

Production of Recombinant Proteins in Transgenic Plants: Practical Considerations

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Received 13 January 1997; accepted 18 April 1997

Abstract: This review is based on our recent experience in producing the first commercial recombinant proteins in transgenic plants. We bring forward the issues that have to be considered in the process of selecting and developing a winning transgenic plant production system. From the production point of view, transcription, posttranscription, translation, and posttranslation are important events that can affect the quality and quantity of the final product. Understanding the rules of gene expression is required to develop sound strategies for optimization of recombinant protein production in plants. The level of recombinant protein accumulation is critical, but other factors such as crop selection, handling and processing of transgenic plant material, and downstream processing are equally important when considering commercial production. In some instances, the cost of downstream processing alone may determine the economic viability of a particular plant system. Some of the potential advantages of a plant production system such as the high levels of accumulation of recombinant proteins, glycosylation, compartmentalization within the cell, and natural storage stability in certain organs are incentives for aggressively pursuing recombinant protein production in plants. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 56: 473–484, 1997.

Keywords: transgenic plants; recombinant protein; gene expression; downstream processing

INTRODUCTION

Recombinant protein production systems utilized today range from prokaryotic systems such as *Escherichia coli* (Georgiou, 1988; Georgiou and Bowden, 1991; Kudo, 1994) and *Bacillus* (Ebisu et al., 1996; Udaka and Yamagata, 1993), to eukaryotic systems such as yeast (Ha-

rashima, 1994; Marino, 1991), *Aspergillus* (Archer, 1994), mammalian (Warren and Krivi, 1991; Werner and Thomae, 1994) and insect cell cultures (Goosen, 1993; Luckow and Summers, 1988), transgenic animals (Lubon et al., 1996; Velandar et al., 1997), and plants. *E. coli* is the workhorse of the biotechnology industry but is not well suited for expressing eukaryotic genes, particularly if the protein product must be glycosylated and terminally processed. In addition, the recombinant protein can be toxic to bacteria, form inclusion bodies or be degraded by proteases (Georgiou, 1988; Georgiou and Bowden, 1991; Hughes and Qoronfleh, 1991; Kudo, 1994). Mammalian and insect cell cultures are currently used for producing many important pharmaceutical proteins because of their ability to perform glycosylation and to process the recombinant protein similar to that of the native host (Goosen, 1993; Jenkins et al., 1996; Lehman et al., 1993; Luckow and Summers, 1988; Werner and Thomae, 1994). Transgenic animals have recently emerged as promising systems for producing human proteins in milk, because mammary glands are capable of performing correct posttranslational modification including complex glycosylation and γ -carboxylation (Jenkins et al., 1996; Lubon et al., 1996; Velandar et al., 1997).

Transgenic plants are potentially one of the most economical systems for large-scale production of recombinant proteins for industrial and pharmaceutical uses (Austin et al., 1994; Krebbers et al., 1992; Pen et al., 1993a; Whitelam et al., 1993). Advantages of plant systems include the low cost of growing plants on large acreage; the ease in scale-up (increase of planted acreage); the availability of natural protein storage organs; and the established practices for their efficient harvesting, transporting, storing, and processing (Whitelam et al., 1993). Other potential advantages that plants offer include: (1) the elimination of the purification requirement when the plant tissue containing the recombinant protein is used as a food or feed supplement; and (2)

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Contract grant sponsor: Center for Crops Utilization Research at Iowa State University

Contract grant sponsor: Pioneer Hi-Bred International, Inc.

the possibility to compartmentalize recombinant proteins in different organelles (Goddijn and Pen, 1995; Ponstein et al., 1996). The present disadvantages of plant systems are the low accumulation levels of recombinant proteins, insufficient information on posttranslational events, and the lack of data on downstream processing (Goddijn and Pen, 1995).

The production of recombinant proteins in plants can be divided into four areas: (1) cloning and expression of the gene of interest in plant cells; (2) regeneration and selection of the plants; (3) recovery and purification of the protein; and (4) characterization of the final product. By far, most of the work to date has been focused on cloning and expression. Many different proteins have been produced in plants primarily to improve agronomic performance. Examples of proteins with potential industrial applications are beginning to accumulate (Table I). The level of accumulation varies considerably in these examples and the reasons for this are discussed in the next section. The examples of a recombinant phytase (Verwoerd et al., 1995), which accumulated in the extracellular space of tobacco leaves at about 14% of the total soluble protein, and of a recombinant hirudin (Parmenter et al., 1995), which was produced at 1% of the canola seed weight, give great hope that commercially attractive enzymes and proteins could accumulate to levels that would make transgenic plants economically viable pro-

duction systems. This review, based on our recent experience in producing the first commercial recombinant proteins in plants, brings forward a whole set of issues to be tackled in the process of selecting and developing a winning transgenic plant system.

OPTIMIZATION OF PROTEIN INTEGRITY AND ACCUMULATION

Historical Perspective

Since the first transgenic plant was introduced in 1983 (Fray et al., 1983; Zambryski et al., 1983), considerable effort has been directed to developing reliable transformation systems and expression vectors that permit high levels of gene expression and to direct recombinant proteins to specific plant tissues. Recombinant proteins from a wide variety of sources were produced in transgenic plants such as tobacco, tomato, petunia, potato, corn, alfalfa, canola, and soybeans, to name a few. Proteins with molecular weights as small as 0.6 kD (Turpen et al., 1995) and as large as 80 kD (Verwoerd et al., 1995) per subunit have been produced (Table I). Although reported accumulation levels are difficult to compare because different plant expression systems

Table I. Proteins of potential commercial interest produced in transgenic plants.

Recombinant protein	Molecular weight (kD/subunit)	Origin	Host	Production level	Reference
α-Amylase	55.2	<i>Bacillus licheniformis</i>	Tobacco	0.3% of soluble leaf protein	Pen et al., 1992
Aprotinin	6	Bovine	Corn	0.07% of soluble seed protein	Zhong G. Y. (personal communication)
Avidin	15	Chicken	Corn	6.0% of soluble seed protein	Hood et al., 1997
Chymosin	30	Calf	Tobacco, potato	0.1–0.5% of soluble protein	Willmitzer et al., 1992
Cyclodextrin glucanotransferase	76	<i>Klebsiella pneumoniae</i>	Potato	<0.01% of soluble tuber protein	Oakes et al., 1991
Enkephalin	0.5	Human	Arabidopsis	0.1% of soluble seed protein	Vandekerckhove et al., 1989
Erythropoietin	37	Human	Tobacco	0.0026% of soluble protein	Matsumoto et al., 1995
Glucosylase	74	<i>Aspergillus niger</i>	Potato	Not available	Van den Elzen et al., 1992
β-Glucuronidase	68	<i>Escherichia coli</i>	Corn	0.7% of soluble seed protein	Witcher, D. (personal communication)
Growth hormone	21	Trout	Tobacco	0.1% of soluble leaf protein	Bosch et al., 1994
Heat-labile enterotoxin B	39	<i>Escherichia coli</i>	Tobacco, potato	0.001% of soluble leaf protein, 0.01% of soluble tuber protein, resp.	Haq et al., 1995
Hepatitis B surface antigen	24	Hepatitis B virus	Tobacco	0.0066% of soluble leaf protein	Mason et al., 1992
Hirudin	11	<i>Hirudo medicinalis</i>	Canola	1% of seed weight	Parmenter et al., 1995
γ- and κ-chains hybridoma	45 and 27	Mouse	Tobacco	1.3% of soluble leaf protein	Hiatt et al., 1989
β-Interferon	20	Human	Tobacco	0.000017% of fresh weight	Eldelbaum et al., 1992
Levansucrase	49.9	<i>B. subtilis</i> , <i>Streptococcus mutans</i>	Tobacco, potato	Not detectable	Ebskamp et al., 1994; Van der Meer et al., 1994
Lysozyme	14.4	Chicken	Tobacco	0.003% of fresh leaf tissue	Trudel et al., 1992
Malarial epitopes	0.6	<i>Plasmodium</i>	Tobacco	0.4–0.8% of virion weight	Turpen et al., 1995
Phytase	80	<i>Aspergillus niger</i>	Tobacco	14.4% of soluble leaf protein	Verwoerd et al., 1995
Ricin	34	Castor bean	Tobacco	0.25% of soluble leaf protein	Sehnke et al., 1994
Serum albumin	66.5	Human	Potato	0.02% of soluble leaf protein	Sijmons et al., 1990
Xylanase	37	<i>Clostridium thermocellum</i>	Tobacco	4.1% of soluble leaf protein	Herbers et al., 1995

and extraction procedures were used, production levels above 1% of soluble protein were reported in several instances ([Herbers et al., 1995](#); [Hiatt et al., 1989](#); [Hood et al., 1997](#); [Verwoerd et al., 1995](#)).

