The Production of Foreign Proteins from Genetically Modified Plant Cells

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While traditionally used to produce natural products, plant suspension cultures can also be utilized for the production of foreign proteins. Production of these high-value products in plant cells is an economically viable alternative to other systems, particularly in cases where the protein must be biologically active. There are several advantages to using plant cells for the large-scale production of secreted proteins. Plant cell media are composed of simple sugars and salts and are therefore less expensive and complex than mammalian media. Consequently, purification of secreted protein is simpler and more economical. Additionally, plant cell derived proteins are likely to be safer than those derived from other systems, since plant cell pathogens are not harmful to humans. In this chapter, we will review foreign protein production from plant cells. To begin, we will discuss the behavior of plant cell cultures, products produced by plant cells, protein secretion and its relationship to purification, and the performance of plant cells as compared to whole plants and other alternative hosts. After a brief discussion of gene transfer techniques, we will present strategies to overcome the limitations of protein production, including protein stabilization, novel production schemes, modeling, and scale-up considerations. To conclude, we will discuss implications for future development of this technology.

Keywords. Plant cell, Foreign protein, Transgenic, Secretion, Increased production

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1 Introduction

1.1 Overview

This chapter addresses the emerging use of plant cell culture as a vehicle for foreign protein production. This technology is a combination of the techniques used to produce natural products using cultured plant cells and those used to produce recombinant proteins in whole plants or other hosts. Plant cell culture is a long-established technique for producing flavors, colors, and other important natural products. For example, plant cell culture has been used industrially to produce shikonin, a red pigment with anti-inflammatory properties [1]. However, the lower growth rates and the lower productivity of plant cells limit the applicability of using cultured plant cells to produce such products. In gen-

eral, economic factors limit the use of large-scale plant cell culture to the production of rare, high-value substances. Based on this need, therapeutic protein production is a good candidate for the utilization of plant cell cultures.

The expression of foreign proteins in plant cells is conceptually similar to protein expression in other host systems, but differs in the details. These differences are the source for both the "virtues" and "vices" of the plant cell production system. Tobacco and other plant cells may be genetically transformed by well-defined means and are highly stable. Since they are derived from higher organisms, plant cells have the capacity to carry out any desired post-transcriptional or post-translational modifications on the transcribed RNA or protein of interest, including intron removal, glycosylation, disulfide bond formation, and protein folding events. As high eukaryotic cells they are also capable of recognizing and cleaving protein leader sequences, a trait that facilitates protein-targeting strategies such as secretion or localization within organelles. Unlike many other higher cell types, plant cells grow easily in suspensions and can be grown to very high biomass densities. However, plant cells exhibit slow growth rates and more complex control of gene expression pathways. These (and perhaps other unknown) traits lead to low protein expression levels, typically in the range of 0.1 – 1 mg/l, for plant cell based systems.

The history of work in plant cell protein expression can be divided into three phases. The first phase of research focused on confirming the feasibility of this technology: developing usable constructs, DNA-transfer techniques, and expressing simple reporter proteins. The second phase focused on establishing the usefulness of these methods: improving early techniques, establishing standard protocols, and expressing proteins with measurable biological activity and economic value. The third and most critical phase of the research will be demonstrating economic feasibility: increasing the expression level of useful protein products, carrying out complete purification of plant-produced proteins, conducting successful *in vitro* and clinical studies, and scaling up these procedures for cost comparison with other available hosts. To meet these challenges it will be crucial to combine current knowledge with innovative strategies.

1.2 Significance

Plant cell culture is a safe and useful production scheme and an important alternative to other protein production systems. Plant cells are uniquely suited for the safe production of protein-based drugs because, unlike microbial and mammalian cells, they are not a potential source of dangerous pathogens. Because of their high economic value, recombinant proteins are among the best candidate products for commercial production in plant cell culture. The accumulating body of research over the past two decades suggests that plant cell culture is a workable system for producing a variety of gene products. Almost without exception, plant-produced proteins are correctly folded and biologically active. Thus, at its present state, plant cell culture could be used as an in-house protein production source for protein characterization studies or to produce sufficient material for early clinical trials. The study of protein production in plant cells is

also justified as a means for understanding the behavior of protein production mechanisms in plant cells and for identifying plant-derived therapeutics – including proteins. In fact, several useful plant-derived proteins have already been identified. Plant cell culture would be particularly appropriate for the production of these "natural" therapeutic plant proteins, such as ribosome inactivating proteins (RIPs), which have a variety of potentially useful pharmaceutical activities such as antiviral and antitumor activity [2]. If production levels can be increased, plant cells may also be an important future source for the large-scale production of therapeutic proteins for human use.

2
Foreign Protein Production in Plant Cells

2.1
Background

The term "plant cell culture" refers to the propagation of any plant-derived cell tissue in gently agitated liquid media. Plant cultures can be classified as unorganized cultures (such as callus, suspension, or protoplast culture) or organized cultures (such as root or embryo cultures) depending on the tissue source and level of differentiation [3]. Cultures are first initiated by taking an explant from specific regions of a seed or plant and sterilizing it with hypochlorate or peroxide to minimize the risk of contamination. The sterile explant is treated with cell wall degrading enzymes, stimulated with hormones, and subsequently grown in medium (consisting of nutrients, salts, vitamins, and growth factors) until cell callus forms. After several generations, the callus becomes established (or friable) and may be transferred to liquid media where the cells will grow as a suspension. Initially these cultures grow slowly as large clumps of cells and must be cultivated in small flasks or six-well plates agitated by a gyratory shaker. After the new suspension has been subcultured into new media the cells become more evenly distributed and eventually develop into a fine suspension. Healthy, established plant suspension cultures can be easily cultivated in larger bioreactors. If desired, the cells can be treated again with cell wall degrading enzymes and grown as protoplasts. However, protoplasts have greater shear sensitivity and will regenerate a cell wall over time.

Beginning in the 1970s significant effort was invested to produce high-value secondary metabolites in plant cell culture. Despite the fact that some metabolites were produced at concentrations equal to or greater than those present in the corresponding plant tissues, only a few selected natural products could be produced economically in plant cells. The main limitation is the high capital costs of fermentation equipment compared to the moderate sale price and small market size for these substances. Around the same time, technology was developing for the manipulation of DNA and subsequent protein production in foreign host cells. With the successful marketing of early recombinant protein products such as insulin and human growth factor, it became clear that important and extremely valuable products could be produced using these methods.

For this reason, over the past two decades the production of recombinant proteins using plant cell culture has received considerable attention as a new research area. The feasibility of recombinant protein production also stems from the development of the *Agrobacterium* transformation technique [4] and other DNA-transfer methods, which incorporate the desired DNA into the plant genome. Plant cells may also be genetically transformed by several methods for the stable production of foreign proteins. The first transgenic plants were produced almost 20 years ago [5], followed closely by the first transgenic cell suspensions. These early studies focused on producing simple marker proteins or proteins from other plant species. Through further work, it became evident that producing commercially useful proteins with plant cells is a worthwhile goal because they can be grown on simple, protein-free media and are able to produce even the most complex protein products [6]. A significant number of proteins have already been produced on a laboratory scale by this method.

2.2 Growth Characteristics of Plant Cells

The growth pattern of plant cells in suspension culture is remarkably similar to the behavior of lower cell types such as bacteria and yeast. Figure 1 shows the typical growth performance for plant suspension cultures in terms of fresh weight and dry weight. These measurements (along with the settled cell volume and packed cell volume) are frequently used to monitor plant cell growth because counting individual cells within large aggregates to obtain a cell number is extremely difficult. The growth pattern of plant cells consists of three main phases: a lag phase, an exponential growth phase, and a stationary phase. There is also typically an acceleration phase and a deceleration phase separating these respective main phases. Doubling times for plant cell suspensions generally range from 1–4 days during exponential growth, depending on the cell type and culture conditions. These doubling rates correspond to total batch culture times of 7–28 days. Maximum cell concentrations typically range between 10 and 18 g l^{-1} dry weight or 200 and 350 g l^{-1} fresh weight.

