goods, resulting in both a lower cost of raw material and the potential for rapid scale-up with limited facility requirements. Plants offer the least expensive source of biomass. In field environments, the major inputs come from sunlight, rain, and air. Although other inputs are added to many commercial crops, they are a relatively minor expense compared to input requirements for non-plant production systems. Moreover, animal and microbial production systems require plant material as the primary carbon source used in converting energy into useful proteins.

Because plants can be rapidly propagated from seeds, they do not have the limitations found in the raising of large herds, as is the case for transgenic animal systems. Nor is it necessary to build production facilities to generate the raw material, as is the case for cell culture systems, for which scale-up can take years and hundreds of millions of dollars. The shorter scale-up times and reduced up-front capital expenditures for facilities for plant production contribute to a lower cost of goods.

There are several additional advantages to plant-based products, particularly when used for direct delivery. Direct delivery refers to applications in which the plant material can be used directly in food or feed or as an industrial feedstock without purification. In some cases, the plant material itself may have value in the application independent of the recombinant proteins contained in it.

Orally delivered human-health products avoid the cost and safety issues associated with injectables. This has been studied mostly as it relates to oral vaccines (Streatfield, 2002; Streatfield and Howard, 2003a,b). With oral delivery, there is no requirement for needles or syringes, and medically trained personnel are not necessary for administration. The costs of delivery are reduced, and concerns about contamination stemming from incomplete disposal of needles are removed. The lower cost and convenience should result in more patients being vaccinated. This is true not only in developing countries, where access to medical staff may be limited, but also in developed countries, where patient compliance requires booster inoculations.

Plant systems are particularly well suited to yielding large amounts of a desired product in a relatively small area. For example, a current corn-based vaccine candidate targeting Enterotoxigenic *Escheria coli* (ETEC) can yield over 200,000 doses from a single acre of cultivation. Thus, the world's supply could be obtained from a single small farm (Streatfield and Howard, 2003b). Moreover, because some plant tissues, such as seeds, can store proteins for years without loss of activity under ambient conditions, a ready supply of material can be manufactured into final form on an as-needed basis. Rapid scale-up, large volumes, and long term storage are particularly advantageous for industrial enzymes. Low cost and the ability to use the raw material directly for industrial processes encourage development in this direction.

These potential advantages have led to a recent increase in interest in using this technology for the production of new biologics. This chapter is focused on evaluating the different plant technologies with respect to their characteristics in producing proteins for plant-made pharmaceuticals and plant-made industrial products.

II. TECHNOLOGY OPTIONS

The choice of plant to be used 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 outcrossing. Current systems include corn, soybean, canola, alfalfa, *Lemna*, tobacco, and safflower. These crops may be wild plants (e.g., *Lemna*), domesticated non-food crops (e.g., tobacco and alfalfa), or food crops (e.g., rice, corn, soybean, potatoes, canola, and safflower).

The type of tissue that is used for protein accumulation is often chosen based on the type of plant used, or vice versa. For example, *Lemna*, alfalfa, and tobacco are leafy crops, thus seed or fruit would not be appropriate for use as production vehicles. Potatoes are a root crop, and thus the tubers are used. Fleshy fruits such as tomatoes or bananas can be used for production. However, grain crops such as corn, soybeans, canola, and safflower have the most stable production vehicle, the seed. While there are multiple interrelated factors affecting protein accumulation and crop choice, for discussion purposes we have divided the technology into two parts, (1) generating the transgenic material and (2) protein expression.

A. 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, (4) the choice of a genotype that has agronomic or horticultural relevance, and (5) 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. This is true whether the methods are used by scientists in industry or in academic institutions. Additionally, particularly for industry, the method must be available for use either because it is in the public domain or because it can be licensed.

The transfer of foreign DNA into plant cells has been going on for centuries. The most obvious example of natural DNA transfer to plants with which we are familiar today is in *Agrobacterium tumefaciens*, the perpetrator of crown galls. Until very recently, the result of this DNA transfer had gone unnoticed. However, after some astute observations, the “tumor-inducing principle” was hypothesized in 1958 (Braun, 1958) and its molecular nature was discovered in the mid-1970s (Chilton *et al.*, 1977). The stage was set to begin developing DNA transfer technology for crop improvement.

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 human-directed foreign DNA transfer into plant nuclei have been developed over the past 20 years, including *Agrobacterium*-mediated transformation and several direct gene transfer methods, e.g., microprojectile bombardment, electroporation, silicon carbide fibers, electrophoresis, and microinjection (Hood, 1999; Songstad *et al.*, 1995). Microprojectile bombardment has also been used to transform chloroplasts of solanaceous plants (Daniell *et al.*, 2002; Maliga, 2002), and viral vectors are used to transiently express genes in plants, primarily tobacco (Lindbo *et al.*, 2001). Of these methods, the *Agrobacterium*-mediated nuclear transformation and microprojectile bombardment-mediated nuclear and chloroplast transformation are the methods most often used today. Viral vectors are being used for commercial development of therapeutic proteins from tobacco (Grill *et al.*, 2002; Lindbo *et al.*, 2001). Notable is the lack of recent papers in which the other direct gene transfer methods are used. However, modifications of some of these techniques developed in the early 1980s have gained some favor (Southgate *et al.*, 1998). The operative word here is variety. Crop plants span many species in many genera, families, and classes, and no “one size fits all” method is appropriate for gene transfer.

Selection of transformed tissues requires the 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 nontransformed cells, or through the starvation of unwanted cells because selective growth of transformed cells is supported.

Nature provided the first example of selectively different plant cells—hormone independent growth in *Agrobacterium*-incited tumors. The first engineered plant cell selection system was antibiotic resistance (see [Chilton, 2001](#)). In the early 1980s, several labs raced to generate tobacco tissue that could be selected on kanamycin or G418 ([Bevan et al., 1983](#)). Subsequent experimentation has focused on refinement of these techniques, broadening of the host range, and broadening of the species amenable to transformation and selection (reviewed in [Wilmink and Dons, 1993](#)).

Selectable markers have recently been deemed undesirable traits because of their perceived danger to public health due to the potential of allergenicity of the protein or the potential of resistance gene transfer to gut microorganisms (antibiotic resistance). Their maintenance in the plant after establishment of the desired trait is assumed to be unnecessary because 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. These 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. Thus, a discriminating assessment of marker gene value versus risk should be undertaken before wholesale removal of the trait is endorsed.