Now that the transformation of major crops is becoming more routine, the choice of a plant for heterologous protein production will be increasingly governed by the ease of production of the plant, proper posttranslational processing, the plant's capabilities for protein accumulation, and the availability of methods for extraction and purification of the recombinant protein ([Krebbers et al., 1992](#); [Whitelam et al., 1993](#)).

Although efforts have been made to quantify and characterize the recombinant proteins produced in plants, the emphasis has been on characterization of the desired trait and not the protein itself. In the majority of instances, no attempt was made to determine the absolute quantity of protein accumulation or whether there were any changes in the amino acid sequence, processing, or glycosylation. A complete characterization of produced proteins is required for commercial applications because a single amino acid modification could render a pharmaceutical product unacceptable even if it has a similar function.

Several studies that have characterized the final product shed some light on the issues related to heterologous gene expression and protein production in plants. Although no generalization is possible to date because different genes, different regulatory sequences, and different plants were used in these studies, several useful points can be made.

Integrity and Stability of rDNA

Key steps to maximize the level of recombinant protein synthesis are to optimize the quality and quantity of the rDNA incorporated in plant cells and to determine its fate after integration into the plant genome. Several studies have shown that the transgene can undergo inactivation (gene silencing) to prevent the expression of the rDNA. The observed rDNA inactivation has been associated with multiple copy integration at one or more sites, different base-composition between rDNA and the integration site, detrimental effects of sequences adjacent to the rDNA integration site, and overexpression effects ([Finnegan and McElroy, 1994](#)). A common feature of many rDNA inactivation cases is the presence of repeated homologous sequences ([Meyer and Saedler, 1996](#); [Park et al., 1996](#)). rDNA methylation is often correlated with gene inactivation, but there are silencing phenomena, such as cosuppression, that were not associated with detectable changes in DNA methylation ([Meyer and Saedler, 1996](#)). A recent report by [Park et al. \(1996\)](#) indicated that gene silencing, based on the presence of homologous sequence at the coding region, is a posttranscriptional process that is meiotically reversible. On the other hand, gene silencing, based on the presence of a homologous sequence at the promoter region, is a transcriptional event associated with increased promoter methylation that is meiotically heritable. Ways to prevent or avoid rDNA

inactivation, as summarized by [Finnegan and McElroy \(1994\)](#), include: screening/selection for plants with single copy rDNA; developing methods for single-copy integration; avoiding repetitive homologous sequences; flanking rDNA with scaffold attachment regions (SAR); selection and screening for stable rDNA expression; and developing site-specific recombination systems. For more information on homology-dependent gene silencing in plants see the review by [Meyer and Saedler \(1996\)](#).

The obvious problem of gene silencing for commercial production of proteins from transgenic plants suggests that, to obtain the commercial line of interest, one must select not only high-expressing lines, but also for lines that are stable over many generations. In the future, to alleviate some of these unpredictable gene silencing events an integrated approach may be used. For example, combining a single-copy integration method with SAR flanking should dramatically reduce the likelihood of homology dependent gene silencing ([Allen et al., 1996](#)). Also, to overcome the methylation-induced silencing, one could select germplasm low in methylation activity. In conclusion, there are several theoretical ways that can be proposed to stabilize rDNA integrity, but currently one still must rely on generating many transformants and selecting events that give the desired results.

Transcription

Perhaps the best studied aspect of gene expression has been transcription. The assumption is that increasing levels of transcription will result in increased protein synthesis. Although there are clearly other factors, one can argue that this oversimplified view of gene expression has led to much success in increasing the levels of recombinant proteins. Recent reviews on the regulation of transcription include discussions on transacting factors such as MYB ([Boulikas, 1994](#)) and leucine zippers ([Hurst, 1994](#)). The ability to regulate transcription, not merely to increase its rate but to control when transcription occurs, has a practical application in the production of recombinant proteins. The control of protein synthesis in plants could decrease production cost and provide qualitative advantages for when and where the protein is synthesized ([Benfey and Chua, 1989](#)).

The reported susceptibility of plant promoters to feedback regulation by the product ([Sheen, 1994](#)) has important implications for heterologous protein production because many of the synthesized proteins could be enzymes that may find suitable substrates in plants. A possible way to avoid the feedback control is to synthesize the enzyme in a compartment different from the compartment where the substrate is produced. Unfortunately, there is little information on the feedback regulation in plants, and predictions have to be based on how the enzymes behave in other hosts.

Posttranscriptional Processing

Posttranscriptional processing can have a major impact on the levels of protein produced in the cell. The factors that

can affect the expression levels include pre-mRNA processing (capping, splicing, and polyadenylation) and transcript stability. Introns occur naturally in many eukaryotic genomic DNAs ([Lambowitz and Belfort, 1993](#)) and must be spliced from a pre-mRNA nucleic acid. Processing of the RNA depends on the accurate recognition of the splice sites and the proper splicing machinery ([Brown et al., 1993](#); [Luehrsen et al., 1994](#); [Solymosy, 1990](#); [Solymosy and Pollak, 1993](#)). The introduction of genes into plants by rDNA methods has demonstrated that introns can actually increase the stability of the RNA ([Topfer et al., 1993](#)), which in turn generates a dramatic increase in the ultimate level of protein produced in the transgenic plants. The use of introns has been particularly successful in elevating the levels of expression in monocots such as maize ([Callis et al., 1987](#); [Maas et al., 1991](#)).

Polyadenylation sites also strongly influence the stability of the message and, ultimately, the level of gene expression in plant cells ([Hunt, 1994](#); [Ingelbrecht et al., 1989](#)). In addition, there are specific recognition sites that enhance RNA decay ([Sullivan and Green, 1993](#)). Some of these sites have been identified and are thought to correspond to specific binding sites ([Taylor and Green, 1995](#)). To increase the accumulation of recombinant protein, it may be necessary to screen for these sites and modify the gene to remove them whenever possible. A significant effort has been made in the last few years to better understand stability and decay mechanisms of plant mRNA. The current knowledge of the factors affecting mRNA stability has been summarized by [Abler and Green \(1996\)](#).

Translation

Initiation is the first step in translation, and there are several models on how this may work in eukaryotes ([Kozak, 1992](#)). For recombinant DNA, it seems that the Kozak sequence is the preferred model for efficient rates of initiation, although the optimum sequence for plants may be slightly different than that for animals ([Cavener and Ray, 1991](#); [Lutcke et al., 1987](#)). In general, initiation is considered the rate-limiting step in translation. After initiation, the rate of translation may become a limiting factor due to a lack of suitable tRNAs. This problem may be resolved by changing the codons of the inserted gene to simulate usage more closely aligned with the host species. This approach should increase the overall rate of translation ([Murray et al., 1989](#)), which was recently illustrated by the example using a Bt protein ([Cornelissen et al., 1993](#)) where codon usage was a key factor in obtaining dramatic increases in the accumulation of Bt protein in plants.

The RNA leader sequence greatly influences the translation of different RNAs. In particular, viral leaders have been shown to greatly increase the accumulation of recombinant proteins when compared with random leader sequences or no leader sequences ([Dowson Day et al., 1993](#)). Plant RNA leaders have also been shown to increase recombinant protein levels in plants ([Brown and Santino, 1995](#)). Therefore,

to maximize the efficiency of translation, no general rule exists and the leader sequence must be custom-tailored for each gene and plant combination. Recent reviews by [Fütterer and Hohn \(1996\)](#) and by [Gallie \(1996\)](#) provide in-depth discussions of the features and factors affecting translation processes in plants.

Because amino acid levels are regulated in the cell, they can be a limiting factor in the synthesis of particular proteins ([Matthews and Hughes, 1993](#), [Singh and Matthews, 1994](#)). For example, when a methionine-rich protein was added to soybeans, the level of other methionine rich proteins decreased ([L. Beach, personal communication](#)). The results of in vitro experiments have shown that the level of methionine-rich proteins can be increased when plants are supplemented with methionine. Therefore, to achieve high production levels for specific proteins, amino acid biosynthetic pathways may have to be altered as well.

Posttranslational Processing

Posttranslational modifications can have dramatic effects on the end product, but detailed analysis has been omitted in most studies dealing with the expression of plant genes. There are examples that demonstrate that properly processed recombinant proteins can be produced in plants, as well as examples of incomplete processing (Table II). Because this aspect is critical to the integrity (quality) of the final product, a discussion of some of the issues and representative examples is appropriate.