Typically, the production of foreign proteins in plant cell culture is growth related, due to the use of unregulated strong promoters [7] such as the cauliflower mosaic virus (CaMV) 35S promoter. This strategy simplifies the optimization of protein production, because a reactor optimized for growth will likely also be near optimum conditions for product formation. As shown in Fig. 1, peak protein production is typically observed during exponential growth. The timing and level of maximal protein production are influenced by culture conditions such as cell concentration at inoculation and aeration [8]. Apparently, constitutive promoters are most active when the cell is actively dividing. However, this strategy of continuous protein production may burden the cells, increasing the likelihood of genetic drift or gene silencing. Also, the protein may be more susceptible to degradation, inhibition, instability, and other losses when it is produced gradually, since the average residence time for protein in the media will be several days. Inducible promoters can be used as an alternative to constitutive protein production. With this type of production

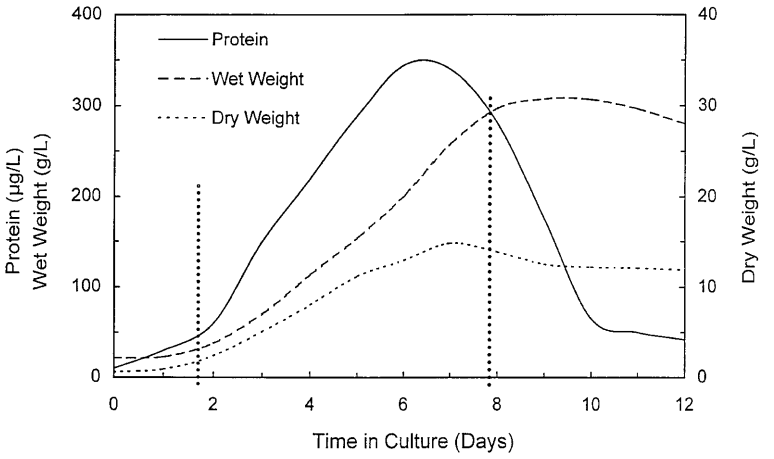


Fig. 1. Batch growth and antibody production of *Nicotiana tabacum* (BY-1) cells in terms of µg l^{-1} protein, wet cell concentrations, and dry cell concentrations. *Dashed vertical lines* represent the boundaries between lag, exponential, and stationary growth phases. The 4-day offset between peak concentrations for biomass and protein product indicates that growth and product formation are not directly linked

scheme, cell growth and protein production steps are de-coupled. This production method can reduce the genetic burden to the transformed cells and the exposure of protein to degrading influences. These aspects of protein production will be discussed in more detail later in this chapter.

Unlike lower cell types, plant cells seem to effect changes in the pH of their growth environment during batch growth. In experiments conducted in our laboratory (unpublished results) attempts were made to overcome this effect by growing cells in media buffered with 0.1 M PIPES [piperazine-*N,N′*-bis(2-ethanesulfonic acid)] and by resetting the pH to 6.5 at the end of every day. Despite the increased regulation of pH in these latter two culture conditions, the pH tends to become more acidic during the exponential growth phase and less acidic during the deceleration and stationary phases. Although plant cell cultures are resistant to pH adjustment during batch culture, pH control is still possible through semi-continuous addition of acid or base in response to changes in pH. The need for pH control, and thus the effort that should be invested, will depend on the specific application. The option of pH control should be pursued in cases where the protein of interest is less stable under mildly acidic conditions or during semi-continuous protein harvest if the yield of the first purification step is sensitive to pH.

2.3 Product Range and Quality

A significant number of proteins have been successfully produced in plant cell suspensions. Table 1 presents a summary of important proteins along with re-

Table 1. Expression levels, activity, and quality of important proteins produced in plant cell culture

Protein of interest	Expression level	Activity or function	Protein quality	Ref
Erythropoietin	26 ng g^{-1} total protein	*In vitro* erythroid colony formation	Homogeneous, glycosylated	[9]
Human Interleukin 2	0.10 mg l^{-1} secreted	Cell proliferation	Not reported	[10]
Human Interleukin 4	0.18 mg l^{-1} secreted	Cell proliferation	Heterogeneous	[10]
Guy's 13 murine monoclonal antibody	18 μg g^{-1} soluble protein	Growth inhibition of *Streptococcus mutans*	Heterogeneous	[11]
Human Granulocyte-Macrophage colony-stimulating factor	0.25 mg l^{-1} secreted	Cell proliferation	Heterogeneous	[12]
Anti-arsonate antibody (murine heavy chain)	0.36 mg l^{-1} secreted	Antigen matrix binding Protein G binding	Heterogeneous	[6]
Anti-TMV antibody (murine full-size)	9 μg g^{-1}soluble protein	TMV binding	Homogeneous	[13]
Single-chain Fv (α phytochrome)	5 μg g^{-1}soluble protein	Antigen binding	Homogeneous	[14]
Chloramphenical acyl transferase (CAT)	17 units ml^{-1}	Positive CAT assay	Not reported	[15]
Preproricin	1 μg g^{-1}soluble protein	*In vitro* translation inhibition	Homogeneous, glycosylated dimer	[16]
Human α_1-antitrypsin (AAT)	22 mg l^{-1}	Inhibition of porcine pancreatic elastase	Heterogeneous	[17]

ported expression levels and the quality, activity, or characterization of the product. In every measurable case, plant-produced proteins demonstrate biological activity that is nearly indistinguishable or in a few cases higher than the activity of commercial standards. This tendency for good *in vitro* biological activity is an indication of proper folding, post-translational processing, and protein transport of the plant-produced protein. Despite the fact that plants and animals are from different biological kingdoms, their common status as "high" eukaryotes is reflected by their similar synthesis and processing of protein products.

Because of these findings, there is great optimism that plant proteins will exhibit *in vivo* activity as human therapeutic agents. However, some plant-produced proteins do exhibit a degree of heterogeneity in their molecular weight and chemical nature. In these instances, a few or even several different mass

species appear on Western blots of unpurified protein product. The mass differences are usually attributed to differing levels of glycosylation, product dimerization, or enzymatic cleavage. For use as an injectable therapeutic the protein product should be homogeneous after purification. Therefore, the source of this variation must be identified and eliminated. Otherwise, it may be necessary for one protein species (perhaps the one with the highest activity, largest concentration, or least risk of antigenicity) to be purified away from the others.

2.4 Product Secretion and Purification

One major advantage of plant cell culture as an expression system is the ability of plant cells to produce and secrete biologically active proteins. The term "protein secretion" refers to protein transport through both the plasma membrane and cell wall into the extracellular media. This is a metabolically dependent process [18] that can be directed by both native [6] and plant [14, 19] leader sequences. It is somewhat surprising that the native (mammalian) signal sequence is so effective in directing protein secretion in plant cells. One study suggests that protein secretion in plants follows a "default" pathway [20]. In this study, several non-secretory enzymes were directed for secretion using an endoplasmic reticulum (ER) directing signal. It may be that direction of the protein to the ER is necessary and sufficient to initiate a chain of events leading to protein secretion.

While the capacity of protein targeting and secretion is a hallmark trait of plant cells (along with other higher cell types), complete secretion (or export) of protein products beyond the cell wall and into the extracellular media has been problematic in some early and anecdotal cases. For example, plant-produced erythropoietin penetrated the plasma membrane of the cells but was confined within the cell wall during cell culture [9]. Following treatment with cell wall degrading enzymes, the protein was secreted at low levels during protoplast culture, due to the removal of the cell wall as an obstacle to secretion. Some efforts to secrete fully assembled antibodies from plant cells were also unsuccessful [21] with the assembled product also confined mainly to the apoplastic space. It has been postulated that proteins penetrate the plasma membrane by leader sequence directed export events and the cell wall by simple pore diffusion. As a corollary, it is hypothesized that penetration of the cell wall is the limiting step for protein secretion and that protein export is limited by the cell wall's pore size distribution for large proteins. Based on studies of cell wall structure the passage of large proteins through the cell wall into the extracellular media will be limited, perhaps with a cutoff as low as 20 kDa [22]. However, the structure and pore size distribution of plant cell walls varies for different species and even for different cell lines within a species. For cells that are well adapted to suspension culture, this cutoff appears to be significantly higher [6]. Protein transport through the cell wall may also be increased through treatment with dimethyl sulfoxide (DMSO) [23] or other permeabilizing chemicals.