Alternative methods have addressed the development of less objectionable selectable markers that have less perceived risk, such as the pflp gene ([Chen et al., 2000](#); [You et al., 2003](#)) and positive selection ([Wang et al., 2000](#)). However, when the risk is determined to outweigh the benefit of retaining the selective trait gene, the methods being sought for their removal should be employed. Such methods include (1) co-transformation into unlinked sites then removal through breeding ([Komari et al., 1996](#)) and (2) inclusion of recombination sites to selectively remove genes (reviewed by [Hare and Chua, 2002](#)). A slate of multiple selectable markers can improve the ability of researchers to maximize recovery of transformed, regenerated plant materials that are close to the final product line. In the future, it may also be desirable for non-food crops to use a selectable marker different from food crops of the same species. This would help to keep the two crops segregated and easily distinguished in the field.

While there are options for removal of the selectable marker, it is also possible to express the marker protein in a non-target tissue of the crop. When seeds are used as a source of recombinant protein, markers may be

designed to be expressed in cell cultures or leaves without presenting a new protein in the harvested crop. Even when the marker protein may end up in the harvested crop, the presence of the marker gene or protein may be inconsequential if the protein product is used in industrial applications or if it requires purification before being used as a pharmaceutical.

B. PROTEIN EXPRESSION

Protein expression is the single most important factor for most recombinant proteins and can dictate the economics of the product as well as regulatory issues. Expression of the protein depends on many factors, including transcription, translation, targeting, and the ability of the plant to accumulate the protein.

Native proteins are expressed in all plant tissues and organs. Some basic “house-keeping” proteins are often present in most tissues, e.g., ubiquitin, and are thus expressed from genes regulated by constitutive promoters. 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 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), which is primarily limited to embryo-specific expression (Hood *et al.*, 2003; Woodard *et al.*, 2003).

Localization of native protein into subcellular compartments combined with the tissue-specific expression of their genes allows cells to differentiate with unique identities and collectively form a eukaryotic organism. 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. For example, targeting to a glyoxysome in a leaf or root would not be as useful as targeting to this organelle in a cotyledon that stores oil. An example of the effect of alternative targeting on protein accumulation is shown in Table I. 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). General pathways for targeting gene products into specific subcellular compartments are diagrammed in Fig. 1. The rough endoplasmic reticulum contributes protein to the several compartments that receive their member proteins from the membrane/secretion pathway. 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 for a protein to arrive on the cell surface (Vitale and Denecke, 1999).

Table 1
Effect of Promoter and Targeting Signal on Protein Accumulation in Maize Seed

Protein location	Protein example	Targeting sequence	Highest T1 seed ^c
Embryo	1	BAASS	0.8%
cell wall ^a			
Constitutive	1	BAASS	0.19%
cell wall ^a			
Constitutive	1	BAASS KDEL	0.12%
ER ^a			
Constitutive	1	None	0.07%
cytoplasm ^a			
Endosperm	1	BAASS	0.0085%
cell wall ^a			
Embryo ER ^a	1	BAASS KDEL	No events recovered
Constitutive	2	SS vacuole	10%
vacuole ^b			
Constitutive	2	BAASS	2%
CW ^b			
Constitutive	2	BAASS KDEL	0.08%
ER ^b			
Constitutive	2	NLS	0.02%
nucleus ^b			
Constitutive	2	None	0.0008%
cytoplasmic ^b			

^aData from Hood *et al.* (2003a).

^bData from Streatfield *et al.* (2003).

^cPercentage given as a percent of total soluble protein.

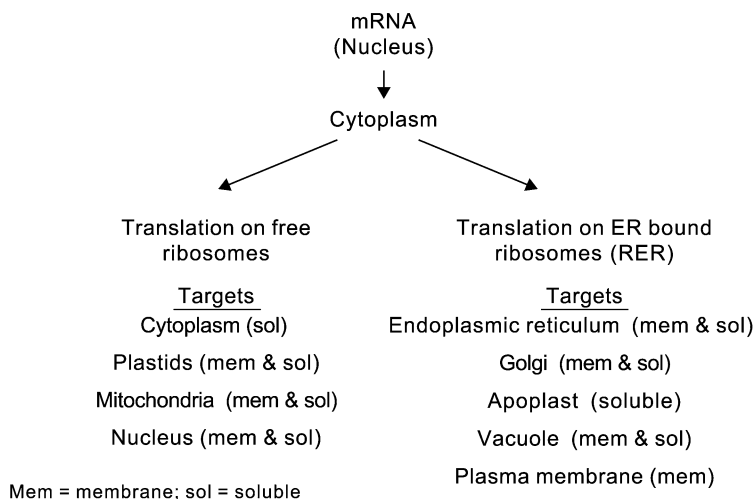


Figure 1 Targeting pathways for plant proteins.

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); they include chloroplasts, chromoplasts, amyloplasts, and elaioplasts. The import process for chloroplast proteins encoded by nuclear genes was reviewed by Keegstra and Cline (1999). The underlying principle of this process 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 plastids (Wan *et al.*, 1996).

Transit peptides and signal sequences have been used 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. In very few cases has subcellular *in situ* localization of the target protein been performed. In one case, Lt B, the bacterial endotoxin from *Escherichia coli*, 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. This includes not only the obvious problem of protease attack, but also more subtle attributes. Specific proteins can have different stabilities depending on their specific environments. 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, their ability to accumulate 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.

III. PRODUCTION OPTIONS

Production of recombinant proteins refers to the growing, harvesting, transport, storage, and tissue processing of the crop, as well as the extraction and purification (Fig. 2). With thousands of species to select from and a wide variety of possible products, it is highly unlikely that any one system will work best for each of these steps. Each plant production system has its own distinct characteristics that may or may not prove advantageous, depending on the product, so it is important to select the plant system that is best for each product. Since it is impractical to have thousands of different production

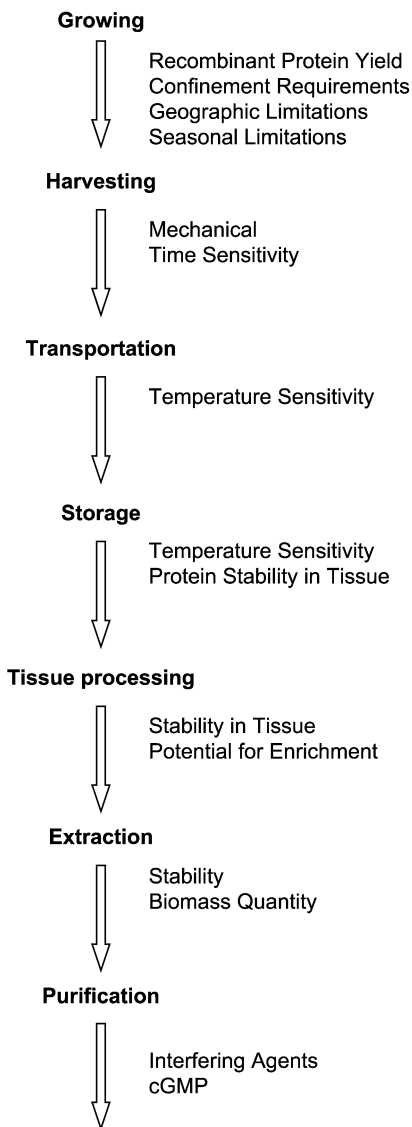


Figure 2 Steps in production with key considerations.

systems, it is preferable to adapt a given system to the needs of the various products. Fortunately, there are some common features that apply to most products, enabling a few systems to accommodate most products. These key features 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 safety and environmental safety (Delaney, 2002; Nikolov and Hammes, 2002). These are discussed in the following sections as to how they relate to the overall efficiency of the system as well as to the regulatory aspects.

