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Heterologous Protein Production in Transgenic Plants

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Introduction

For the purposes of this review, a heterologous protein is defined as being the product of a gene isolated from a species different from the receiving organism and is further restricted to proteins which do not exist as closely similar analogues in the donor and receiver species. Justifications for the production of such heterologous proteins in plants fall into two basic categories: (1) 'factory farming', in which the unique properties of plants can be exploited in the production of desirable proteins, and (2) 'phenotype modification', in which the heterologous protein may be used to alter the characteristics of the plant itself.

Factory farming

It is difficult to envisage a protein production system, more economical and more amenable to large-scale production than plant agriculture. All that is required is a suitable plant, sunlight, mineral salts from the soil (or fertilizers) and water. Furthermore, modern agricultural machinery allows efficient harvesting of large quantities of plant material. Traditional agriculture exploits these characteristics of plants in the production of immense quantities of materials including, for example, the proteins of wheat and soybean.

All of these advantages apply equally to the production of heterologous proteins and, particularly where very large-scale production is contemplated, the plant system represents a realistic alternative to microbial or animal

Abbreviations: BiP, binding protein; CaMV, cauliflower mosaic virus; cDNA, complementary DNA; CDR, complementarity determining region; C_H, constant domain of the heavy chain; CL, constant domain of the light chain; EPSP synthase, enolpyruvylshikimate-3-phosphate synthetase; ER, endoplasmic reticulum; HAMA, human anti-mouse antibody; HSA, human serum albumin; IgG, immunoglobulin G; IgM, immunoglobulin M; mRNA, messenger RNA; PCB, polychlorinated biphenyl; PCR, polymerase chain reaction; TMV, tobacco mosaic virus; V_H, heavy-chain variable region; V_L, light-chain variable region.

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cell-based systems for heterologous protein production. Furthermore, as eukaryotes, plants are capable of the post-translational processing of proteins of eukaryotic origin (plant or animal) which may be essential to their proper functioning and which may present problems in bacterial and other prokaryotic systems. The ultimate determinant of the utility of an expression system is, of course, the overall cost of production. In this context, the existence of an established efficient infrastructure and facilities for the growth, harvesting and processing of plants will represent a significant component in the cost equation.

Although it may well turn out to be of transient concern, any programme aiming to exploit transgenic technology must ultimately come to terms with consumer acceptability. Here again plants offer advantages in that the general public appears to be less concerned about the production and exploitation of transgenic plants than transgenic animals or micro-organisms. Presumably the ease with which the layman can identify with experimental animals and the association of micro-organisms with disease, along with the benign though alien image of plants, are significant factors here.

High-value proteins for which there is a sustained demand and limited supply by conventional means represent the most attractive prospects for factory farming. Examples of such proteins which plants have already been shown to be capable of synthesizing are human serum albumin and leu-enkaphalin (Krebbers and Vanderkerckhove, 1990; Sijmons *et al.*, 1990). Plants have also been shown to be capable of expressing functional antibodies and their derivatives (discussed in detail below), and these important therapeutic and diagnostic reagents represent another of the many potential candidates for factory farming.

Additional to the ability to synthesize and post-translationally modify such proteins is, of course, the requirement for yields of products which are both sufficient to be economical and are amenable to extraction and downstream processing. These two crucial requirements are linked to the site of accumulation of the product and are discussed in detail below.

An intriguing suggestion which illustrates the potential sophistication of the heterologous protein farming approach is the suggestion that a microbial α-amylase, an enzyme involved in processing potato starch, could be secreted to the potato apoplast where it has no access to starch in the living tissue. After-harvest processing, however, would result in substrate and enzyme being brought together, thus obviating the step in conventional processing in which exogenous amylase is added (Pen *et al.*, 1993).

An attractive possibility which may be viewed as a variant of factory farming is to use plant expression as a means of distributing and delivering oral vaccines. This currently requires specialist knowledge and often specialized storage including refrigeration, often creating difficulties in the third world. It should be possible to express the appropriate vaccine proteins in plants and take advantage of the plant's ability to conveniently package proteins in seeds as a means of simplifying storage and distribution. Expression of vaccine proteins in fruits or vegetables may also be envisaged as perhaps the ultimate arrangement for distribution and administration of the

vaccine. Only seeds (usually very robust and long-lasting) need to be transported and distributed and even in the most hard-pressed countries, provided that the seeds themselves are inedible, the most likely outcome is that plants grown from them will be eaten.

It is hoped that this brief survey of but a few of the potential applications of factory farming will serve to show that where large-scale production is required, plants are worthy of consideration for the expression of any high-value protein.