Targeting

Target peptide sequences can be added to proteins to direct them to a specific location in the cell ([Bednarek and Raikhel, 1992](#)). These sequences direct proteins to the mitochondria, vacuoles, chloroplast, or endoplasmic reticulum and are usually cleaved after the protein reaches its destination. Targeting may allow the protein of interest to be compartmentalized into specific organelles where it can be sequestered from being rapidly degraded or interfering with cell metabolism. Target sequences can play a vital role in protein accumulation. Adding target sequences has led to increased levels of protein expression in some studies ([Fiedler and Conrad, 1995](#); [Pen et al., 1993a](#); [Schouten et al., 1996](#); [Sijmons et al., 1990](#); [Wandelt et al., 1992](#)), whereas, in other studies, targeting has lowered the levels of recombinant proteins ([Denecke et al., 1990](#)). Recombinant proteins that could not be expressed because their prior toxicity to the cell were produced at high levels when targeted to specific organelles ([Hood et al., 1997](#)). Targeting to endoplasmic reticulum is essential for glycosylation ([Iturriaga et al., 1989](#)) and disulfide bridge formation ([Bruyns et al., 1996](#); [Shani et al., 1994](#); [Vitale et al., 1993](#)). In all instances, the target sequence must be properly cleaved, otherwise incomplete processing will affect the ultimate quality of the recombinant protein.

Table II. Pre- and prosequences used for recombinant protein production in transgenic plants.

Recombinant protein	Origin	Host	Presequence	Prosequence	Processing	Reference
α -Amylase	<i>Bacillus licheniformis</i>	Tobacco	α -amylase	—	Proper	Pen et al., 1992
α -Amylase	<i>B. licheniformis</i>	Tobacco	Tobacco PR-S	—	Proper	Pen et al., 1992
α -Zein	Corn	Tobacco	α -Zein	—	Proper	Ohtani et al., 1991
β -Zein	Corn	Tobacco	β -Zein	—	Proper	Hoffman et al., 1987
Hybridomas of γ - and κ -chains	Mouse	Tobacco	Native	—	Proper	Hiatt et al., 1989
2S Albumin	Brazil nut	Tobacco, canola	Native	Native	Proper	Altenbach et al., 1989, 1992
Chymotrypsin inhibitor I	Tomato	Nightshade, tobacco, alfalfa	Native	Native	Proper	Narvaez-Vasquez et al., 1992
CryIA(c)	<i>B. thuringiensis</i>	Tobacco	Modified <i>ats1A</i>	—	Proper	Wong et al., 1992
Enkephalin-2S albumin fusion	—	Canola	<i>at2S1</i>	<i>at2S1</i>	Proper	Vandekerckhove et al., 1989
Lectin	<i>Pisum sativum</i>	Potato	Native	—	Proper	Edwards et al., 1991
Legumin	<i>Pisum salivus</i>	<i>Nicotinia plumbaginifolia</i>	—	—	—	Ellis et al., 1988
Modified α -zein	—	Tobacco	α -Zein	—	Proper	Ohtani et al., 1991
Modified 2S albumin	Brazil nut	Tobacco, <i>Brassica napus</i> , <i>A. thaliana</i>	<i>AT2S1</i>	—	Proper	De Clercq et al., 1990
Modified glycinin	—	Tobacco	Native	Native	Proper	Utsumi et al., 1993
Modified proglycinin	—	Potato	Native	Native	Incomplete	Utsumi et al., 1994
Native proglycinin	Soybean	Potato	Native	Native	Incomplete	Utsumi et al., 1994
Normal glycinin	Soybean	Tobacco	Native	Native	Proper	Utsumi et al., 1993
Phytase	<i>Aspergillus niger</i>	Tobacco	Tobacco PR-S	—	Proper	Pen et al., 1993b
Pro-serum albumin	Human	Potato	Human serum albumin	Human serum albumin	Improper	Sijmons et al., 1990
Prohevein	<i>Hevea brasiliensis</i> (rubber tree)	Tomato	—	Native	Incomplete	Lee and Raikhel, 1995
Serum albumin	Human	Potato	Tobacco PR-S	No prosequence	Proper	Sijmons et al., 1990
Thionin	Barley, wheat	Tobacco	Native	Native	Proper	Carmona et al., 1993; Florack et al., 1994
Type 1 fish antifreeze protein	Fish	Tobacco	Native	Native	Proper, but not efficient	Kenward et al., 1993

Proteolytic Processing

The outcome of proteolytic processing of various recombinant proteins produced in transgenic plants is summarized in Table II. In several instances, recombinant proteins were not properly cleaved, which resulted in the accumulation of pro-proteins instead of mature proteins. Normal and modified prepro-glycinins were not correctly processed in tubers of transgenic potato (Utsumi et al., 1994), but were correctly processed in seeds, leaves, and stems of transgenic tobacco (Utsumi et al., 1993). The incorrect processing of the recombinant glycinins in potato tubers indicates that the vacuoles of potato tubers lack the enzymes necessary for proteolytic cleavage of proglycinins to mature glycinins (Utsumi et al., 1994). Similar to the processing of the normal and modified prepro-glycinins, prepro-human serum albumin (HSA) was only processed to pro-HSA in tobacco and potato. The apparent problem that stemmed from the lack of serine proteases in the plant has been resolved by directly fusing the HSA to the signal sequence of the tobacco P-RS protein. This fusion allowed the formation of mature HSA indistinguishable from the authentic human protein (Sijmons et al., 1990).

Tobacco and *Brassica napus* have successfully performed a several-step proteolytic processing of recombinant thionins (Carmona et al., 1993; Florack et al., 1994), Brazil nut 2S albumin (Altenbach et al., 1989, 1992) and human protein C (Cramer et al., 1996). The cleavage of both the aminoterminal signal peptide and the carboxyterminal peptide of prepro-thionins appeared to be important for mediating the transition of the recombinant protein into the endoplasmic reticulum and the correct folding of mature thionins, respectively (Florack et al., 1994).

In summary, plants possess the machinery necessary for required proteolytic processing, but not all plant tissue and gene combinations give the same results as the native host. To take full advantage of plant systems more work is needed to develop strategies for achieving proper processing.

Glycosylation

One of the advantages of the plant production systems is the ability to perform posttranslational modifications such as protein glycosylation. There are only a few studies that have

completely characterized proteins produced in transgenic plants, and no definitive judgment can be made on how accurately the transgenic plants can glycosylate foreign proteins. The current data indicate that the level and/or the pattern of glycosylation of recombinant proteins may vary.

Recombinant α -amylase produced in tobacco was glycosylated in contrast to the wild-type α -amylase from barley (Pen et al., 1992; Pen et al., 1993a). Treatment of the recombinant α -amylase with endo- β -*N*-acetyl glycosaminidase H and trifluoromethanesulfonic acid showed that complex-type carbohydrate chains were attached to the recombinant protein (Pen et al., 1992). Avidin was glycosylated in transgenic maize with a similar but not identical pattern to that of chicken eggs (Hood et al., 1997). A single carbohydrate chain was attached to the Asn-17 amino acid residue in maize as it was for avidin from eggs. *Aspergillus niger* phytase produced in transgenic tobacco was glycosylated (MW 67 kD) but differently from that of the wild-type enzyme with a MW of 80 kD (Pen et al., 1993b). The deglycosylated recombinant phytase had the same molecular weight of 60 kD as the deglycosylated wild-type enzyme. Differential glycosylation of recombinant β -phaseolin was also observed in tobacco (Hoffman et al., 1988). The majority of β -phaseolin produced in the transgenic tobacco contained two high-mannose carbohydrate chains, whereas the rest contained only one high-mannose carbohydrate chain. An inducible tobacco expression system has been used to produce active glycosylated human glucocerebrosidase, a lysosomal enzyme (Cramer et al., 1996).

Proteins that have possible glycosylation sites should be further characterized to determine what effect glycosylation will have on their properties and, when glycosylation alters activity in a detrimental way, sequence changes may be necessary to prevent it. However, in many cases, a plant expression system offers a significant advantage over bacterial expression systems when protein glycosylation is important for a particular recombinant protein to function properly. Properties of the recombinant protein such as biological activity, protein folding, stability, and solubility could be affected by glycosylation (Marino, 1991). For pharmaceutical purposes, recombinant proteins have to be identical to the wild-type protein even though different forms may have exactly the same characteristics as the wild-type. If the recombinant proteins produced for pharmaceutical purposes do not have the same composition, new regulatory approval for the recombinant proteins must be obtained. This adds a tremendous amount of time and money to product development before it can be marketed. For industrial applications, protein glycosylation is not a major concern as long as the enzymes have the same functional characteristics needed for their end use.