A. GROWTH

One of the first decisions to be made when selecting a crop for production is whether working with a cultivated species would be preferred. Cultivated species have several advantages, including a higher yield of biomass compared to their wild relatives. An understanding of agronomic practices will lead to a more reliable supply than that from non-domesticated species, most of which have not undergone selection for higher yields and pest resistance in past centuries. These characteristics practiced on domesticated crops have the net effect of a greatly reduced cost of producing the raw material compared to competing technologies. Another advantage of cultivated species is that many have been evaluated for antinutritional, allergenic, or toxic agents. In addition, ample infrastructure for downstream processing and storage of cultivated crops is established along with the experience of handling the crop.

Plants only grow well in specific environments due to light, temperature, and soil conditions. 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.

Non-cultivated crops have an advantage in that they are unlikely to be mistaken for food crops and therefore are unlikely to be inadvertently mixed with the food supply. Unfortunately, they would be more likely than food crops to outcross with native plants in the environment, which may pose a larger hazard. Matching the yield of cultivated species with that of wild species would require decades of research, assuming the crop had the potential. Finally, the impact of these relatively unknown species on product safety is unknown. Determination of the degree of this impact would undoubtedly require extensive effort and time.

One consideration for foreign protein production is whether to select a food crop or a non-food crop. One advantage of food crops is that we know they are safe for consumption. This is a dramatic advantage in cases where the final product can include the plant tissue as well as the recombinant protein. Examples include orally delivered products, such as vaccines, for which the

protein product is not purified from the plant tissue; rather, a formulated product made from the food crop is orally administered. The use of non-food crops for orally delivered products would demand that we understand the potential for toxicants, allergens, or antinutritional agents of these uncharacterized systems. In the case of therapeutics where the protein is purified from plant tissue there is also an advantage to using food crops. A greater safety margin can be obtained with known food crops since any host protein that co-purifies with the recombinant protein would already be part of the food chain. The only obvious potential advantage for using a non-food crop is that it may be less likely to inadvertently mix with the food crop.

Another choice to be made is whether to use an open-pollinated or a self-pollinating plant. The advantage of using self-pollinating plants is that there is a much lower risk that pollen will unintentionally transfer onto other plants of the same species. Controlled pollen shed of open-pollinated crops can be used to help alleviate this concern by either physical or genetic means to prevent outcrossing onto weedy species or related food or feed crops.

For most cultivated crops, self-pollination usually means that the seed planted by the grower can be saved every year and replanted the following season. However, it is more difficult to maintain control of the seed and more likely for a contaminant to accumulate when the same seed source is used to grow crops season after season. Therefore, the advantage of self-pollinating crops is partially offset by the potential for amplification of contaminating plants. This is in contrast to the case of open-pollinated crops that are produced as hybrids, in which subsequent production is not from plants in the production fields but from parent seed stocks. Growers do not save seed because of the poor yield compared to the hybrid seed. Therefore, it is unlikely that any amplification of contaminating plants will occur.

One additional possibility that exists for plant production systems is that of plant cell culture systems or hydroponics. These systems have the advantage of being physically contained and avoid the potential disadvantage of outcrossing with species that are grown in an open environment. Unfortunately, other factors such as higher cost and unknown product safety present major hurdles for product development.

B. HARVESTING/TRANSPORT/STORAGE

With the exception of some specialty crops, most crops today are harvested mechanically. Therefore, collecting the plant material itself is usually not a problem. The problem with harvesting concerns how time sensitive the plant material is. If fresh fruit is used as the source tissue, it may be critical to harvest the crop within a narrow time window to avoid degradation of the crop and/or the protein product. The next related concern is how to move

this tissue into a storage facility without protein degradation. Will the tissue require refrigeration for any length of time? Fresh fruit and green leafy material can be a problem in that even if they are harvested at the right time, they can still degrade during transport or storage. In many cases, protein degradation can be greatly reduced upon immediate refrigeration. Some leafy material can be dried; assuming this drying does not degrade the protein, this option is very appealing. With regard to harvest, seeds are a preferred choice since they are not time sensitive and do not require special handling conditions to prevent degradation of the recombinant proteins.

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. The storage concerns of the host tissue mimic those for transporting the tissue but may be magnified, since the raw material may be in storage for years until it is fully processed to its final form. The length of time between harvesting and processing can vary significantly depending on the plant tissue. If the protein source is fresh tissue, then the protein will be at risk for degradation due to the plants' active metabolic machinery. Fruit crops are at a disadvantage with regard to storage because of natural degradation by native enzymes. This limitation can be overcome if the fruit is immediately processed in some form. For example, the fruit can be dried, thus providing a useful storage system. Alternatively, fruit can be made into a juice that could be further processed immediately, thereby eliminating the storage of the host tissue in its native form. This means either that processing must take place shortly after harvest with no storage or that storage will require refrigeration. In many cases, both are required.

In contrast, in plant storage organs, e.g., seeds or tubers, the plant part is in a dormant state with little metabolic activity. In this regard seed tissue has a distinct advantage because seeds store proteins for years without degrading proteins. Examples of this phenomenon include the demonstration that recombinant proteins can remain stable in seed tissue for months to years (Kusnadi *et al.*, 1998a; Lamphear *et al.*, 2002; Stoger *et al.*, 2000). This stability may be due to the high concentration of protease inhibitors in seed, the low water content, or the carbohydrate available to stabilize the protein. Thus, seeds can be stored under ambient conditions to allow greater flexibility for processing options.