If successful, transport of the protein out of the cell greatly simplifies downstream purification of the desired product because of the simple and protein-

free composition of plant culture media. Even at relatively low expression levels, the protein of interest comprises a significant percentage of the total secreted protein. Also, protein secretion eliminates the need for cell disruption. This omission reduces the difficulty of subsequent filtration or centrifugation steps because whole cells are simpler to process than cell debris. Since these steps are capital intensive, simplifying or eliminating them will significantly reduce purification costs. Despite these advantages, some aspects of protein recovery following secretion are still problematic. The low concentration of secreted protein in medium (due to limited protein expression levels) may increase the cost and difficulty of purification steps such as extraction and concentration.

2.5 Comparison with Alternate Host Systems

Microbial cells are excellent hosts for protein production because of their simplicity. Bacteria such as *Escherichia coli* grow rapidly (doubling time approximately 20 min) and accumulate high levels of protein (on the order of grams/l) without the product inhibition typical of other cell types. If every protein could be produced in an active form from microbial cells there might be no need for other hosts. However, the production of eukaryotic proteins in prokaryotic cells is limited because they lack the cellular machinery to fold, process, and secrete proteins. In general, eukaryotic gene products produced in prokaryotes are unfolded and inactive. Large quantities of unfolded protein accumulate as insoluble inclusion bodies that must be solubilized, refolded, and in some cases enzymatically treated to produce active protein. These steps are difficult, time consuming, and often have low yields. Even if they can be performed successfully, these steps greatly complicate protein purification leading to longer processing times and higher capital costs.

Some simple eukaryotic proteins can be produced using transgenic yeast cells. Protein yield from yeast cells is typically high – often in the range of 100 – 1000 mg l^{-1}. Commercial production equipment design and conditions for large-scale yeast culture are already well characterized by the beer industry. DNA can be transferred easily to yeast cells by well-established means using plasmid vectors or integrating vectors for small segments or yeast artificial chromosomes (YACs) for larger (> 100 kb) segments [7]. As eukaryotes, yeast cells are able to perform some post-translational functions that are impossible for bacterial cells. However, since yeast cells do not consistently remove introns, they are typically transfected with cDNA rather than natural genes. In addition, yeast cells carry out some post-translational modifications in a different manner than higher cells. Glycosylated groups on proteins produced by yeast are larger and contain more mannose than the correct patterns produced in mammalian cells. In some cases these enlarged and highly branched groups significantly hinder the desired activity of the protein of interest. Even in cases where *in vitro* activity is retained, *in vivo* activity may be lost due to systematic elimination of the foreign protein triggered by these antigenic differences in glycosylation. Eliminating potential glycosylation sites through point mutations in

the protein sequence can circumvent these problems. Since glycosylation is generally not required for biological activity, the modified protein will retain its desired activity. However, modifying the protein sequence to remove glycosylation sites is time consuming and labor intensive. Furthermore, the unglycosylated protein may differ significantly in its level of activity and stability *in vivo* compared with the native protein. Therefore, the most complex protein products (requiring glycosylation, assembly of multiple subunits, or other specialized modifications) may be better produced in "high" eukaryotic cells – cells originating from multicellular organisms.

For these "high" eukaryotic proteins, the most widely and commonly used host systems are mammalian cell culture and insect (or baculovirus based) cell culture. Mammalian cell culture produces the highest protein yields of these options – often in the range of 10 – 50 mg l^{-1}. Traditional mammalian cells, such as baby hamster kidney cells (BHK), are anchorage dependent or shear sensitive and must be cultured in flask-type growth chambers or on microcarriers in complex and expensive serum-containing media. These growth conditions result in slow growth and product that is difficult to purify from contaminating protein in the media. Newer and more robust cell lines, such as protein-free Chinese hamster ovarian cells (PF CHO) or hybridoma, have been successfully cultivated in spinner flasks and serum-free media. However, acclimation of mammalian cell lines to protein-free media requires several passages and careful attention. Since spinner flasks are inadequate for culture volumes beyond a few liters, large-scale cultivation of mammalian cells is typically limited to expensive perfusion or membrane reactor systems. Despite these challenges, the efficacy and appropriateness of commercial protein production using mammalian cells are well established for antibodies and other naturally occurring protein products. Insect cell culture produces lower protein yields than mammalian culture – typically in the range of 1 – 10 mg l^{-1} – but it is a more rapid and flexible production system. Through the use of the *Autographa californica* polyhedrosis virus, baculoviral constructs can be constructed by replacing the polyhedrin viral coat gene with a gene for the protein of interest [7]. Using this modified virus, untransformed insect cells can be directly induced to produce a protein of interest through the simple mechanism of viral infection. Since the viral DNA carries the foreign gene of interest, production levels for this system are very stable and predictable provided that infection conditions (and viral stock characteristics) remain constant. The major disadvantage of baculoviral expression is the destruction of insect cells during the course of infection. Cell lysis, a natural consequence of viral infection, ends the reproductive potential of the production culture and releases contaminating protein into the media along with the desired product. In comparison to these more established systems, plant cells express significantly lower levels of protein than prokaryotic, yeast, mammalian and even insect cell based production. This difference is mitigated by lower media costs, simpler purification, and the lack of human pathogens in the plant cell system. Given a complete economic analysis of both protein production and purification, plant cell culture may be competitive with these more established systems in some cases. Obviously, if protein yields for plant cell culture were closer to mammalian cell production levels, this would

be the preferred mode of production for proteins that are not amenable to production in yeast or bacterial hosts.

2.6 Obstacles and Limitations

At present the predominant obstacle preventing the commercial implementation of plant cell culture for foreign protein production is low expression levels. Since protein production in plant cells is a new technology, there is cause for optimism. Several of the following sections will highlight new approaches that may lead to significant increases in protein productivity. There is also some question about the exact chemical nature of glycosylation groups added by plant cells. While studies indicate that plant-produced products are biologically active *in vitro*, studies of the *in vivo* activity and possible immunogenic behavior of plant-produced proteins have been scarce. The techniques needed to definitively answer these questions about protein structure, behavior, and activity require larger quantities of purified material than the amounts typically produced in plant cell experiments. Increased protein expression, increased reactor scale, and full purification of plant-produced proteins should provide answers to these important questions. There are also some unresolved problems concerning the homogeneity of plant-produced proteins. Western blot analysis reveals multiple forms of plant proteins, which may be problematic for the application of plant-produced proteins as injectable pharmaceuticals. Other limiting factors include longer times needed to produce and isolate stable transgenic plant cell clones (because of slower doubling times) and the lack of dependable cryopreservation protocols for plant cell suspensions. These unsolved problems should be viewed as challenges that, if overcome, will provide insights toward the improvement of all protein production systems.

3 Foreign Protein Production in Plants

3.1 Background

"Plant-based" protein production refers to the production of a protein of interest in whole, intact plants (or plantlets) as opposed to undifferentiated plant tissue. The stable transfer of foreign DNA to the main genome of plants dates back to 1983 [24]. Since that time, the transfer of single or multiple genes to plants has been utilized primarily to confer pathogen resistance or other favorable agricultural traits. In these studies the whole plant (or a specific organ, fruit, or cereal) was the desired product and genetic modifications were used to improve the quality, performance, or robustness of the plant. The production of specific genes as products in whole plants began in 1989 with the production of antibodies in tobacco leaves [25]. Subsequent studies have produced a wide variety of proteins (from α-amylase to xylanase) with a wide range of activities and sizes [5]. In these studies the desired product is large, concentrated amounts of

the single recombinant protein rather than plant biomass for its own sake. While productivity varies for different products and host species, reported production levels are typically near 1% of the total protein in the harvested plant or tissue. In most cases, the absolute amount of protein is not reported so it is difficult to compare these expression levels with other production methods.