Folding and Assembly

Folding and assembly of secreted proteins are necessary for their efficient exit from the endoplasmic reticulum and sub-

sequent transport (Chrispeels, 1991). One of the potential concerns for recombinant protein production is, will folding and assembly of synthesized proteins in transgenic plants be similar to that in the native host? Several studies involving recombinant proteins produced in plants have shown that the proteins can undergo self-assembly (Beachy et al., 1985; Hiatt et al., 1989; Hoffman et al., 1988; Ma et al., 1995; Shani et al., 1994; Utsumi et al., 1993, 1994). Wheat high-molecular-weight glutenin subunits were able to assemble into multimeric form in transgenic tobacco (Shani et al., 1994). Also, normal and modified glycinin produced in transgenic tobacco and potato, self-assembled into hexameric molecules similar to that of the wild-type glycinin in soybean seeds (Utsumi et al., 1993, 1994), but only in potato tubers and tobacco seeds and not in tobacco stems or leaves. When different subunits are needed for the accumulation of the final product, as in the case of antibody formation, the area for concern is the coordinated level of gene expression. A complex secretory immunoglobulin consisting of four polypeptide chains assembled into a functional antibody in tobacco plants that expressed all four polypeptides simultaneously (Ma et al., 1995). Several studies demonstrated that the antibody complex accumulated only when all subunits were expressed (Hiatt et al., 1989; Ma and Hein, 1995). Any excess of one of the subunits of the antibody complex presumably led to protein degradation, because only very low levels of free subunits were observed.

Evidence to date suggests that the requirements for protein assembly, whether the protein is a monomer, homopolymer, or heteropolymer, are present in plants. Seemingly critical factors for efficient assembly include glycosylation, signal sequences, and sufficient amounts of each subunit in the appropriate cellular compartment (Hiatt et al., 1989; Hoffman et al., 1988).

Protein Degradation

The level of protein accumulation is the consequence of not only the synthesis of the protein but the degradation as well. There are known systems which aid in protein degradation such as the ubiquitin system (Hondred and Vierstra, 1992; Huffaker, 1990; Parsell and Lindquist, 1993; Vierstra, 1993). In general, it is believed that smaller peptide sequences will degrade faster. There have also been examples that have demonstrated that recombinant proteins can be degraded at rates which result in a significantly reduced level of the end product in the cell (Hoffman et al., 1988; Ohtani et al., 1991; Utsumi et al., 1993).

There are several potential ways to reduce protein degradation: (1) to express the recombinant protein in seed tissues where levels of protease inhibitors may slow the rate of protein degradation; (2) to target the protein to the endoplasmic reticulum (Fiedler and Conrad, 1995; Wandelt et al., 1992); (3) to engineer proteins without protease-specific

sites, thereby making them less susceptible to digestion; and (4) to screen for germplasm low in protease activity.

In summary, each recombinant protein has its own requirements and presents its own unique expression obstacles. Consequently, each recombinant protein must be evaluated individually, at least until we collect enough data to make better predictions.

COMMERCIAL PRODUCTION OF RECOMBINANT PROTEINS IN PLANTS

There are many factors to consider when selecting a system for protein production. As discussed previously, the level of protein accumulation is critical, but other factors such as germplasm, handling and processing of transgenic plant material, and protein purification are equally important when considering commercial production. This section focuses on the factors that have to be taken into account when considering commercial production of proteins in transgenic plants.

Crop Selection

The germplasm source can affect the quality, quantity, the ease of purification and, consequently, the cost of the final product. With all other parameters equal, one can estimate the relative cost of producing the raw protein material. Figure 1 shows a representative example of seven crops that have significantly different protein contents, established handling and processing technologies, and different production costs. The dollar amount was calculated based on the commodity price of the crop, the weight fraction of the total protein in the crop, and the assumption that each crop could produce the target protein as 10% of the total protein. One factor that has a major impact on the cost is the amount of protein in the harvested product. In general, crops that have a higher protein content are more cost-effective. Soybeans (40% total protein) have a very high protein content, which

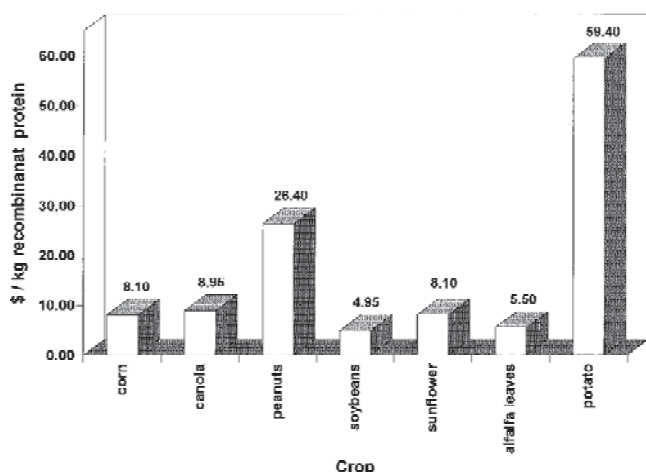


Figure 1. Cost of producing a recombinant protein assuming accumulation of 10% (w/w) of the total crop protein.

gives them a considerable advantage over other crops in which the protein content is only 2% or less (potato tubers). In addition to the cost of raw material, the higher protein content requires less crop to be grown and harvested, which is important when considering the cost of crop handling, processing, and protein extraction (i.e., the less the amount of biomass per unit weight of recombinant protein, the less the extraction cost will be). We have estimated that the cost of producing 1 kg of recombinant protein from the crops given in Figure 1 is 10 to 50 times lower than the cost of producing the same amount by *E. coli* fermentation, assuming that recombinant protein is 20% of total *E. coli* protein (Datar and Rosen, 1990; Petridis et al., 1995).

Directing protein accumulation to a specific part or tissue of the transgenic plant that is harvested is another factor that should be considered. Having specific tissues only expressing the protein may reduce the downstream processing cost (see below), reduce the potential toxicity of the recombinant protein, or reduce the regulatory concerns for plants carrying proteins in the environment via pollen. From the crop-handling viewpoint, one of the more appealing choices within the plant is the seed, although the recombinant protein could be directed to any specific cell or tissue (Edwards and Coruzzi, 1990). Seeds and tubers act as natural storage organs and deposit proteins for long periods of time without degradation. Seeds may be preferred to green tissues which cannot be stored after harvesting without cooling or immediate isolation (Fiedler and Conrad, 1995; Hood et al., 1997; Lorenz and Kulp, 1991; Salunkhe et al., 1992; Smith, 1975). As demonstrated by transgenic alfalfa processing, the green tissue extract had to be immediately processed and stabilized after harvesting to reduce product degradation (Austin et al., 1994). The protein storage stability in transgenic seeds and tubers is important because it eliminates the requirement of locating the downstream processing facility adjacent to the harvesting location and the crop processing plant.

The opportunity to further fractionate the transgenic material (tissue) containing the target protein before the extraction step has several potential advantages. These include: (1) decrease in extract complexity and contamination with interfering plant compounds; (2) increase in the extraction yield and recombinant protein concentration; (3) reduction in the extract volume to be handled in the subsequent steps; and (4) potential revenues from selling fractionated material (corn germ, grits, fiber, oil, starch, etc.). Another criterion for crop selection is the end use of the final product. For example, food crops may be an attractive source for enzymes used in the food industry, whereas feed crops could be used for enzymes and vaccines in the feed industry (Ponstein et al., 1996). In such instances, minimal or no protein purification would be required. In contrast, if the protein product is to be purified, the advantage of using well-established crop processing procedures together with the ease of extraction may be critical and may favor seed-based rather than green tissue-based production methods.

Not only are there differences between different crops,

but a significant variation can occur within a crop. Although it is a difficult task to sort out which crop or variety could be the best for glycosylation, processing, degradation, and high levels of recombinant protein accumulation, there is reason for optimism. With other systems, most noticeably bacteria, several strains have been identified that are optimal for protein production. There already exist a wide array of germplasm for certain crops that have been successful for many agronomic characteristics but not for their ability to produce recombinant proteins. This collection of germplasm and mutants should provide ample opportunity to find exactly what is needed.

Downstream Processing Considerations

Downstream processing for any production system refers to all unit operations after product synthesis. The unit operation steps usually fall into two main categories, recovery and purification, although this division is somewhat arbitrary and varies among different protein production systems (Lesser and Asenjo, 1992; Wheelwright, 1991). Thus, protein recovery from plants would include processing/fractionating of the plant tissue, protein extraction, solid-liquid separation, and concentration, whereas purification would include traditional separation methods such as precipitation, liquid-liquid extraction, membrane filtration, chromatography, etc.