C. TISSUE PROCESSING

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 total biomass. Fresh fruit is generally at a disadvantage because it is relatively low in overall protein content (1–3%) and high in water content. In contrast, most seeds are high in total protein (10–40%) and low in water content (Koehn, 1978). Mechanical processing of seeds to flour is common and suitable for extraction. In the case of leaves, maceration can be employed before grinding the tissue. For fresh fruit or fresh leaf tissue, it may be necessary to extract the product immediately after processing to avoid protein degradation. In this instance, tissue processing and extraction need to be considered together. Therefore, seeds offer protein stability and allow for processing dry tissue and a relatively low biomass to give an overall advantage prior to extraction.

Tissue can also be processed and separated into fractions enriched with recombinant proteins by mechanical means. For 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 a much higher protein content than the whole grain due to the expression technology used. Furthermore, the remaining part of the grain can be used for other industrial applications. This has been done for maize for both orally delivered product candidates and purified proteins. In these cases, the germ fraction contained a 5- to 10-fold enrichment of the recombinant protein on a dry-weight basis compared to the whole grain, due in part to the type of promoter used (Kusnadi *et al.*, 1998b; Streatfield *et al.*, 2003). The amount of biomass required for extraction is reduced between 5-fold and 10-fold, thereby reducing the cost of extraction. This leaves 90% of the grain available for other industrial applications, such as ethanol production. In this way, the cost of the raw material can be reduced since it is offset with byproduct credits. Finally, the waste from the process is greatly reduced, thereby reducing the cost of waste disposal. In this example, tissue processing is not only a required step, but also an opportunity to reduce downstream costs.

D. EXTRACTION/PURIFICATION

Extraction is relatively easily done 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 (Bai and Nikolov, 2001; Evangelista *et al.*, 1998). Fresh tissue will most likely require refrigeration to prevent protein degradation. Seeds, however, usually have the advantage of

endogenous protease inhibitors, which allow 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. For pharmaceutical products, this will require a cGMP (current Good Manufacturing Practices) facility. Many control points will be similar to those already used for other systems, but a few characteristics that are more pertinent to plant-based systems will be discussed.

The need to test for animal pathogens in the final product should be greatly reduced, if not eliminated, when using plant material. Unfortunately, this concern may be replaced with the need to test or validate protocols to demonstrate that no pesticides used in growing the crop will be present in the final product. Often, pesticides are not present on the harvested portion of the crop. Moreover, the small molecule pesticides will separate easily from proteins, even if they are present on the harvested material, due to their vastly different physical properties. Acceptance that the final product is free of pesticides will most likely require validation before it gains acceptance from regulatory agencies.

Plants make a number of phenolics, alkaloids, and other secondary metabolites that can interfere with protein purification. These small molecules sometimes bind to proteins, making purification difficult, or they may interfere with the applications when the proteins are used directly without purification. Green leaf material is generally high in these compounds, although some seeds can also have a high content. Care must be taken either to select material that is low in these compounds or to validate that there is no interference with purification or applications.

The advantages and disadvantages for a variety of plant types when considering all of these characteristics are summarized in [Table II](#). 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 be beneficial. 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.

Combining these characteristics into a single crop would require starting with a food crop. Because of the perceived danger of intermixing recombinant protein-containing crops with commodity crops, specific preventive measures can be taken. For example, a colored marker could be used to differentiate between the industrial grain and the commodity crop. The public’s acceptance of the crop may change for open-pollinated crops if a

Table II
Characteristics of Plant Systems

Crop	Advantages	Disadvantages
Wild species	■ Clearly distinguishable from crops	■ Low yield ■ Outcross to native plants ■ Little known about safety
Domesticated species	■ High yields ■ Experience in growing ■ Infrastructure and experience exist	■ Potential to intermix with crops used for other purposes
Food	■ High margin of safety for human health products	■ Greater potential to intermix with food supply
Non-food	■ Less potential to intermix with food supply	■ Greater potential for toxic, antinutritional, or allergenic agents
Fresh tissue	■ Abundant biomass	■ Harvest/transport/storage
Seed or dry tissue	■ Harvest/transport/storage ■ High protein content	
Hydroponics, cell cultures	■ Limited exposure to environment	■ High cost ■ Limited knowledge of product safety
Field grown	■ Low cost ■ Infrastructure in place	■ Higher potential to intermix
Modified food/feed grain designed for industrial applications	■ Clearly distinguished by color/shape ■ Non-transferable genetics ■ Low cost ■ Infrastructure and experience transferable from commodity crop	■ Not yet developed

male sterile system is used. Alternatively, a self-pollinating crop could be used. Chemically induced promoters could also be employed such that expression is only present when an exogenous chemical is applied. By incorporating these measures, the valued experience and product safety attributes of food quality grains can be kept and at the same time, the potential for an unintentional environmental impact can be minimized.

While a general discussion of different plant types provides a framework, it is also possible to compare actual systems used today. We have summarized the abilities of several different plant species to be host production systems (Table III). This is a very controversial topic, and a number of assumptions must be made to even begin to compare them. Therefore, this

Table III
Ratings for Selected Crops as Suitable Protein Production Systems

Crop	Product safety	Cost	Environmental safety	Lab ease	Growing ease	Harvest transport storage	Process purification	Byproduct credits
Tobacco	C	B	A	A	B	C	B	C
Rice	B	A	B	B	A	A	A	A
Cell cultures	B	C	A	A	B	C	C	C
Sunflower	B	A	C	B	A	A	B	B
Canola	B	A	C	A	A	A	B	B
Banana	A	C	B	C	B	C	C	C
Potato	A	B	B	A	B	B	C	B
Alfalfa	B	A	A	B	A	B	C	C
Safflower	A	A	A	B	A	A	B	C
<i>Lemna</i>	C	C	A	B	C	C	C	C
Hydroponics	C	C	A	B	C	C	C	C
Sorghum	A	A	C	C	A	A	A	A
Maize	A	A	B	B	A	A	A	A
Tomato	A	C	B	A	B	C	C	C
<i>Arabidopsis</i>	C	C	B	A	C	C	C	C
Soybean	B	A	B	C	A	A	A	B
Peanut	B	A	B	B	B	A	B	C
Modified industrial grain	A	A	A	A	A	A	A	A

should only be used as a general guide. To understand the ratings, the criteria are listed below as they relate to how the art is practiced today.