The production of foreign proteins in whole plants is a more advanced field than protein production in plant cell culture. In fact, several products are under development by companies such as Integrated Protein Technologies (a unit of Monsanto) [26], Applied Phytologics [27], and Prodigene, Inc. [5, 28]. Protein production in whole plants is particularly appropriate in cases where a plant tissue such as a fruit, tuber, etc. can be used to deliver the protein of interest. For example, oral vaccines are currently being developed in potatoes and other edible vegetables [29]. Protein production using established field crops is inexpensive and extremely scalable using current agricultural methods. The screening and preservation of good clones (or transgenic "cultivars") may also be accomplished through established agricultural means.

3.2
Product Range and Quality

In general, plant-produced proteins are correctly folded, glycosylated, homogeneous, and biologically active [5]. In several cases, their activity has been shown to be nearly indistinguishable from their mammalian counterparts [30]. This is almost certainly due to the commonalties in protein synthesis, folding, and post-translational modification pathways shared by plant and mammalian cells. To date hundreds of proteins, including more than 30 distinct types with direct economic value [5], have been produced in plants. These numbers continue to climb steadily as new research accumulates in this area. As mentioned above, these proteins span a wide range of source species and activities. One unique characteristic of protein production in whole plants is their natural capacity for long-term protein storage. Recombinant proteins confined within the leaves or seeds of plants may be stored for several weeks, months or perhaps years with only minimal losses in protein activity [30]. Whole plants are recognized as an excellent potential source of recombinant antibodies [31]. That is why, as mentioned above, several companies are advancing plant-produced products toward FDA approval and marketing.

3.3
Obstacles and Limitations

Despite this area's promise there are some obstacles and limitations that must be solved, or at least considered, as plant-based proteins move toward the marketplace. The most intrinsic drawback for protein production in plants is time. When a protein is expressed in a whole plant, its production is linked directly to the development of that plant, often from seedling (or plantlet) to mature plant. In cell culture techniques, where cells are growing at maximal rates in an artificial environment, the time scale for producing a batch of protein is on the

order of days. For protein production in plants, where plant tissue is cultivated during the course of a natural growing season (or perhaps in a greenhouse environment), the time scale for producing a batch of protein is on the order of months. This difference in time scale is also present during the initial regeneration of transformed plantlets and the selection of high producing plant clones. This disadvantage is mitigated by the enormous amount of biomass (and accompanying protein) that may be produced in an "agricultural" sized batch.

Another potential disadvantage for protein production in whole plants is gene inactivation. Cell cultures are often stable for long-term protein production, especially with the periodic use of selecting agents or recurrent use of low passage cell stocks. In contrast, transgenic plants may lose their ability to produce protein product due to gene-silencing events. Incidences of gene inactivation are correlated with several factors, including high protein expression levels, repetitive homologous promoter sequences, repetitive homologous coding sequences, and multiple copy gene integration [5]. A high incidence of gene-silencing events in the field could prove economically deleterious because of energy wasted on harvesting and processing crops that contain little or no protein. Field contamination with non-transgenic or closely related crops would pose a similar economic threat.

The most important limiting factor for protein production in whole plants may be purification. Recombinant protein expression levels in whole plants are typically quite high. However, even for "secreted" products, this protein will be inevitably trapped within the plant's tissue. In some cases protein may be recovered through direct extraction, which may supply inadequate yields, or tissue infiltration [32], which is likely not to be scalable. Therefore, the first purification steps in most cases will be plant (or organ) harvest, tissue homogenization, protein extraction (if appropriate) and clarification by filtration or centrifugation [5]. These steps are capital intensive and result in contamination of the plant product with protein impurities (natural proteins from the host tissue) and degrading influences such as proteases, phenolics, and (in the worst case) shear and foaming.

3.4 Comparison with Production in Plant Cells

In reality, the technologies of protein production in plant cells and in whole plants are almost inextricably linked. Transgenic plants are typically regenerated from genetically modified tissues or suspensions and suspension cell lines may be derived from transgenic plants [30]. The two technologies may be viewed as two variations on the same theme, each with their own strengths and weaknesses. Production in whole plants tends to give higher protein yields, but this occurs at the cost of longer cultivation times and a more difficult purification task. Similarly, secreted proteins are more easily recovered and purified from suspension cultures, but they tend to accumulate at lower levels. Even active researchers working in the area of protein production in whole plants recognize the advantages of using cell suspensions [33]. Their well-defined growth conditions, controlled sterility, and simpler purification methods miti-

gate the lower protein expression levels. For example, the production of ricin (a potential anticancer agent) by cultured tobacco cells led to more consistent yields and more favorable processing [16] than its production in whole tobacco plants [34]. As these technologies mature, it is likely that they will continue to compliment one another well.

4
Gene-Transfer Techniques

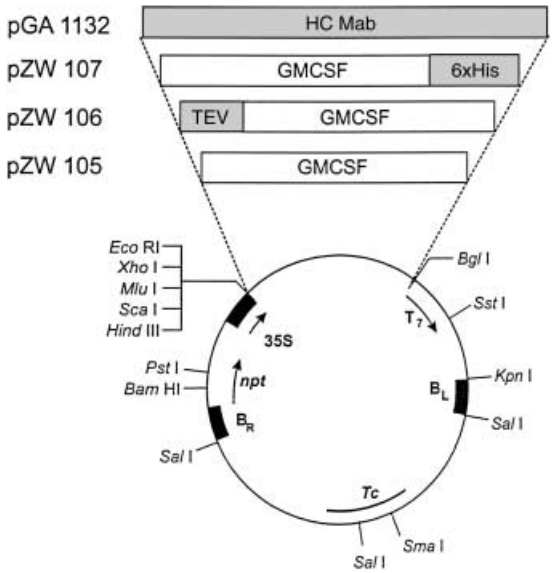
The construction and transfer of DNA to plant tissue is a critical step for successful protein production in plant cells. Decisions made during the planning and execution of this phase will affect not only direct considerations such as the transformation efficiency and the difficulty in selecting clones, but also indirect considerations such as the ultimate performance of the cells and production level of the final product. Bearing this in mind it is not surprising that some of the most important intellectual property in the realm of protein production in plant cells falls into this category. Care must be taken in selecting the elements of the transfer DNA, the method of DNA transfer, and the strategy that will be used for clonal selection. In an informative review article, Birch suggests that the essential requirements for a gene-transfer system are a source of compatible target tissue, a method of DNA introduction, and a procedure for selecting and cultivating transformed cells [35]. To this list we would add developing an appropriate DNA construct as an additional required element. The essentials of these four tasks will be discussed in the sections that follow.

4.1
Target Tissue for DNA Transfer

For the transformation of suspension cells, a wide range of cell types is available. The most widely used cell type is tobacco, particularly the BY1 and BY2 cell lines derived from *Nicotiana tabacum*. These cell lines have excellent growth characteristics and are easily transformed by co-culture with *Agrobacterium* (discussed in more detail below). In general, dicotyledons, such as tobacco, have excellent growth characteristics and are easily transformed while monocotyledons, such as corn, are often less robust in culture and are more difficult to transform. However, since some studies indicate that plant species carry out different degrees of post-translational modification and levels of expression [26], the transformation of both monocotyledons and dicotyledons or of several alternative species in parallel may be advantageous. Beyond these considerations, candidate tissue for transformation should have favorable growth characteristics and should be pathogen free.