A generalized approach to extraction and purification of plant proteins and enzymes has been outlined by Jervis and Pierpoint (1989). Depending on the plant source, seed grinding or green tissue homogenization is usually the first step in the recovery process followed by cold buffer extraction and centrifugation. The major problems that have been encountered during extraction of plant enzymes from plant tissue, and that will likely be encountered with transgenic plant systems, are the efficiency of protein extraction, the possible structural modification of the product due to secondary reaction with phenolics, and proteolytic degradation. The amount of phenolics varies dramatically among the crops and among different tissues within species, but in general the green leaf material contains a greater amount of phenolic compounds than seeds.

The underlying principles of protein purification are the same, regardless of the source of the target protein, and the selection of a purification sequence does depend on the scale and to some extent on the tissue from which the protein has been extracted (Jervis and Pierpoint, 1989). The process scale-up could also raise problems such as reduced extraction and purification yields and activity loss due to foaming and/or shear (Porter et al., 1991).

To our knowledge, there are very few examples where extraction and purification of recombinant proteins from transgenic plants have been studied and quantified (Austin et al., 1994; Fuchs et al., 1993; Herbers et al., 1995; Hood et al., 1997; Kusnadi et al., 1997; Mason et al., 1996; Vandekerckhove et al., 1989; Van Rooijen and Moloney, 1995). Usually, recombinant proteins were extracted to-

gether with other seed or tissue proteins to determine accumulation levels, to establish protein functionality, and to confirm the expected amino acid sequence. Depending on the transgenic plants and the nature of the recombinant protein, different extraction solutions were used. Beyond control of ionic strength and pH, extraction has been aided by use of detergents (Mason et al., 1992, 1996; Sehnke et al., 1994; Thanavala et al., 1995), reducing agents (Fuchs et al., 1993; Voelker et al., 1989), and protease inhibitors such as phenylmethanesulphonyl fluoride (PMSF) (Hiatt et al., 1989; Sijmons et al., 1990; Mason et al., 1992, 1996; Thanavala et al., 1995) or protease inhibitor mixtures containing PMSF, leupeptin, and benzamidine (Fuchs et al., 1993). Unfortunately, the purification of recombinant proteins produced in transgenic plants has been performed mainly on a laboratory scale using methods not differing greatly from those traditionally used for isolation of plant proteins (Fuchs et al., 1993; Hood et al., 1997; Kusnadi et al., 1997; Mason et al., 1992, 1996; Ohtani et al., 1991; Sijmons et al., 1990; Vandekerckhove et al., 1989). Most laboratory-developed procedures may not be directly scalable because of the high cost of chemicals, the difficulties in controlling the process shear and foaming, and the altered characteristics of the processed transgenic material (particle size, moisture content, etc.) caused by the differences between the laboratory and plant equipment.

The feasibility for recovering recombinant enzymes from transgenic alfalfa has been evaluated recently by Austin et al. (1994). The recovery steps included juice extraction by maceration, clarification, concentration, and stabilization of the soluble protein concentrate. Further fractionation and purification was not investigated but losses of enzymatic activity in the extract were observed. We have compared the laboratory-scale extraction of recombinant β -glucuronidase (GUS) from transgenic maize, soybeans, and canola seeds and determined that, except for canola (40% oil content), the presence of oil and/or starch in the ground seed samples did not affect the extraction yield (unpublished data). The extraction was carried out only with 0.05 M phosphate buffer (pH 7.5) and no proteolytic degradation was observed. Further purification of GUS by ion-exchange and hydrophobic interaction chromatography was not affected by the soluble starch or corn oil in the clarified extract (Kusnadi et al., 1997). Similarly, the extraction and affinity purification yields of recombinant avidin from transgenic maize were not significantly affected by the complex maize extract (Hood et al., 1997).

Transgenic plants offer unique opportunities to simplify recombinant protein extraction and purification. One avenue worth exploring is the targeting of the recombinant protein synthesis to a tissue or organelle that can be easily separated from the rest of the plant material. For example, by using the maize germ (10% of the seed) as a starting material for the extraction, we have increased the concentration of GUS in the extract almost ten times, because 90% of the extractable enzyme activity was located in the germ. Recently, Van Rooijen and Moloney (1995) demonstrated

the effectiveness of combining the organelle fractionation (oil bodies in canola) and the fusion-tail technology. The fusion protein consisting of GUS and oleosin (oil-body protein) was adsorbed on the surface of the oil-bodies and was recovered from the oil fraction of the aqueous canola seed extract.

Clearly, transgenic plants provide a wide array of options that could facilitate protein purification and these options should be considered along with the other presented criteria in selecting the best crop system. Needless to say, each plant-recombinant protein system has to eventually be addressed and optimized separately.

CONCLUSIONS

Current transgenic plant systems with accumulation levels at or below 1% of the total protein may not be ready yet to compete with other recombinant protein production systems, but advantages such as potentially high accumulation levels of the protein, glycosylation, targeting, compartmentalization, and natural storage stability in certain organs are important incentives to aggressively investigate heterologous protein production in plants. To take full advantage of posttranslational processing abilities of plants, improvements and a better understanding of the rules of protein glycosylation, processing, and trafficking will be required.

The cost of downstream processing will play a major role in determining whether a particular plant production system is competitive with traditional fermentation systems. For partially purified protein products, transgenic crops can provide a significant cost reduction by selling coproducts such as oil, starch, fiber, and protein. In instances where highly purified product is needed, the low production cost of recombinant protein (see Fig. 1) might be counterbalanced by the high downstream processing cost, which could easily amount to greater than 80% of the final product cost.

Transgenic plants also offer unique solutions to biocatalysis. For example, several enzymes could be produced and activated upon "request" (controlled biocatalysis). One way of achieving enzyme activation is to produce the proenzyme in one compartment and the corresponding specific protease in another, and then during protein extraction the proenzyme would be activated by the specific protease activity. Alternatively, activation of the enzyme could be accomplished by using temperature, pH, or other required factors for activity. Although still to be proven, an area of undisputed advantage of plant systems is the oral delivery of pharmaceuticals as well as feed and food enzymes.

In spite of these advantages, only two purified protein products (avidin and GUS) to date are ready to be sold. We believe that this will change dramatically over the next few years as we learn to integrate all aspects of producing proteins in plants.

We thank Drs. E. E. Hood and J. M. Jilka of ProdiGene, Inc., and D. R. Witcher and G. Y. Zhong of Pioneer Hi-Bred International, Inc., for helpful comments during preparation of this manuscript.

We also thank Professor C. E. Glatz of Iowa State University for commenting on downstream processing. Lisa Baker of ProdiGene, Inc., also helped with manuscript preparation. This is Journal Paper No. 17105 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project No. 3017.