- *Product safety* refers to what we know about the crop as it relates to antinutritional, allergenic, or toxic agents. An “A” rating means a crop is a food source with no known problems. A “B” rating means the crop can be used as a food source; the known toxins and antinutritional or allergenic agents associated with it are not severe. A “C” rating means there are known toxins, allergens, or antinutritional properties or there is no information known.
- *Environmental safety* refers to the potential to outcross with weedy species or food crops and the potential to intermix with the food supply. An “A” rating means there is little outcross potential with weeds or food crops. A “B” rating is given to those species that, as practiced today, have reduced this risk to near zero. A “C” rating is given to species for which there is a significant risk as practiced today.
- *Cost* is defined as the commodity price of the harvested crop, which was used to provide an index to determine its relative production cost. The percentage of total protein in the harvested tissue was then used to calculate the relative amount of recombinant protein that could be present if expression, based on percent of total protein, was the same in all cases. An “A” rating was given to the low cost crops, “B” ratings refer to species that were significantly higher, and “C” ratings were given to species that were an order of magnitude or more higher than the “A”-rated crops.
- *Laboratory ease*: This represents the ease of transforming and targeting protein accumulation in the harvested tissue. An “A” was given to crops for which this is routine and can be done in most laboratories. A “B” was given to crops for which protein accumulation is routine but does require additional skills and is only practiced in specialized laboratories. A “C” was given to crops for which this process is known to be difficult.
- *Field knowledge and experience*: An “A” rating refers to crops for which we have ample experience and infrastructure to grow the plant and for which we have a series of variants that can be useful if specific problems arise. A “B” rating is given to those for which we have some experience but the crop experiences are not as well characterized. A “C” refers to those crops for which we have little experience in growing them.
- *Harvest/storage/transport*: An “A” rating refers to crops that can be harvested at almost any time, and for which transport and storage is stable at ambient temperatures for extended times. A “B” rating is for species that under certain conditions can be stored at cool temperatures but for which there is flexibility in the harvest date. “C” ratings are for those crops that easily spoil and that are time-sensitive to harvest, transport, and storage conditions with or without refrigeration.

- *Tissue processing/extraction/purification*: An “A” rating is for plant tissue in which processing can be optimized for extraction and purification. The tissue would contain low levels of phenolics that can interfere with protein purification and low protease activity that can degrade the product during purification. “B” ratings are for those crops that are easily processed but require a larger biomass to extract the same amount of protein. “C” ratings are for those crops that are difficult to process, are low in protein content, and have protease activity or phenolics that could interfere with protein purification.

While each crop has its unique features, [Table III](#) illustrates a few key points. First, no one plant species exists today that is best under all conditions. There has been no attempt to weight each of these categories, because the importance of each category may be different for different product types. Therefore, we will consider each of the categories separately and point out what products are most appropriate for that category and what can be done to improve a perceived disadvantage.

While some advocate the use of weeds to produce recombinant protein products, no specific examples have been suggested. Weeds in general score poorly in all categories. The suggestion of using weeds arises from the fact that weeds can be easily differentiated from food crops and serve no other useful purpose. However, they would often be able to outcross with native weeds, which gives them a lower rating for the environment. Little is known about how weeds may fair in downstream processes or product safety. It would require decades of research and cost millions of dollars to fully characterize these systems. At the end of that time, it may be clear if weeds could be a viable production system—but the answer may be that they are unacceptable.

Tobacco is one of the favorite plants used in the laboratory; this is undoubtedly why it was one of the first crops used for plant production systems. In addition, there is little need for concern about its effect on the environment or intermixing with food. One disadvantage in using tobacco for the direct delivery of human health products is its high alkaloid content. It may also have some antinutritional properties, and it is generally not palatable to humans. For larger acreage and low-cost products such as industrial enzymes, tobacco would not be a good choice because of its relatively high cost of production. This crop fits best with parenteral products that have higher profit margins and high purification requirements compared to industrial enzymes. In a unique situation, by using a viral vector system, tobacco can be one of the best systems to deliver small-scale therapeutics in record time. This is being explored for single-chain antibodies and other small proteins ([Grill et al., 2002](#)).

Cell cultures and hydroponics are more suited for containment than all other plant choices. The physical facility required for growing material is

analogous to microbial or animal cell culture systems, making this one of the most expensive ways to produce raw material from plants. It is highly unlikely that this cost disadvantage could be overcome any time soon. These systems may be some of the best when expressing proteins that are toxic or deleterious if released.

Sunflower and canola are good choices in most categories. There are some known allergenic problems associated with sunflower (Zitouni *et al.*, 2000), which slightly lowers its rating for product safety. This is probably only a significant factor when producing orally delivered products. Sunflower, canola, and sorghum have the potential to outcross with weedy species; therefore, they have the worst rating for safety, which is the ability to intermix with food and the ability to outcross with weedy species. This safety issue can be controlled by management practices, depending on how and where the plants are grown, although they will increase the cost of production slightly and limit the flexibility of areas in which the crops can be grown. While it may be possible to overcome this limitation, it will be difficult to convince the public and regulatory agencies that practices are in place such that these crops pose little risk. The recent entry of safflower as a recombinant protein production vehicle may help to limit some of the unwanted characteristics and provide an alternative to sunflower.

Bananas and tomatoes may be good choices for orally delivered products since they are highly palatable, assuming that a dose can be obtained in convenient amounts of plant tissue. These crops fall short in producing parenterals because of their low protein content, which impacts purification costs, and because they are difficult to harvest, transport, store, and extract. They also do not have the infrastructure or the cost advantages needed to grow large volume industrial products.

Soybeans are one of the better choices for industrial products, in theory, based on their high protein content and low cost. They can also be used for human health products, although they have a slightly lower rating for product safety because of some known allergens and antinutritional agents (Kleine-Tebbe *et al.*, 2002; Rihs *et al.*, 1999). This should not be a major disadvantage for industrial products, however, and its cost advantages and self-pollination aspects make this crop a good candidate. The major disadvantage of soybeans is that they are very difficult to work with in the laboratory. This limitation will undoubtedly be reduced with time, and soybeans may become one of the preferred crops for industrial products in the future.

Maize has been the most used crop to date for the production of recombinant proteins in plants (Hood *et al.*, 1999). The reasons become apparent when examining the data in Table III. Maize is well accepted as a safe product (GRAS) and is widely used in food, feed, and industrial applications today (Watson, 1988). The production cost of maize is very low, and the

infrastructure can handle large or small acreages for industrial or pharmaceutical products. Storage and transport of seed and protein purification from flour are very compatible and flexible with current practices without special handling. In addition, maize has an advantage in that the kernels can be mechanically separated to yield a germ fraction with enriched protein and an endosperm fraction with enriched carbohydrate (Watson, 1988). This allows for easy use of the carbohydrate fraction for industrial applications such as ethanol production. In this way, not only is the cost of the raw material reduced but the waste products are handled as well. There are no known agents in maize that generally interfere with protein purification. Finally, the grain can be processed with little or no heat inactivation steps without affecting the nutritional or taste properties, making this a good candidate for orally delivered products.