4.2
Construction of Transfer DNA

The construct requirements for the transfer of DNA to plant cells vary depending on the transfer method. Obviously, the foreign DNA must be genetically compatible with the plant cell host and there must be some means of differen-



Scheme 1. Design elements of a typical plant cell expression vector, including promoter sequence (*35S*), terminator sequence (*T7*), resistance gene (*npt*), right and left borders (*Br* and *Bl*), and insertion point for genes of interest. This binary vector is suitable for plant and *Agrobacterium* host cells

tiating between transformed and untransformed tissue. Therefore, at minimum, the transgene must include an effective promoter sequence, the gene of interest, a terminator sequence, and a selectable marker. Scheme 1 shows a typical plant cell expression vector.

For *Agrobacterium* transformation, the gene of interest must be carried in a binary vector with appropriate bacterial T-DNA elements. For other transformation methods the requirements may be less stringent. However, even in these cases, the DNA of interest is typically amplified in bacteria. The choice of a promoter is of particular importance, as this element will dictate both the expression level and the time course of protein expression. The inclusion of additional elements, such as enhancers, 5′ and 3′ untranslated regions, and fusion elements (e.g. fluorescent or 6-histidine tags), should also be considered for improved expression, detection, and downstream processing.

4.3 DNA-Transfer Methods

4.3.1 *Agrobacterium Transfer*

The transfer of foreign DNA to plant tissue material is most commonly achieved using the natural gene transfer function of *Agrobacterium tumefaciens*, a "parasitic" soil bacterium.

Agrobacterium utilizes a highly adapted gene-transfer and integration technique to integrate single-stranded DNA into plant cells [35]. The mechanism for this transfer is not well understood. However, this method is widely used since it gives predictable results. *Agrobacterium* transformation produces genetically transformed plant cells or tissue that is highly stable [36] because the foreign gene is inserted into the main plant genome. The typical procedure for *Agrobacterium*-mediated transformation includes co-culture of plant cells with the bacteria, antibiotic treatment to disinfect the culture and kill the contaminating bacteria, selection of transformed plant cells using the conferred resistance trait, and identification of high producing clones through callus culture under continued selection pressure. In all, this procedure can yield stable clones for further research in approximately 2 months for cells co-cultured with *Agrobacterium* and 1 year for leaf disk transformation [33].

4.3.2 *Microprojectile Transfer*

Microprojectile bombardment (or BIOLISTIC transformation) is also a common and effective method of DNA transfer. In this method, gold microcarriers are coated with plasmid DNA and delivered to plant tissue by means of a particle gun. At the time of particle delivery, DNA enters the cell by mechanical means and, in a small percentage of events, is able to incorporate into the plant genome. It is likely that particle delivery also damages the host DNA by shearing. Such a disruption would give the opportunity for foreign gene incorporation by the plant's DNA repair mechanisms. Damage to the host DNA also places the viability of the tissue at risk. Because of damage caused by bombardment, the newly transformed plant tissue is initially fragile and must be cultivated under selection-free conditions until signs of visible growth are observed. Because of its basis on mechanical delivery, microprojectile transformation can be used to deliver multiple plasmids simultaneously [37]. However, to achieve balanced expression of multiple genes, it is advisable that each separate plasmid carries a unique selectable marker. The major advantage of this method is wide applicability – even some of the most "recalcitrant" species can be transformed [35]. The main disadvantages of this method are low transformation efficiency, DNA fragmentation, and collateral genetic damage.

4.3.3 *Other Methods*

Between the methods of *Agrobacterium* and microprojectile transfer, nearly every plant species can be transformed effectively [35]. However, these methods are covered by patent claims and may result in limited transformation efficiency for some cell types. For this reason, the use of alternative gene-transfer methods is an active area of research for all cell types. Alternative gene-transfer techniques include electroporation, microinjection, liposome fusion, direct transfer into protoplasts, and laser treatment [38]. In electroporation, DNA is transferred into the cell using a high-voltage electrical pulse [39]. Standard

techniques for the production of transgenic plant cells by electroporation often include a pretreatment with cell wall degrading enzymes [40]. In general, electroporation yields a high transfection frequency and stable integration of the desired transgene. Microinjection utilizes a microscopic delivery system to directly inject genetic material into host tissues. Since this method is exacting and requires specialized equipment it is generally avoided.

New research efforts also include transient protein expression in plant cells using viral expression systems. Essentially, this strategy mimics the pattern of baculoviral expression using insect cell culture. A natural virus infects the cell, usurps the cellular machinery to reproduce its viral components, and causes cell lysis to distribute and propagate the new viral particles. By infecting cultured plant cells with a virus carrying the gene for the protein of interest, production of the desired protein product will accompany the natural course of infection. Transient (virally induced) expression generally has the advantage of higher protein expression levels (in terms of protein concentration per liter), but the disadvantage of a limited production interval and destruction of the host cells. A discussion of the implications for transient protein production in plant cells will be included in our discussion of new schemes for protein production.

4.4 Cell Selection and Screening

Following DNA transfer, transformed plant cells must be selected and, subsequently, high producing cell lines must be identified. The effectiveness of this selection and screening procedure is an important aspect of successful protein production. For genetic stability, it is critical that true plant cell clones are isolated. For good productivity it is critical that the highest producing cells are identified from among the full distribution of possible clones. At the conclusion of transfection, the plant cells exist as a mixture of transformed and untransformed cells. In the case of *Agrobacterium* transformation the cells are co-cultured with transgenic bacteria also containing the selectable marker. In this case, the culture must be decontaminated by the use of cefotaxime or other microbe-specific antibiotic agents.

The selection of transformed cells is achieved using a conferred selectable trait, most commonly antibiotic resistance. Only transformed cells with the conferred resistance trait are able to survive and reproduce. However, the use of antibiotics as a media component for commercial protein production is problematic for at least two reasons. First, residual antibiotic would be a highly undesirable contaminant in the final product. Second, the recurrent use of antibiotics increases the likelihood that antibiotic-resistant pathogen strains will develop. Recent studies indicate that antibiotic selection is not necessary during every passage (or generation).

An interesting alternative method for the selection of transgenic plant cells utilizes the principle of "positive selection" [41]. This method uses a novel glucuronide cytokinin derivative as a selective agent and the *E. coli* β-glucuronidase gene (GUS) as the selectable gene. Only transformed cells carrying the GUS gene are capable of cleaving the cytokinin into its active form. Since plant

cells cannot grow without stimulation from cytokinins or other hormones, only transformed cells will be stimulated to grow. A similar new method of selection is based on the use of phosphomannose isomerase (PMI) as a selectable marker [42]. Normally, plant cells cannot metabolize mannose as a carbon source. However, transformed plant cells with the PMI gene can convert mannose to fructose and then metabolize that carbon source by natural pathways. Untransformed plant cells will not grow due to carbon starvation.

Regardless of the method, the goal of selection is to eliminate untransformed cells, restricting the population to those with potential to produce the protein of interest. Following selection, a screening (or cloning) strategy must be used to isolate high producers from among the full population of cells. Because most DNA-transfer strategies insert the gene of interest in a "random" location, the gene of interest will be transcribed at different frequencies (and even at different periods of the cell cycle) corresponding to the genomic location of the point of insertion [12]. At the level of translation, the population of cells will produce corresponding levels of protein. Screening is most commonly accomplished by plating a low density of selected cells onto solid selective media so that distinct "clones" of callus form on the plate. Once they are established, these calli are transferred to fresh plates and assayed for protein production levels by ELISA, Western blotting, or other methods. Fusion of the gene of interest with a reporter element such as green fluorescent protein (GFP) or other easily detectable markers can greatly simplify the identification of transformed plant cells and the quantification of the desired product [43]. However, for many applications, this fusion element must be removed during product purification. Once the protein of interest has been quantified, the cells exhibiting the highest levels of protein expression are isolated and transferred to liquid media.