References

- Abler, M. L., Green, P. J. 1996. Control of mRNA stability in higher plants. *Plant Mol. Biol.* **32**: 63–78.
- Allen, G. C., Hall, G., Jr., Michalowski, S., Newman, W., Steven, S., Weissinger, A. K., Thompson, W. F. 1996. High-level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. *Plant Cell* **8**: 899–913.
- Altenbach, S. B., Kuo, C. C., Staraci, L. C., Pearson, K. W., Wainwright, C., Georgescu, A., Townsend, J. 1992. Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol. Biol.* **18**: 235–245.
- Altenbach, S. B., Pearson, K. W., Meeker, G., Staraci, L. C., Sun, S. S. M. 1989. Enhancement of the methionine content of seed proteins by the expression of a chimeric gene encoding a methionine-rich protein in transgenic plants. *Plant Mol. Biol.* **13**: 513–522.
- Archer, D. B. 1994. Enzyme production by recombinant *Aspergillus*, pp. 373–393. In: Y. Murooka and T. Amanaka (eds.), *Recombinant microbes for industrial and agricultural applications*. Marcel Dekker, New York.
- Austin, S., Bingham, E. T., Koegel, R. G., Mathews, D. E., Shahan, M. N., Straub, R. J., Burgess, R. R. 1994. An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. *Ann. NY Acad. Sci.* **721**: 235–244.
- Beachy, R. N., Chen, Z. L., Horsch, R. B., Rogers, S. G., Hoffmann, N. J., Fraley, R. T. 1985. Accumulation and assembly of soybean β -conglycinin in seeds of transformed petunia plants. *EMBO J.* **4**: 3047–3053.
- Bednarek, S. Y., Raikhel, N. V. 1992. Intracellular trafficking of secretory proteins. *Plant Mol. Biol.* **20**: 133–150.
- Benfey, P. H., Chua, N. H. 1989. Regulated genes in transgenic plants. *Science* **244**: 174–181.
- Bosch, D., Smal, J., Krebbers, E. 1994. A trout growth hormone is expressed, correctly folded and partially glycosylated in the leaves but not the seeds of transgenic plants. *Transgen. Res.* **3**: 304–310.
- Boulikas, T. 1994. Myb proteins 'talking' to their DNA (review). *Int. J. Oncol.* **5**: 101–109.
- Brown, S. M., Santino, C. G. 1995. Enhanced expression in plants, U.S. patent 5,424,412.
- Brown, J. W. S., Simpson, C. G., Simpson, G. G., Turnbull-Ross, A. D., Clark, G. P. 1993. Plant pre-mRNA splicing and splicing components. *Phil. Trans. Roy. Soc. Lond. B* **342**: 217–224.
- Bruyns, A.-M., de Jaeger, G., de Neve, M., de Wilde, C., van Montagu, M., Depicker, A. 1996. Bacterial and plant-produced scFv proteins have similar antigen-binding properties. *FEBS Lett.* **386**: 5–10.
- Callis, J., Fromm, M., Walbot, V. 1987. Introns increase gene expression in cultured maize cells. *Genes Devel.* **1**: 1183–1200.
- Carmona, M. J., Molina, A., Fernandez, J. A., Lopez-Fando, J. J., Garcia-Olmedo, F. 1993. Expression of the α -thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant J.* **3**: 457–462.
- Cavener, D. R., Ray, S. C. 1991. Eukaryotic start and stop translation sites. *Nucl. Acids Res.* **19**: 3185–3192.
- Chrispeels, M. J. 1991. Sorting of proteins in the secretory system. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 21–53.
- Cornelissen, M., Ellebogten, H., Soetaert, P., Eekhoekstraat, L., Stam, M., Uilenstede, A. A., Dockx, J., Tentoonstellingslaan, G., Van Aarssen, R., Zwignjaardssesteenweg, G. 1993. Modified genes and their expression in plant cells, international patent WO 93/09218.
- Cramer, C. L., Weissenborn, D. L., Oishi, K. K., Grabau, E. A., Bennet, S., Ponce, E., Grabowski, G. A., Radin, D. N. 1996. Bioproduction of

- human enzymes in transgenic tobacco. *Ann. NY Acad. Sci.* **792**: 62–71.
- Datar, R., Rosen, C.-G. 1991. Downstream processing economics, pp. 741–792. In: J. A. Asenjo (ed.), *Separation processes in biotechnology*. Marcel Dekker, New York.
- De Clercq, A., Vandewiele, M., Van Damme, J., Guerche, P., Van Montagu, M., Vandeckerckhove, J., Krebbers, E. 1990. Stable accumulation of modified 2S albumin seed storage proteins with higher methionine contents in transgenic plants. *Plant Physiol.* **94**: 970–979.
- Denecke, J., Botterman, J., Deblaere, R. 1990. Protein secretion in plant cells occur via a default pathway. *Plant Cell* **2**: 51–59.
- Dowson Day, M. J., Ashurst, J. L., Mathias, S. F., Watts, J. W., Wilson, T. M. A., Dixon, R. A. 1993. Plant viral leaders influence expression of a reporter gene in tobacco. *Plant Mol. Biol.* **23**: 97–109.
- Ebisu, S., Takagi, H., Kadowaki, K., Yamagata, H., Uda, S. 1996. The efficient production of human epidermal growth factor by *Bacillus brevis*. *Ann. NY Acad. Sci.* **782**: 115–122.
- Ebskamp, M. J. M., van der Meer, I. M., Spronk, B. A., Weisbeek, P. J., Smeeckens, S. C. M. 1994. Accumulation of fructose polymers in transgenic tobacco. *Bio/Technology* **12**: 272–275.
- Edwards, G. A., Hopher, A., Clerk, S. P., Boulter, D. 1991. Pea lectin is correctly processed, stable and active in leaves of transgenic potato plants. *Plant Mol. Biol.* **17**: 89–100.
- Edwards, J. W., Coruzzi, G. M. 1990. Cell-specific gene expression in plants. *Annu. Rev. Genet.* **24**: 275–303.
- Eldebaum, O., Stein, D., Holland, N., Gafni, Y., Livneh, O., Novick, D., Rubinstein, M., Sela, I. 1992. Expression of active human interferon- β in transgenic plants. *J. Interferon Res.* **12**: 449–453.
- Ellis, J. R., Shirsat, A. H., Hopher, A., Yarwood, J. N., Gatehouse, J. A., Croy, R. R. D., Boulter, D. 1988. Tissue-specific expression of a pea legumin gene in seeds of *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* **10**: 203–214.
- Fiedler, U., Conrad, U. 1995. High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technology* **13**: 1090–1093.
- Finnegan, J., McElroy, D. 1994. Transgene inactivation: plants fight back. *Bio/Technology* **12**: 883–888.
- Florack, D. E. A., Dirkse, W. G., Visser, B., Heidekamp, F., Stiekema, W. J. 1994. Expression of biologically active hordothionins in tobacco. Effects of pre- and pro-sequences at the amino and carboxyl termini of the hordothionin precursor on mature protein expression and sorting. *Plant Mol. Biol.* **24**: 83–96.
- Fraleigh, R. T., Rogers, S. G., Hoersch, R. B., Sanders, P. R., Flick, J. S. 1983. Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. USA* **80**: 4803–4807.
- Fuchs, R. L., Heeren, R. A., Gustafson, M. E., Rogan, G. J., Bartnicki, D. E., Leimgruber, R. M., Finn, R. F., Hershan, A., Berberich, S. A. 1993. Purification and characterization of microbially expressed neomycin phosphotransferase II (NPTII) protein and its equivalence to the plant expressed protein. *Bio/Technology* **11**: 1537–1541.
- Fütterer, J., Hohn, T. 1996. Translation in plants—rules and exceptions. *Plant Mol. Biol.* **32**: 159–189.
- Gallie, D. R. 1996. Translational control of cellular and viral mRNAs. *Plant Mol. Biol.* **32**: 145–158.
- Georgiou, G. 1988. Optimizing the production of recombinant proteins in microorganism. *AIChE J.* **34**: 1233–1248.
- Georgiou, G., Bowden, G. A. 1991. Inclusion body formation and the recovery of aggregated recombinant proteins, pp. 333–356. In: C. Ho, A. Prokop, and R. Bajpaj (eds.), *Recombinant DNA technology and applications*. McGraw-Hill, New York.
- Goddijn, O. J. M., Pen, J. 1995. Plants as bioreactors. *Trends Biotechnol.* **13**: 379–387.
- Goosen, M. F. A. 1993. Insect cell culture engineering: an overview, pp. 1–16. In: M. F. A. Goosen, A. Baugulis, and P. Faulkner (eds.), *Insect cell culture engineering*. Marcel Dekker, New York.
- Haq, T. A., Mason, H. S., Clements, J. D., Arntzen, C. J. 1995. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**: 714–716.
- Harashima, S. 1994. Heterologous protein production by yeast host-vector systems. *Bioproc. Technol.* **19**: 137–158.
- Herbers, K., Wilke, I., Sonnewald, U. 1995. A thermostable xylanase from *Clostridium thermocellum* expressed at high levels in the apoplast of transgenic tobacco has no detrimental effects and is easily purified. *Bio/Technology* **13**: 63–66.
- Hiatt, A., Cafferkey, R., Bowdish, K. 1989. Production of antibodies in transgenic plants. *Nature* **342**: 76–78.
- Hoffman, L. M., Donaldson, D. D., Bookland, R., Rashka, K., Herman, E. M. 1987. Synthesis and protein body deposition of maize 15-kd zein in transgenic tobacco seeds. *EMBO J.* **6**: 3213–3221.
- Hoffman, L. M., Donaldson, D. D., Herman, E. M. 1988. A modified storage protein is synthesized, processed, and degraded in the seeds of transgenic plants. *Plant Mol. Biol.* **11**: 717–729.
- Hondred, D., Vierstra, R. D. 1992. Novel applications of the ubiquitin-dependent proteolytic pathway in plant genetic engineering. *Curr. Opin. Biotechnol.* **3**: 147–151.
- Hood, E. E., Withcher, D. R., Maddock, S., Meyer, T., Baszczyński, C., Bailey, M., Flynn, P., Register, J., Marshal, L., Bond, D., Kulisek, E., Kusnadi, A., Evangelista, R., Nikolov, Z., Wooge, C., Mehig, R. J., Hernan, R., Kappel, W. K., Ritland, D., Li, P. C., Howard, J. A. 1997. Commercial production of avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. *Molec. Breeding (in press)*.
- Huffaker, R. C. 1990. Proteolytic activity during senescence of plants. *New Phytol.* **116**: 199–231.
- Hughes, J., Qoronfle, M. W. 1991. Perspectives in plant genetic engineering and biopharmacy. *BioPharm* **May**: 18–28.
- Hunt, A. G. 1994. Messenger RNA 3' end formation in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **45**: 47–60.
- Hurst, H. C. 1994. Transcription factor 1: bZIP proteins. *Prot. Profile* **1**: 123–124.
- Ingelbrecht, I. L. W., Herman, L. M. F., Dekeyser, R. A., Van Montagu, M. C., Depicker, A. G. 1989. Different 3' end regions strongly influence the level of gene expression in plant cells. *Plant Cell* **1**: 671–680.
- Iturriaga, G., Jefferson, R. A., Bevan, M. 1989. Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco. *Plant Cell* **1**: 381–390.
- Jenkins, N., Parekh, R. B., James, D. C. 1996. Getting the glycosylation right: implications for the biotechnology industry. *Nature Biotechnol.* **14**: 975–981.
- Jervis, L., Pierpoint, W. S. 1989. Purification technologies for plant proteins. *J. Biotechnol.* **11**: 161–198.
- Kenward, K. D., Altschuler, M., Hildebrand, D., Davies, P. L. 1993. Accumulation of type I fish antifreeze protein in transgenic tobacco is cold-specific. *Plant Mol. Biol.* **23**: 377–385.
- Kozak, M. 1992. A consideration of alternative models for the initiation of translation in eukaryotes. *Crit. Rev. Biochem. Mol. Breed.* **27**: 385–402.
- Krebbers, E., Bosch, D., Vandeckerckhove, J. 1992. Prospects and progress in the production of foreign proteins and peptides in transgenic plants, pp. 315–325. In: P. R. Shewry and S. Gutteridge (eds.), *Plant protein engineering*. Cambridge University Press, London.
- Kudo, T. 1994. *Escherichia coli* secretion vector using the *kil* gene, pp. 291–299. In: Y. Murooka and T. Amanaka (eds.), *Recombinant microbes for industrial and agricultural applications*. Marcel Dekker, New York.
- Kusnadi, A., Evangelista, R., Nikolov, Z. L., Howard, J. 1997. Recovery of recombinant β -glucuronidase from transgenic corn. In: L. E. Erickson (ed.), *The proceedings of the 26th annual Biochemical Engineering Symposium* (pp. 143–148), Kansas State University, Manhattan, KS.
- Lambowitz, A. M., Belfort, M. 1993. Introns as mobile genetic elements. *Annu. Rev. Biochem.* **62**: 587–622.
- Lee, H.-I., Raikhel, N. V. 1995. Prohevein is poorly processed but shows enhanced resistance to a chitin-binding fungus in transgenic tomato plants. *Brazil. J. Med. Biol. Res.* **28**: 743–750.
- Lehman, D. J., Roof, L. L., Brideau, R. J., Aeed, P. A., Thomsen, D. R., Elhammer, A. P., Wathen, M. W., Homa, F. L. 1993. Comparison of