The disadvantage of maize is the potential for intermixing with the food supply. This one feature has brought other crops of lesser strength to the forefront. Intermixing potential can be handled by management practices, but the public's confidence needs to be gained. While skeptics may claim no food source can ever be used to produce pharmaceutical products, we must remind ourselves that both eggs and yeast are used to make pharmaceuticals. Not only is there not a problem with intermixing in these instances, but also the public has accepted these as distinctive production systems from the food system. Maize as well as other plant systems must build an infrastructure dedicated to pharmaceutical protein production, which is as distinct from food production as edible eggs are from vaccine production.

IV. PRODUCTS

When should one plant system be chosen over another system, and for what specific types of products? Table IV summarizes some of the key characteristics of production as they apply to different types of products.

Table IV
Production Characteristics of Different Product Types

	Acreage	Product safety requirement	Relative cost of raw material	Relative overall production cost
High purity	Low	Med	Med	High
Human health				
Oral delivery	Low	High	High	Med
Industrial	Med-high	Low	Low	Low

The following sections describe progress on these types of products and the features that are most critical in choosing the best production system.

A. HIGH-PURITY HUMAN HEALTH PRODUCTS

Most therapeutic proteins were historically derived from animal tissue because they could only be produced by animals. The advent of recombinant DNA technology has led to many new hosts for therapeutic protein production and the design of new therapeutic proteins. The recent explosion of antibodies as a class of therapeutics, a class that did not exist 20 years ago, has propelled these proteins to become the major class of new protein therapeutics. Antibodies also represent a challenge for the industry (Chu and Robinson, 2001; Houdebaine, 2000; Schwartz, 2001). How can these types of products be produced inexpensively to realize their great potential? Currently, the preferred production system is animal cell culture because microbial systems are an inadequate host for making complex mammalian proteins. However, the cost of production from animal cell culture is quite high, resulting in antibody products that are too expensive for many applications. This has opened up the possibility for plants to play a major role for these products because plants can adequately synthesize these complex proteins and their production costs are lower than animal cell culture systems (Schillberg *et al.*, 2003).

Another class of proteins that fit the plant technology platform is that of blood proteins. In many cases, extremely large quantities are needed and at a very inexpensive price. Proteins such as human serum albumin have been a target for a low-cost supply for years to increase its applications. In addition, these proteins can be made in an animal-free system, which is a great concern when considering the amount of blood required from unknown sources to obtain the products. Additional proteins, if made in quantity and at a low cost, may be the basis for an eventual blood replacement. Both hemoglobin (Merot *et al.*, 2002) and human serum albumin (Farran *et al.*, 2002; Sijmons *et al.*, 1990) have already been expressed in plants.

Glycoproteins have different glycan structures depending on the host used for expression. One significant difference is the addition of sialic acid on some mammalian proteins, which does not occur in other systems. While this represents a very limited number of proteins, it does represent an important class of proteins used in the pharmaceutical industry. Sialic acid allows therapeutic glycoproteins to have a prolonged clearance time in the blood stream (Rasmussen, 1991). One of the major limitations is that plants do not usually make sialic acid, although a recent study demonstrated that at least limited sialylation may be possible (Shah *et al.*, 2003). This option is not practical for recombinant proteins until plants are better equipped with both the specific neuraminic acid transferase and the enzymes required to make

sialic acid. While this seems possible, it is not without technical challenges and will require several years to accomplish. Alternatively, if the therapeutic proteins are not toxic, it may be possible to use a higher dose to overcome clearance time and achieve the same physiological results without sialic acid. While this may add cost to the product, the plant source may still cost less than alternative systems and easily accommodate the increased cost needed for a larger dose.

In addition to sialic acid, plant glycoproteins have other subtle differences from their mammalian counterparts (Faye *et al.*, 1989; Lerouge *et al.*, 1998), including the addition of xylose and the change in fucose from an $\alpha(1,6)$ to an $\alpha(1,3)$ linkage. These carbohydrates have been implicated in the allergic response as it relates to pollen allergens (Garcia-Casado *et al.*, 1996), and they may also be responsible for allergic reactions with recombinant proteins. (van Ree *et al.*, 2000). In several cases, recombinant proteins expressed in plants contained carbohydrate structures that have been characterized with no apparent effect on activity (Bakker *et al.*, 2001; Cabanes-Macheteau *et al.*, 1999; Ma *et al.*, 1998; Samyn-Petit, 2001). In a few examples, these proteins also were examined for their possible role in allergic response. The minor changes in glycosylation, however, did not appear to be implicated in an allergenic response (Chargelegue *et al.*, 2000; van Ree *et al.*, 1994).

Commercial high-purity recombinant protein products from plants were first demonstrated with the diagnostic products avidin (Hood *et al.*, 1997) and β -glucuronidase (GUS) (Witcher *et al.*, 1998) expressed in corn grain. The large-scale production of a foreign protein in a recombinant plant has recently been achieved for bovine trypsin, also expressed in corn grain (Woodard *et al.*, 2003). These first products represent the prototype for most high-value therapeutics. In these instances, the raw material is only a small cost of the final product. What is critical is the concentration of the protein in the starting material before extraction. As is the case for all types of products, higher levels of expression and accumulation are the key drivers. Unlike industrial enzymes, however, the price for obtaining the high concentrations can be easily absorbed, even if this causes a significant loss in agronomic yield. This is important because there are several variant lines of crops that could cause an increase in recombinant protein as a percent of dry weight but that may result in a net decrease in overall agronomic yield. One example may be breeding recombinant protein genes into opaque mutants of corn. The yield of opaque corn is significantly lower than normal hybrid maize. However, the concentration of recombinant protein can be as much as 3–5 times higher in this grain, allowing significant savings in extraction costs. This tradeoff is easily acceptable, not only because of the relatively low cost of raw material compared to the final product but also because of the relatively low overall acreage requirement.

B. ORALLY DELIVERED PRODUCTS

In general, oral delivery of vaccines or therapeutics is favored because of the convenience of this method. The economic advantages of oral delivery when using plant-based production systems that eliminate the need for purification are also compelling. The key driver is to obtain sufficient levels of expression to enable oral delivery. The required dose must be in an amount of plant material that is manageable for consumption at a single sitting. If the material is not particularly palatable, it could be presented in pill form. If the material is palatable, a single dose may be in the form of a wafer or other processed food-like substance. There are several processing alternatives depending on the available food or feed industry procedures for the chosen plant material. A few alternatives have been explored, for example, with corn (Watson, 1988). Selecting tissues that contain high levels of protein as measured by the concentration of the protein per gram of tissue is advantageous for oral delivery rather than as a percent of total soluble protein, as is the case for highly purified products. This favors plant tissues that are low in water content, as opposed to tissues such as fresh fruits. Preferred plant tissues include seeds, which are low in water content and rich in protein, and leaf tissue, if it can be dried to reduce water content without interfering with the product. Preferred crops include dried alfalfa and edible grains such as corn or rice (Streatfield *et al.*, 2001, 2002, 2003). Potatoes have been used but may cause some problems with digestion when eaten raw by humans (Tacket *et al.*, 1998), although recent studies using corn germ appeared to overcome these mild adverse symptoms (Tacket *et al.*, 2004). In general, plant tissue that is consumed as food or feed should provide an extra level of comfort with regard to orally delivered product safety.