5
Increasing Protein Production in Plant Cell Culture

5.1
A Multistep View of Protein Production

In simple organisms it is somewhat safe and effective to focus optimization efforts on just a few or even one area to affect a desired improvement in productivity. For example, to increase protein production in *E. coli* it may be sufficient to increase the plasmid copy number or to add a chaperone or folding aid. However, in higher organisms, changes in behavior due to these types of strategies are not as predictable or easy to control. This is due in part to the fact that there are several additional steps along the protein production "pathway" that are unique to higher cells. The successful production of a protein from any higher cell type is a multistep process, requiring efficient transcription, post-transcriptional processing, translation, post-translational processing, and secretion [5]. Any of these may be a limiting step for protein production in a given system. The present body of research does not suggest a single limiting step or "bottleneck" for plant cell systems in general. In fact, significant improvements in protein expression and recovery have resulted from work on

several of these fronts. These include, increased translation using the tobacco etch virus 5′ untranslated region (TEV UTR) [12], secretion directed by native [10] or plant signal sequences [14, 19] in conjunction with exogenous stabilizers [6], transcription using scaffold attachment regions (SARs) [44], or even novel procedures in isolating clones (unpublished results). From this information one can conclude either that the limiting step varies depending on the protein of interest, or that many steps in the protein expression cascade must be optimized. Bearing this in mind, each step along the protein production pathway may be viewed as a frontier for new research.

5.2 Instability of Protein Products

In recent years, increasing attention has been directed toward protein stability problems as a target area for increasing the yield of protein products. The degradation, or loss, of protein product after synthesis may occur by a number of mechanisms; including enzymatic breakdown, aggregation, denaturation, and adsorption. Regardless of the cause, any event that significantly reduces the amount of functional or active protein constitutes a costly loss of product. In general, protein instability is caused by unfavorable interactions between the protein and its chemical environment. As such, it can be addressed either by modifying the structure of the protein or by modifying its chemical environment. Several studies have noted that the plant growth medium is not an ideal environment for dilute concentrations of recombinant protein. As result, work in our group and other laboratories has focused on modifying growth media with additives to increase the stability of secreted proteins.

5.3 Methods To Enhance Protein Stability and Production

Due to the occurrence of unfavorable environmental conditions, the loss of protein due to product instability during plant cell culture and subsequent purification significantly influences product yields. Protein stability following secretion can be improved with the addition of appropriate chemical agents (or stabilizers) to the growth media or storage solution. Previous work in our laboratory demonstrates increased recovery of a mouse monoclonal antibody heavy chain by the addition of DMSO [45], gelatin [46] and polyvinyl pyrrolidone (PVP) [6, 47]. As shown in Fig. 2, the level of secreted protein is significantly increased with the addition of these exogenous stabilizers.

Similar results were observed for the stabilization of an antibody produced in hairy root culture [11]. In contrast, some proteins are more resistant to stabilization efforts. As shown in Fig. 3, plant-produced granulocyte-macrophage colony-stimulating factor (GM-CSF) and Interleukin 4 (IL-4) are only moderately stabilized with the addition of bovine serum albumin (BSA). The use of known stabilizers such as PVP and gelatin was ineffective for these proteins. This difference highlights the need for more general protein stabilizers for use in plant cell cultures.

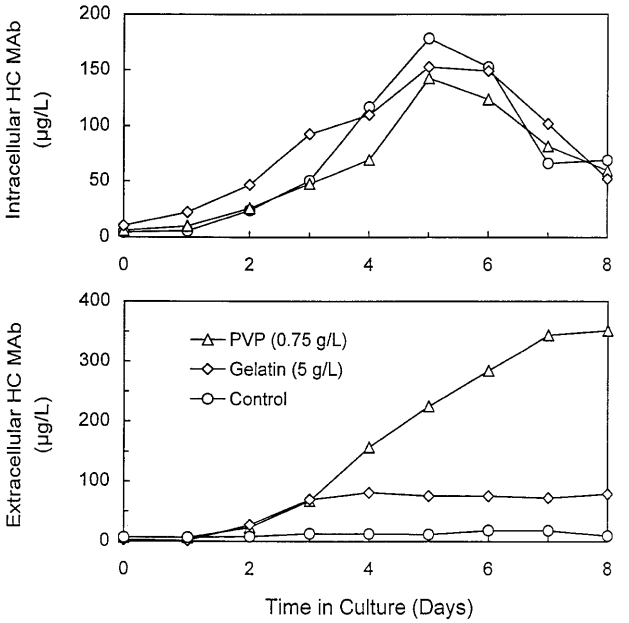


Fig. 2. Effect of exogenous gelatin [46] and PVP [47] on the production and stability of a murine antibody heavy chain. *Triangular symbols* indicate PVP, *diamonds* indicate gelatin, and *circles* indicate no stabilizer (control). In the upper portion of the figure, the intracellular protein is unaffected by the addition of exogenous stabilizers. In the lower portion of the figure, added PVP and gelatin significantly increase the level of extracelluar protein product

One interesting new stabilizer that may help fulfill this need is the protein bacitracin, which has been shown to enhance cell growth [48] and inhibit the degradation of plant-produced proteins [49]. The use of stabilizers to increase protein stability is not exclusively limited to plant cell culture. Other published examples include the stabilization of acidic fibroblast growth factor by nucleotides [50], malate dehydrogenase by sulfobetaines [51], and ribonuclease A by sorbitol [52]. By using this strategy, protein yields can be increased dramatically with the addition of inexpensive chemical agents. However, in the ideal case, the stabilizing agent should not complicate downstream purification of the product.

5.4
New Schemes for Protein Production

Even with the use of stabilizing agents and "optimized" transfer DNA, research in our laboratory suggests that there is a maximum protein yield for plant cells using standard production conditions [12]. Given this apparent limitation, the most promising new methods seek to increase protein production by devising new production strategies that will overcome product instability and inhibition or that will increase the rate or level of protein expression. Emerging schemes include the use of new promoter sequences, semi-continuous protein harvest to

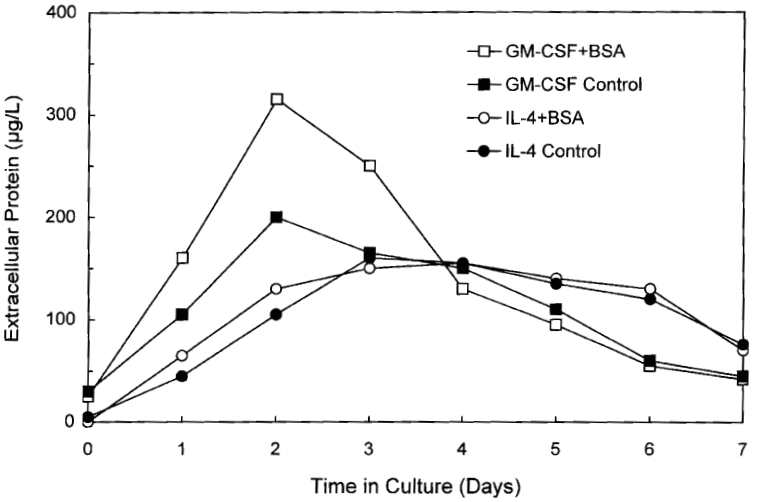


Fig. 3. Effect of exogenous BSA on the production and stability of plant-produced human GM-CSF [12] and IL-4 [10]. *Square symbols* represent GM-CSF production and *circles* represent IL-4 production. *Open symbols* indicate added BSA. Sterilized BSA was added to media prior to inoculation at a concentration of 100 mg l^{-1} For GM-CSF, adding BSA increases the level of extracellular protein. For IL-4 there is no significant effect

eliminate protein stability or inhibition problems, and virally induced protein production. Each of these novel methods seeks to circumvent the traditional limitations of protein production in plant cells by changing the behavior of the system.

5.4.1 Inducible Promoter Systems

Several new expression strategies utilize inducible promoter systems in an effort to de-couple cell growth and protein production. This is a potentially powerful production scheme, especially if it can be combined with cell immobilization (discussed below). Unlike constitutive promoters, which are always active, inducible promoters are triggered by a specific stimulus. This function can be the result of natural promoter sequences or, in certain circumstances, it may be conferred through the use of paired repressor and activator proteins in a scheme mimicking bacterial operon systems such as the lac operon. A wide variety of inducible plant promoter systems appear in the literature including steroid [53], auxin [54], heat shock [55], metal [56], tetracycline [57], salt [58], sugar starvation [59], and ethanol [60] inducible systems. Comparison of different inducible systems indicates that each promoter stimulates different levels of protein production under inducing and baseline levels of the induction agent – even after optimization of induction conditions [61]. Typically, an inducible promoter is chosen to give "tight" control and "strong" promotion of the gene of interest. This means there should be low or negligible protein production

rates under baseline conditions and high protein production rates under induction conditions.