- soluble and secreted forms of human parainfluenza virus type 3 glycoproteins expressed from mammalian and insect cells as subunit vaccines. *J. Gen. Virol.* **74**: 459–469.
- Lesser, E. W., Asenjo, J. A. 1992. Rational design of purification processes for recombinant proteins. *J. Chromatogr.* **584**: 43–57.
- Lorenz, K. J., Kulp, K. 1991. Handbook of cereal science and technology. Marcel Dekker, New York.
- Lubon, H., Paleyanda, R. K., Velandar, W. H., Drohan, W. N. 1996. Blood proteins from transgenic animal bioreactors. *Transfus. Med. Rev.* **10**: 131–143.
- Luckow, V. A., Summers, M. D. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* **6**: 47–55.
- Luehnsen, K. R., Taha, S., Walbot, V. 1994. Nuclear pre-mRNA processing in higher plants. *Prog. Nucl. Acid Res. Mol. Biol.* **47**: 149–193.
- Lutcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., Scheele, G. A. 1987. Selection of AUG initiation codons differs in plants and animals. *EMBO J.* **6**: 43–48.
- Ma, J. K.-C., Hein, M. B. 1995. Immunotherapeutic potential of antibodies produced in plants. *Trends Biotechnol.* **13**: 522–527.
- Ma, J. K.-C., Hiatt, A., Hein, M., Vine, N. D., Wang, F., Stabila, P., van Dolleweerd, C., Mostov, R. K., Lehner, T. 1995. Generation and assembly of secretory antibodies in plants. *Science* **268**: 716–719.
- Maas, C., Laufs, J., Grant, S., Korfhage, C., Werr, W. 1991. The combination of a novel stimulatory element in the first exon of the maize *Shrunken-1* gene with the following intron 1 enhances reporter gene expression up to 1000-fold. *Plant Mol. Biol.* **16**: 199–207.
- Marino, M. M. 1991. Expression of heterologous proteins in yeast, pp. 29–65. In: A. Prokop, R. K. Bajpal, and C. S. Ha (eds.), *Recombinant DNA technology and applications*. McGraw-Hill, New York.
- Mason, H. S., Ball, J. M., Shi, J.-J., Jiang, X., Estes, M. K., Arntzen, C. J. 1996. Expression of Norwalk virus capsid protein in transgenic tobacco and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. USA* **93**: 5335–5340.
- Mason, H. S., Lam, D. M.-K., Arntzen, D. J. 1992. Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* **89**: 11745–11749.
- Matsumoto, S., Ikura, K., Ueada, M., Sasaki, R. 1995. Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. *Plant Mol. Biol.* **27**: 1163–1172.
- Matthews, B. F., Hughes, C. A. 1993. Nutritional improvement of the aspartate family of amino acids in edible crop plants. *Amino Acids* **4**: 21–34.
- Meyer, P., Saedler, H. 1996. Homology-dependent gene silencing in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**: 23–48.
- Murray, E. E., Lotzer, J., Eberle, M. 1989. Codon usage in plant genes. *Nucl. Acids Res.* **17**: 477–498.
- Narvaez-Vasquez, J., Orozco-Cardenas, M. L., Ryan, C. A. 1992. Differential expression of a chimeric CaMV-tomato proteinase inhibitor I gene in leaves of transformed nightshade, tobacco and alfalfa. *Plant Mol. Biol.* **20**: 1149–1157.
- Oakes, J. V., Shewmaker, C. K., Stalker, D. M. 1991. Production of cyclodextrins, a novel carbohydrate, in the tubers of transgenic potato plants. *Bio/Technology* **9**: 982–986.
- Ohtani, T., Galili, G., Wallace, J. C., Thompson, G. A., Larkins, B. A. 1991. Normal and lysine-containing zeins are unstable in transgenic tobacco seeds. *Plant Mol. Biol.* **16**: 117–128.
- Park, Y.-D., Papp, I., Moscone, E. A., Iglesias, V. A., Vaucheret, H., Matzke, A. J. M., Matzke, M. A. 1996. Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. *Plant J.* **9**: 183–194.
- Parmenter, D. L., Boothe, J. G., van Rooijen, G. J. H., Yeung, E. C., Moloney, M. M. 1995. Production of biologically active hirudin in plant seeds using oleosin partitioning. *Plant Mol. Biol.* **29**: 1167–1180.
- Parsell, D. A., Lindquist, S. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Gen.* **27**: 437–496.
- Pen, J., Molendijk, L., Quax, W. J., Sijmons, P. C., van Ooyen, A. J. J., van den Elzen, P. J. M., Rietveld, K., Hoekema, A. 1992. Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. *Bio/Technology* **10**: 292–296.
- Pen, J., Sijmons, P. C., van Ooijen, A. J. J., Hoekema, A. 1993a. Protein production in transgenic crops: analysis of plant molecular farming, pp. 239–251. In: A. Hiatt (ed.), *Transgenic plants fundamentals and applications*. Marcel Dekker, New York.
- Pen, J., Verwoerd, T. C., van Paridon, P. A., Beudeker, R. F., van den Elzen, P. J. M., Geerse, K., van der Klis, J. D., Versteegh, H. A. J., van Ooyen, A. J. J., Hoekema, A. 1993b. Phytase-containing transgenic seeds as a novel feed additive for improved phosphorus utilization. *Bio/Technology* **11**: 811–814.
- Petridis, D., Sapidou, E., Calandranis, J. 1995. Computer-aided process analysis and economic evaluation for biosynthetic human insulin production—a study case. *Biotechnol. Bioeng.* **48**: 529–541.
- Ponstein, A. S., Verwoerd, T. C., Pen, J. 1996. Production of enzymes for industrial use. *Ann. NY Acad. Sci.* **792**: 91–98.
- Porter, J. E., Ladisch, M. R., Herrmann, K. M. 1991. Ion exchange and affinity chromatography in the scaleup of the purification of α -galactosidase from soybean seeds. *Biotechnol. Bioeng.* **37**: 356–364.
- Salunkhe, D. K., Chavan, J. K., Adsule, R. N., Kadam, S. S. 1992. World oilseeds. Chemistry, technology, and utilization. Van Nostrand, New York.
- Schouten, A., Roosien J., van Engelen, F. A., de Jong, G. A. M., Borst-Vrenssen, A. W. M., Zilverentant, J. F., Bosch, D., Stiekema, W. J., Gommers, F. J., Schots, A., Bakker, J. 1996. The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco. *Plant Mol. Biol.* **30**: 781–793.
- Sehnke, P. C., Pedrosa, L., Paul, A. L., Frankel, A. E., Ferl, R. J. 1994. Expression of active, processed ricin in transgenic tobacco. *J. Biol. Chem.* **269**: 22473–22476.
- Shani, N., Rosenberg, N., Kasarda, D. D., Galili, G. 1994. Mechanism of assembly of wheat high molecular weight glutenins inferred from expression of wild-type and mutant subunits in transgenic tobacco. *J. Biol. Chem.* **269**: 8924–8930.
- Sheen, J. 1994. Feedback control of gene expression. *Photosynth. Res.* **39**: 427–438.
- Sijmons, P. C., Dekker, B. M. M., Schrammeijer, B., Verwoerd, T. C., van den Elzen, P. J. M., Hoekema, A. 1990. Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* **8**: 217–221.
- Singh, B. K., Matthews, B. F. 1994. Molecular regulation of amino acid biosynthesis in plants. *Amino Acids* **7**: 165–174.
- Smith, O. 1975. Effect of transit and storage conditions on potatoes, pp. 171–235. In: W. F. Talburt and O. Smith (eds.), *Potato processing*. AVI, Westport, CT.
- Solymosy, F. 1990. Similarities and dissimilarities between plant UsnRNAs and their equivalents in other eukaryotes with respect to structure, complexing with proteins, gene organization and function. *Acta Biochim. Biophys. Hung.* **25**: 67–85.
- Solymosy, F., Pollak, T. 1993. Uridylate-rich small nuclear RNAs (UsnRNAs), their genes and pseudogenes, and UsnRNPs in plants: structure and function. A comparative approach. *Crit. Rev. Plant Sci.* **12**: 275–369.
- Sullivan, M. L., Green, P. J. 1993. Post-transcriptional regulation of nuclear-encoded genes in higher plants: the roles of mRNA stability and translation. *Plant Mol. Biol.* **23**: 1091–1104.
- Taylor, C. B., Green, P. J. 1995. Identification and characterization of genes with unstable transcripts (GUTs) in tobacco. *Plant Mol. Biol.* **28**: 27–38.
- Thanavala, Y., Yang, Y.-F., Lyons, P., Mason, H. S., Arntzen, C. 1995. Immunogenicity of transgenic plant-derived hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **92**: 3358–3361.
- Topfer, R., Maas, C., Horicke-Grandpierre, C., Schell, J., Steinbiss, H. H. 1993. Expression vectors for high-level gene expression in dicotyledonous and monocotyledonous plants. *Meth. Enzymol.* **217**: 66–79.