Processing costs for orally delivered products are greatly reduced compared to purified proteins, and if it were not for tight quality control and regulatory standards, the cost would be similar to industrial proteins. Therefore, most plant systems will be economically viable as long as expression is moderately high. The more critical concern is to demonstrate that during whatever limited processing is necessary, the protein product is not altered. Most processing steps for grain products include a high-temperature step that will most likely inactivate the protein. It has been shown, however, that antigens can be processed in plant tissues at reduced temperatures, retaining their native state while at the same time achieving the desired effect (Streatfield *et al.*, 2002).

Oral delivery can be used with therapeutic proteins, growth hormones, or vaccines. Because of the large cost advantage in plants, obtaining doses of 1000 times the injectable dose is technically and economically feasible. This means that even if the oral product requires a 1000-fold higher dose to be equivalent to an injectable dose, it can be effective and cost the same or less.

Most oral delivery research has focused on vaccines. Immunogenic responses have been observed with several plant-based vaccine candidates in experimental animal systems. Protection from disease symptoms has been observed in target animal trials (Lamphear *et al.*, 2002); this has recently included the possibility of passive immunity for the application to newborns pigs when boosting the lactogenic immunity in sows (Lamphear *et al.*, 2004). Human clinical studies have not yet advanced this far and have focused on inducing an immune response (Kong *et al.*, 2001; Tacket *et al.*, 1998, 2000, 2004).

Presumably, orally consumed proteins are protected by surrounding plant tissue that allows a sufficient dose to survive the digestive processes of the stomach and small intestine (Bailey, 2000). This biological encapsulation, or bioencapsulation, has an effect similar to the use of encapsulating agents such as liposomes with oral vaccines produced from alternative sources. It is not yet known if bioencapsulation is true for all plant tissues or if it is restricted to certain types of tissues. Grains offer the potential for native protease inhibitors, carbohydrates for protein stability, and a granular matrix that may account for some of the proteins' slow release and ability to survive into the gut.

C. INDUSTRIAL ENZYMES

Industrial proteins are used for protein processing and purification, diagnostics, and processes relating to food, feed, and industrial applications. Most commercial industrial enzymes are currently derived from microbial sources, either natural or recombinant, primarily because all other systems are cost prohibitive. However, using plants as a production system is a developing industry that could be competitive in the next few years (Hood, 2002; Hood and Woodard, 2002).

There are several categories of enzymes, including hydrolases, transferases, oxidation/reduction (redox) enzymes, lyases, isomerases, and ligases. Only a few are routinely used in industrial applications. Hydrolases such as amylases and proteases are the most commonly used industrial enzymes. Other enzyme classes, particularly redox enzymes, will be useful in industry when they become less expensive and thus available for testing in various processes.

The principal non-food industrial uses for enzymes are starch hydrolysis (amylases), textile desizing (amylases), leather production (proteases), pulp processing (xylanases), detergent additives (proteases), and animal feeds, which represent substantial markets to date. However, a reason for the limited use of enzymes in certain applications is that no cost-effective source of the enzyme is available. Transgenic plant systems can meet both the scale and cost targets for many new applications.

Using an enzyme as a catalyst in a particular process has advantages that result from the enzyme properties of functioning at ambient temperatures, in aqueous solution, and usually at near neutral or physiological pH values. Most enzymes have a very high specificity and function in low concentrations to produce the desired effect. Enzymes can produce a rapid reaction and have a low level of toxicity. However, these highly active proteins are generally unstable and require a degree of care and expertise in their preparation and use. In addition, extremes of pH and temperature can limit activity levels, even if these conditions do not destroy the enzymes. Enzymes may be inactivated by the presence of various ions, organic molecules, or solvents, components that are often present in organic reaction systems and in large-scale industrial processes. Significant changes in the process may be necessary when switching from chemical to enzymatic catalysts. Alternatively, the enzyme of interest could be engineered using a variety of emerging technologies to more closely fit into the reaction conditions of the current target process. Nevertheless, considering the large market opportunity for industrial enzymes and their environmentally friendly benefits, it is well worth the effort to generate an efficient production system and make process or protein changes to accommodate them.

For achieving low-cost industrial enzymes, plants as a production system have many advantages over current competing technologies. Plants are an excellent system for cost competition because of their protein expression potential and minimal production costs. Various plant systems can be used to produce enzymes, such as tobacco, alfalfa, barley, canola, and corn (Hood and Woodard, 2002). The first recombinant proteins produced and sold from transgenic plants were avidin and GUS (Hood *et al.*, 1999; Witcher *et al.*, 1998). Avidin, the first plant-produced recombinant protein product to be marketed, was first sold in 1997. Applications of these proteins include using the GUS protein as a research reagent and using avidin as a diagnostic reagent and protein purification tool for biotinylated proteins.

Currently, the highest profile plant-based industrial enzyme projects involve enzymes for applications in feed, cleaning agents, processing reagents, the wood products industry, and biomass conversion. These include mainly xylanases and cellulases for the textiles and wood products industries, as well as laccase and trypsin (Bailey *et al.*, 2003; Hood and Woodard, 2002; Hood *et al.*, 2003b; Woodard *et al.*, 2003). For food applications, enzymes are used in the conversion of raw materials to form intermediate products that are more useful in food processing and in food formulations. The treatment of food products with enzymes makes them more palatable or more stable or enables the development of some other desirable property. Enzyme production for the food industry is primarily through microbial fermentation. However, major value from edible plant-based protein or enzyme production is readily apparent. For example, brazzein, a high intensity sweetener, has been

expressed in transgenic maize (Lamphear *et al.*, in press). For food applications, the direct addition of corn meal is possible, allowing for both a bulking agent and a low-calorie sugar substitute. The cost is kept to a minimum since there is no added cost in preparing the sweetener, and the corn meal can be processed using methods similar to conventional methods.