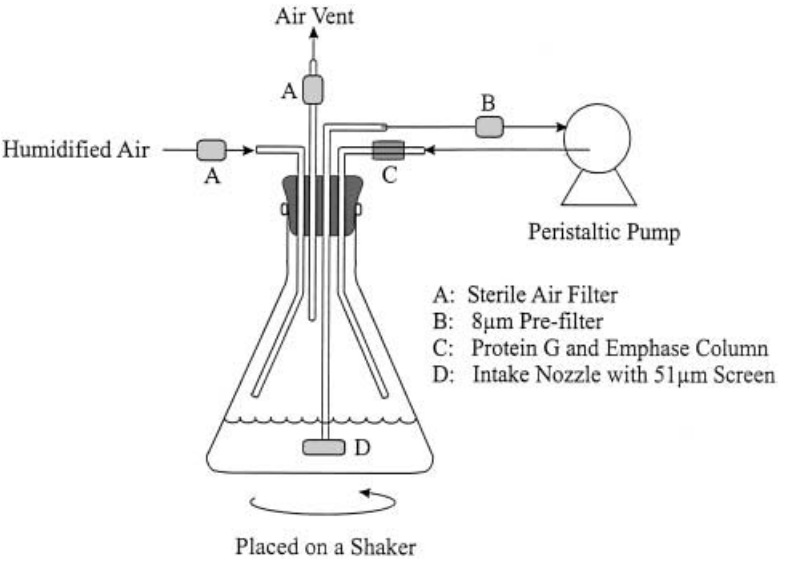
Since inducible promoters are triggered through the addition (or removal) of exogenous chemicals or through changes in environmental or growth conditions, their activation is independent of cell growth. Instead, protein production is triggered at a designed growth stage or even in a separate reactor. In general, the goal of inducible protein production is to stimulate a large burst of production once the cells have reached the determined optimum biomass concentration or growth phase. Using standard suspension culture this strategy requires several unproductive days of cell growth before protein can be produced and, in some cases, a medium exchange to rapidly expose the cells to induction conditions. If immobilized cells are used, beads or carriers can be pre-made with cell concentrations at or near the ideal conditions and then reused several times. Also, since the cells are trapped inside the immobilizing matrix, media replacement proceeds quickly and easily once the beads or carriers have settled.

Inducible protein production may also have advantages over constitutive expression in the area of protein stability. Much of the instability of secreted proteins can be attributed to the harsh, dilute environment of the extracellular media. Although the mechanism of protein loss is unknown, it is clearly a time-dependent process [6]. With the inducible promoter system similar (or larger) amounts are produced over a very short amount of time. This "compressed" production interval allows less time for time-dependent protein loss and leads to a less dilute protein environment even in the absence of stabilizers.

5.4.2
Affinity Chromatography Bioreactor

As discussed earlier, secreted proteins are often unstable in growth media. This behavior, coupled with mechanisms for product inhibition, deactivation, and proteolysis, significantly decreases the yield of recoverable proteins for traditional bioreactor configurations. If the desired product could be harvested before it is degraded, and if product inhibition effects were minimized, protein yields should increase significantly. A novel strategy employed in our laboratory is the use of an affinity chromatography bioreactor or ACBR to simultaneously produce and harvest recombinant proteins from plant cell culture. The features of the ACBR (Scheme 2) include a cell growth chamber where protein is produced, a cell retention screen allowing separation of biomass from the protein harvest stream, an affinity chromatography column used to collect the protein of interest, and a media return loop which returns media to the growth chamber.

Reactors of this type have been used to produce and harvest the heavy chain of a murine monoclonal antibody using protein G affinity and histidine-tagged human granulocyte-macrophage colony-stimulating factor (GM-CSF) using metal affinity chromatography. Implementation of this scheme on a small scale was difficult, but these studies show that the amount of recoverable protein can be increased [62]. As shown in Fig. 4, recoveries of human GM-CSF and a mouse monoclonal antibody heavy chain were increased by three- and seven-fold, re-



Scheme 2. Affinity chromatography bioreactor (ACBR) for the semi-continuous recovery of plant-produced proteins. Media is drawn up through the intake nozzle (*D*) during culture, through a pre-filter (*B*) that removes particulates, and then to a protein-collection column (*C*) using a peristaltic pump. After protein binding, the media is returned to the growth chamber. Humidified air is supplied to the culture through a sterile pre-filter (*A*) [62]

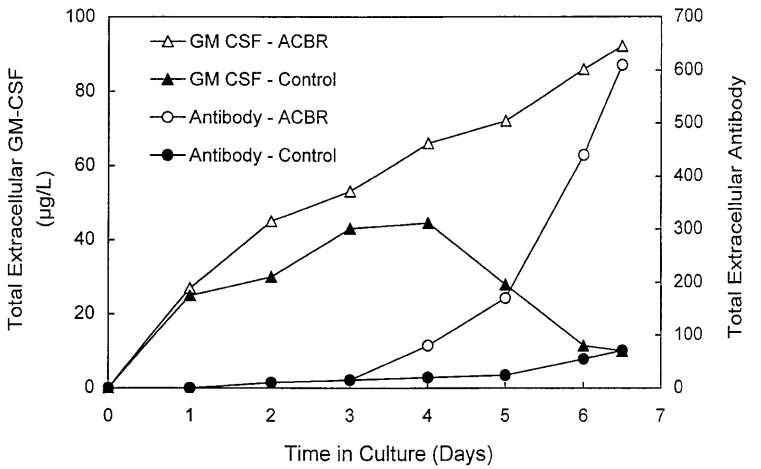


Fig. 4. Increased recovery of plant-produced GM-CSF and murine antibody in the affinity chromatography bioreactor (ACBR). *Triangular symbols* represent GM-CSF production and *circles* represent antibody production. *Closed symbols* indicate normal batch production and *open symbols* indicate ACBR production. For both products, protein recovery is increased several-fold using the ACBR [62]

spectively, as compared to normal batch production. This improvement is actually considerably less than the expected increase. This shortfall is due in part to the short recirculation times used in these experiments and in part to inadequate performance of the chromatography columns over repeated semi-continuous use.

Studies using this strategy clearly demonstrate that product inhibition is a significant limiting factor for protein production in plant cell culture. It is possible that, on a larger scale, reactor configurations of this type will lead to more significant increases in protein yield from plant cell cultures.

5.4.3
Transient Protein Production

Another new method for protein production utilizes plant viruses to stimulate transient protein expression in plant cells. Virally based protein production constitutes both a novel method of DNA transfer (as discussed above) and a novel production scheme. This strategy is essentially the same as baculoviral infection of insect cell cultures for transiently induced protein expression. First, the gene of interest is cloned into the viral genome under the control of a judiciously chosen promoter sequence. This insertion may be accomplished by replacement of a nonessential viral gene, insertion of an independent strong promoter, or fusion with an existing viral gene product [63]. Subsequently, the virus is amplified (using a small-scale infection) to produce a working viral stock and administered to plant tissue for protein production. Viral expression strategies have recently been developed or modified for plant tissue using cauliflower mosaic virus [64], maize streak virus [65], and numerous others. At present, virally driven protein production strategies have been used almost exclusively in whole plants to conduct basic research in plant biology and pathology. More recently, the production of antigenic peptides (epitope presentation) using plant viruses has been investigated for the development of oral vaccines [66]. Further development of these methods for the production of commercially useful protein products and application of virally induced production to plant cell cultures may give impressive results.