- Trudel, J., Potvin, C., Asselin, A. 1992. Expression of active hen egg white lysozyme in transgenic tobacco. *Plant Sci.* **87**: 55–67.
- Turpen, T. H., Reinl, S. J., Charoenvit, Y., Hoffman, S. L., Fallarme, V., Grill, L. K. 1995. Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. *Bio/Technology* **13**: 53–57.
- Udaka, S., Yamagata, H. 1993. High-level secretion of heterologous proteins by *Bacillus brevis*. *Meth. Enzymol.* **217**: 23–33.
- Utsumi, S., Kitagawa, S., Katsube, T., Higasa, T., Kito, M., Takaiwa, F., Ishige, T. 1994. Expression and accumulation of normal and modified soybean glycinins in potato tubers. *Plant Sci.* **102**: 181–188.
- Utsumi, S., Kitagawa, S., Katsube, T., Kang, I. J., Gidamis, A. B., Takaiwa, F., Kito, M. 1993. Synthesis, processing and accumulation of modified glycinins of soybean in the seeds, leaves and stems of transgenic tobacco. *Plant Sci.* **92**: 191–202.
- Van den Elzen, P. J. M., Pen, J., Hoekema, A., Sijmons, P. C., Van Ooyen, A. J. J., Rietveld, K., Quax, W. J. 1992. Transgenic plants having a modified carbohydrate content, international patent WO 92/05259.
- Van der Meer, I. M., Ebskamp, M. J. M., Visser, R. G. F., Weisbeek, P. J., Smeeckens, S. C. M. 1994. Fructan as a new carbohydrate sink in transgenic potato plants. *Plant Cell* **6**: 561–570.
- Vandekerckhove, J., Damme, J. V., Lijsebettens, M. V., Botterman, J., Block, M. D., Vandewiele, M., Clercq, A. D., Leemans, J., Montagu, M. V., Krebbers, E. 1989. Enkephalins produced in transgenic plants using modified 2S seed storage proteins. *Bio/Technology* **7**: 929–932.
- Van Rooijen, G. J. H., Moloney, M. M. 1995. Plant seed oil-bodies as carriers for foreign proteins. *Bio/Technology* **13**: 72–77.
- Velander, W. H., Lubon, H., Drohan, W. N. 1997. Transgenic livestock as drug factories. *Sci. Am.* **276**: 70–74.
- Verwoerd, T. C., van Paridon, P. A., van Ooyen, A. J. J., van Lent, J. W. M., Hoekema, A., Pen, J., 1995. Stable accumulation of *Aspergillus niger* phytase in transgenic tobacco leaves. *Plant Physiol.* **109**: 1199–1205.
- Vierstra, R. D. 1993. Protein degradation in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**: 385–410.
- Vitale, A., Ceriotti, A., Denecke, J. 1993. The role of the endoplasmic reticulum in protein synthesis, modification, and intracellular transport. *J. Exp. Botany* **44**: 1417–1444.
- Voelker, T. A., Herman, E. M., Chrispeels, M. J. 1989. In vitro mutated phytohemagglutinin genes expressed in tobacco seeds: role of glycans in protein targeting and stability. *Plant Cell* **1**: 95–104.
- Wandelt, C. I., Khan, M. R. I., Craig, S., Schroeder, H. E., Spencer, D., Higgins, T. J. V. 1992. Vicilin with carboxy-terminal KDEL is retained in the endoplasmic reticulum and accumulates to high levels in the leaves of transgenic plants. *Plant J.* **2**: 181–192.
- Warren, T. G., Krivi, G. G. 1991. Strategies for production of proteins in mammalian cells, pp. 66–96. In: A. Prokop, R. K. Bajpai, and C. S. Ho (eds.), *Recombinant DNA technology and applications*. McGraw-Hill, New York.
- Werner, R. G., Thomae, K. 1994. Successful products and future business prospects, pp. 573–578. In: R. E. Spier, J. B. Griffiths, and W. Berthold (eds.), *Animal cell technology products of today prospects for tomorrow*. Butterworth-Heinemann, Oxford.
- Wheelwright, S. M. 1991. In: *Protein purification: design and scale up of downstream processing*. Springer, New York.
- Whitelam, G. C., Cockburn, B., Gandeche, A. R., Owen, M. R. L. 1993. Heterologous protein production in transgenic plants. *Biotechnol. Genet. Eng. Rev.* **11**: 1–29.
- Willmitzer, L., Sonnewald, U., Roeber, M., Carlsen, S. K. 1992. Transgenic plants expressing genes for industrial enzymes, international patent WO 92/01042.
- Wong, E. Y., Hironaka, C. M., Fischhoff, D. A. 1992. *Arabidopsis thaliana* small subunit leader and transit peptide enhance the expression of *Bacillus thuringiensis* proteins in transgenic plants. *Plant Mol. Biol.* **20**: 81–93.
- Zambryski, P., Joss, H., Genetello, C., Leemans, J., van Montagu, M., Schell, J. 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. *EMBO J.* **2**: 2143–2150.