Two potential new areas for enzyme applications are biomass conversion and the wood products industries. For biomass conversion applications, enzymes that degrade cell walls will be useful. The resulting products from those enzymes are monomeric components of walls, primarily 5- and 6-carbon sugars, but also amino acids and lignin products. These will be substrates for a variety of applications, including fermentation into ethanol and specialty chemicals. Oxidation/reduction enzymes such as laccase (described previously) and peroxidases (Caramelo *et al.*, 1999; De Jong *et al.*, 1992; Jensen *et al.*, 1996) will find many new markets as new production systems are introduced. These enzymes can potentially replace many applications currently using chemicals that are damaging to the environment. Because of the scale-up potential of the plant system and the low cost of goods, plants will likely be the system of choice for large-scale enzyme production for these industries. It is important to explore many plant systems, enzymes, promoters, and targeting sequences to understand the factors that affect expression levels, and hence the economics of industrial enzyme production in plants. To date, such experimentation has been limited.

V. PUBLIC ACCEPTANCE

GMO (genetically modified organism) products that enter the food supply are generally not labeled, and thus the public is not given a choice about consumption of these products. This has led to a controversy about whether to label GMO products, resulting in a public that is skeptical about their value and safety. While GMO non-food products can be easily labeled and are not intended for the food supply, the skepticism and lack of trust have carried over to them as well. For pharmaceuticals manufactured from any source, however, the public has a clear choice whether or not to use the drug product. If the product is effective, they experience the direct benefit. In this example, the public has been much more accepting. Therefore, there should not be any major concerns about the plant-produced products themselves. In fact, the animal-free source, lower cost of goods, and oral delivery should make the public enthusiastic about these products.

The major public concern is that non-food products produced in plants will not somehow end up in the food supply. The public usually assumes that

GMOs will be grown with commodity practices that do not limit the intermixing of the plants. In practice, however, these plants are grown with regulatory standards that are similar to those used for pharmaceutical and industrial products from non-plant systems. There are new regulations for these types of products that are similar to practices used in non-plant systems. It is hoped that in the future the public will have the same confidence for plants as they now have for eggs or yeast, potential food products that are currently used for pharmaceutical and industrial protein production.

The regulations and containment practices used for plants, as well as for other hosts, do not overlook the remote possibility that unintended exposure may occur, regardless of how insignificant it is. This situation calls for a safety and risk assessment that would be accepted by the industry, regulatory agencies, special interest groups, and the public. The risk assessment needs to be science based and could be similar to what is used for other systems. A system has been proposed for quantification of unintentional exposure that is based on evaluating the risk that is linked to the hazard and exposure. Formulas exist for regulated articles, and it has been suggested that these equations be modified for non-food products produced in plants, allowing a quantitative method of assessing the risk of unintentional exposure (Howard and Donnelly, 2004).

The requirements for producing non-food products in transgenic plants are considerably different from those for producing food products. They include physical isolation, delayed planting times from food crops, agronomic support, dedicated equipment, and frequent monitoring. When taking these practices into account, the amount of a crop that may inadvertently end up in the food supply and the associated risk can be calculated. Aprotinin is an example of a pharmaceutical product that has been produced in plants (Delaney *et al.*, 2003; Zhong *et al.*, 1999). It has been calculated that even without any of the required confinement practices, the amount of aprotinin that could inadvertently end up in the food supply would be well below the level needed to show an effect (Howard and Donnelly, submitted). This means that there is no hazard even if the plants were grown and harvested as a commodity crop. If the required containment practices are used, the calculated level of risk can be a million times below the non-contained exposure levels.

In addition to potential toxicity, some proteins can be allergenic. Using the case of aprotinin, we can calculate how much transgenic corn containing aprotinin used for commercial production must be eaten to induce an immune response. In this example, one would have to eat 350 tacos or 350 bowls of cereal at one time on three different occasions just to ingest the minimum dose needed to observe an immune response.

The above calculations seem inconsistent with the fact that the general public considers these non-food products to be a grave danger. Aprotinin is one case for which even though the product is intended for non-food

applications, it is already in the food supply from natural sources and no problems have ever been documented. Other proteins discussed earlier, trypsin and avidin, are also in the food supply from natural sources, and we would anticipate similar results. Clearly, the fear of products entering the food supply is unwarranted for proteins that are already in the food supply at much higher concentrations than an accidental intermixing would produce. This is not to say that all proteins would have the same risk profile, and it is this that fuels public fears. Thus, a case-by-case assessment is needed to allay public concerns if it is applied to all non-food products.

In conclusion, confinement practices can reduce unintentional exposure for most proposed products to a level that is orders of magnitude below the slightest concern for food safety. However, safety assessment models need to be standardized and accepted by the public, regulatory agencies, and special interest groups. Finally, we need to treat plant-made pharmaceuticals and plant-made industrial proteins with the same considerations as other pharmaceutical production systems such as eggs or yeast and not as value-added agriculture.

VI. CONCLUSIONS AND FUTURE

Plant production technology is still in its infancy. The key to economic feasibility of products derived from this technology lies first with the expression level of the foreign genes. The best plant expression systems are now approaching 1% of the dry weight of tissue. This would enable plants to be the low-cost producer of most proteins compared to other current systems. However, there is no theoretical reason why plant systems cannot achieve levels of expression that are at least an order of magnitude higher. Microbes can produce proteins that comprise as much as 70% of their energy store. For plants, especially grain with storage proteins, it is reasonable to assume that the recombinant protein can constitute or replace other proteins that supply a sink for amino acids. Many advances in technology remain to be made to take this production system to higher levels.

Direct delivery will be essential for many oral vaccines, and in the future this process may deliver nutraceuticals and possibly some therapeutics. The possibility of using products that can be prophylactic, such as specific antibodies, is now approachable using this technology. Feed products easily lend themselves to direct delivery because growers can control the diet and supplements of domesticated animals. Direct delivery of industrial products results in added cost savings for production.

The increase in research efforts for plant-made products will also translate into many more clinical and industrial application trials to demonstrate

efficacy of these products. There will undoubtedly be additional technologies available that will increase the potency of the orally delivered products. However, while the data to date are promising, the anticipated increase in effort will also show that the technology will have limits. These will become clear as the research is completed.

The next several years should also see detailed regulatory guidelines that will pave a clear path for later stage clinical trials and manufacturing of pharmaceutical products. A separate regulatory path for industrial products should be developed. This will help reduce risks and increase acceptance. Regulations currently exist for growing non-food crops, but these will need refinements before large-scale industrial and nutraceutical products can be commercialized.

The most likely near-term commercialization possibilities for pharmaceutical-type proteins from plants will be with proteins currently made from animals or animal cell cultures. These have a much clearer advantage of cost and safety than products made from microbes. This should set the stage for additional orally delivered products and industrial products that can compete with microbial systems for cost. The industry is young, and it is impossible to even imagine the full range of products at this time.

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