5.5
Modeling Considerations

Particularly as protein production schemes become more complex, intuition and experience will be insufficient to predict appropriate conditions and understand the observed behavior for the production of a protein of interest. Because of the large number of variables, the wide range of possible values for these variables, and the long time-scale for experiments, it will also be nearly impossible to determine optimal production conditions through experimental data alone. Any efforts to understand and optimize plant cell culture behavior can be aided greatly with the use of mathematical modeling. In general, a model can be used for two distinct purposes: either to explain the measured behavior of a given system, leading to theoretical mechanisms for the observed

behavior, or to predict the behavior of the system leading to estimates of system performance over a wide range of conditions. Both of these aspects of mathematical modeling are useful for studying plant cell cultures. Early in model development, a basic modeling approach must be chosen.

Traditional models range from simple (unstructured and unsegregated) to very complex (structured and segregated). The full implications of these choices, and subsequent model development, are too involved to cover here in any depth. However, the reader is cautioned that in most cases unstructured models have proved inadequate to describe the complex behavior of plant cell cultures. Structured kinetic modeling, first applied to plant cell cultures by Frazier [67], more adequately describes cell growth (including the lag phase), cell viability, and product synthesis. At present, few researchers have undertaken full modeling efforts to describe protein production in plant cell culture, but the effort can be fruitful. An approach developed in our group successfully modeled plant cell growth, protein production, secretion, degradation, and interactions with exogenous stabilizer molecules [68]. With the implementation of novel production schemes, as described above, modeling approaches will be essential for process optimization.

6
Scale-Up Considerations

6.1
Demand for Alternative Hosts

During the early developmental stages of biotechnology, companies tended to develop expertise for expression in a single host system. More recently, it has become clear that individual proteins are expressed more efficiently, secreted more easily, or perhaps folded and modified more consistently in a given host as compared to other alternative hosts. Following this reasoning the ideal host for expressing a protein of interest will change from case to case depending on issues such as codon usage, difficulty of folding, product homogeneity, and protein modifications. As a result, the biotechnology industry is becoming increasingly open to the idea of comparing alternative hosts for the production of promising therapeutic agents. Legal issues such as intellectual property, patent rights, and licensing agreements also make the development of novel host systems a favorable choice. With continued development, plant cell culture should become a more common candidate host system. The successful application of plant cells as production lines in the commercial arena will, in turn, necessitate cultivation in large bioreactors.

6.2
Plant Cell Bioreactors

For initial studies, plant cell suspensions are typically cultivated in shake flasks agitated by a gyratory shaker, but for the economical production of commercial products, plant suspension cultures must be cultivated in larger bioreactors.

Such reactors are essentially the same as those used for microbial culture [69]. However, the design of suitable bioreactors for plant cell culture also presents some unique challenges because plant cells tend to form large aggregates. Concerns for the scale-up of plant cell bioreactors include shear sensitivity, high viscosity, and risk of contamination. The problem of foaming, once a significant concern, can be mitigated through the addition of small quantities of mineral oil or other antifoaming agents to the reaction vessel [70]. Experimental studies [71] show that plant suspension rheology is a function of cell size and morphology and can be controlled. Traditional plant bioreactors use stirred tank, airlift reactor, or similar configurations. New approaches include the use of membrane stirrers for improved aeration [72]. Plant cells may also be amenable to culturing in hollow fiber or perfusion reactors. For protein expression applications, plant cell bioreactors must be configured and optimized not only for cell growth but also for product formation. The use of bioreactors for large-scale protein production is a relatively new application, but knowledge derived from the production of natural products from plant suspensions will be easily transferred to this new application.

6.3 Cell Immobilization

Plant cell immobilization is an important process alternative for plant cell cultivation. The use of encapsulating gels, such as alginate, to immobilize plant cells is a relatively old technology dating back more than 20 years [73]. It is well known that cell immobilization protects the cells, facilitates the re-use of cells in continuous or semi-continuous culture, and allows higher inoculum percentages than standard methodologies. A significant body of work in the literature has shown that cell encapsulation has a number of favorable effects. The changes in growth environment of the cells can significantly influence the product yield for natural plant products [74]. These encapsulated cells are also less sensitive to shear stresses because of protection from the surrounding gel material. Gel beads have more favorable settling velocities than cells because their diameter is much larger than the individual clumps of cells.

Procedures for plant cell immobilization are represented in the literature for a wide variety of encapsulating agents. These include alginate, agarose, gelatin, carrageenan, polyacrylamide, and combinations of these substances [75]. To form spherical beads, mature (late exponential or early lag phase) cells are mixed with the encapsulating agent until a homogeneous slurry is formed and transferred drop-wise into the gelling reservoir. For most thermogels, this method is not suitable because exposure to high temperatures will significantly reduce cell viability. Instead, the slurry may be rapidly dispersed in a two-phase oil/water system. While this dispersion method allows the use of a wider range of encapsulating materials, it produces a heterogeneous mixture of bead diameters. Recent studies in our laboratory also indicate that plant cell immobilization can lead to increased protein production [76]. As shown in Fig. 5, merely immobilizing the plant cells in alginate beads can increase the production of human GM-CSF.

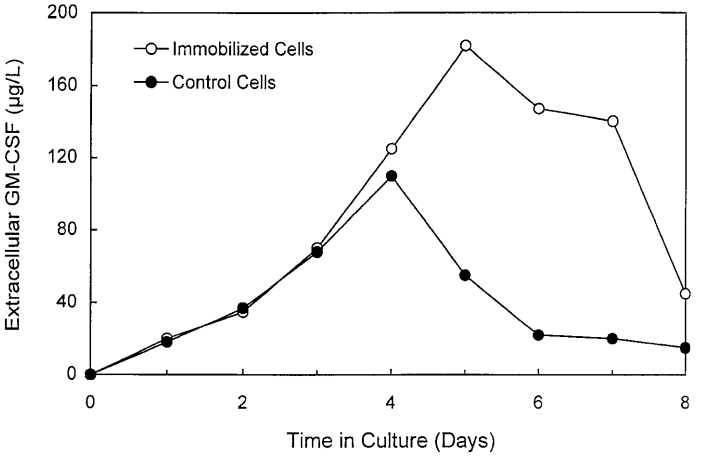


Fig. 5. Increased expression of human GM-CSF by immobilized plant cells [76]. *Closed symbols* indicate normal batch production and *open symbols* indicate cells encapsulated by alginate. The effects of encapsulation increase the concentration of extracellular protein

The use of encapsulating beads may be effectively combined with other strategies as discussed in earlier sections (inducible promoters, affinity bioreactors) to produce highly desirable results.

7 Conclusion

7.1 Summary

Clearly protein production in plant cell cultures is an exciting and promising frontier for new research. Plant cells have favorable growth characteristics and attributes (including the capacity for effecting complex modifications and protein secretion) that should help this technology compete effectively with other host systems. They can be genetically modified by well-established means and, in many cases, are highly stable. Plant cell cultures have demonstrated a capacity for producing a number of useful proteins both safely and effectively and potential for large-scale cultivation. Significant progress has been made in the last decade, but many challenges and unanswered questions remain. The key area for further research will be determining and overcoming the limiting factors in protein production to bring production levels of plant-produced recombinant proteins up to economically feasible levels. Protein production in plant cells is entering the most critical phase of its development, but there is significant cause for optimism. Future efforts must be based around novel approaches and innovative ideas that circumvent the root causes of the current production limits.

7.2
Future Outlook

To some, the production of protein products in plant cell culture may appear as merely an odd curiosity: an interesting area for academic study, but showing little importance or promise for practical applications. More discerning minds may note that, over the development of the biotechnology industry, yeast expression systems found a place among bacterial systems and mammalian and baculoviral expression systems found a place among bacteria and yeast. Plant cell culture can produce functional, high-quality proteins at low to moderate expression levels. Considering the numerous advantages of plant cell culture over its closest competitors, including simplified purification, diverse processing options, and lack of human pathogens, the outlook for this technology is very bright.

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