Phenotype modification

The range of strategies and possibilities for the modification of the plant phenotype through the expression of heterologous proteins is very broad indeed, and encompasses virtually every activity of the plant. A conceptually straightforward application is in the correction of the well-documented deficiencies (from the point of view of human nutrition) of existing crop storage proteins, legumes in sulphur amino acids and tryptophan, and cereals in lysine, threonine and tryptophan. These deficiencies are amenable to correction by transferring genes coding for proteins containing balancing quantities of these amino acids and by the use of appropriate promoters and targeting signals to ensuring that they accumulate in sufficient amounts in the seed storage tissue (Altenbach and Simpson, 1990).

Reversible male sterility is a desirable agronomic trait which is valuable in the production of defined hybrids in outbreeding crops. The expression of the gene encoding the *Bacillus amyloliquefaciens* RNase, Barnase, in the tapetum of developing anthers, which prevents the anther from producing functional pollen but which can be inactivated by the expression of a second heterologous protein, barstar, when required, achieves this aim (Mariani *et al.*, 1990, 1992).

The expression of heterologous enzymes feature in numerous strategies aimed to improve crop value through simple increase in yield of desired product and also through modification of the nature of the product. Changes in assimilate distribution may be brought about by the expression of heterologous proteins which catalyse key steps in a pathway. The accumulation of glucose and fructose rather than sucrose as a result of the expression of yeast invertase in the apoplast of potato indicates that such approaches may be realistic (Sonnewald, von Schaewen and Willmitzer, 1993).

The accumulation of desirable natural products in plants which do not normally produce them is also feasible. The expression of bacterial fructosyl transferases in tobacco results in the accumulation of polyfructans in tobacco, a plant which does not normally accumulate these commercially useful polymers (Smeekens *et al.*, 1991). This example serves to illustrate the potential complexity which may attend execution of an apparently simple strategy. In fructan accumulation, the site to which the transgenic enzyme is targeted plays a vital part in the outcome of expressing the heterologous gene. When targeted to the cytosol or apoplast, the transgenic enzyme (presumably through accumulation of fructans) is damaging to the plant. Targeting the

enzyme to the vacuole, the normal site of fructan metabolism and accumulation, is much less damaging.

Thus, an understanding of the spatial dimension and targeting to appropriate locations is likely to be very significant in the successful implementation of strategies involving heterologous protein production. The characteristics of fructan accumulation in transgenic tobacco also demonstrates that attention should be paid to temporal and developmental aspects of heterologous protein expression. Sink leaves which receive carbon as sucrose store it as fructan, whereas source leaves which accumulate carbon through photosynthesis rather than transport, accumulate starch (in the chloroplasts) as well as fructans.

Bioconversions resulting in the accumulation of compounds not naturally accumulating in any plant species are also possible. Through the expression of genes encoding two bacterial enzymes, transgenic *Arabidopsis* plants have been produced which convert acetoacyl-coenzyme A (a normal metabolite) to polyhydroxybutyrate, a compound not naturally accumulating in plants and which forms the basis for a biodegradable thermoplastic (Poirier *et al.*, 1992). Thus, there is the possibility of the development of entirely novel crops producing a range of materials not even limited by the plants' already remarkable capacity for chemical gymnastics.

In addition to the expression of heterologous enzymes which participate directly in bioconversions, there is also the possibility of modulating the activities of existing metabolic pathways through expression of proteins which influence the activity of pathway enzymes. Enzymes which synthesize or degrade effector molecules such as fructose 2,6-bisphosphate or antibody derivatives which bind to an active site would fall into this category. The activities of regulatory proteins are also accessible to modulation through the expression of antibody derivatives (immunomodulation), and plants expressing an antibody derivative directed against the photoregulatory protein phytochrome show modified phytochrome-mediated photomorphogenic responses (Owen *et al.*, 1992a).

An important area where the expression of heterologous proteins will certainly find application is in plant protection. The expression of proteins which interfere in some way with pathogenicity can extend potential defence mechanisms beyond the plant's normal genetic repertoire, and therefore potentially outside the range of defence mechanisms to which particular pathogens have evolved responses. From the agronomic point of view, heterologous protein-mediated protection (or indeed any modification of plant activities) is particularly attractive because it involves only minor changes to the plant, often the addition of a single heterologous protein gene. Thus the important characteristics of cultivars for which commercial demand and growth, harvesting and processing parameters are already established are likely to remain unchanged.

It is possible to envisage heterologous protein-mediated protection against all manner of pathogens and in a few cases the strategies have already been shown to be successful. For example, resistance to viruses can result from the expression of viral coat proteins and resistance to insects by expression of the

Bacillus thuringiensis toxins and proteinase inhibitors (Sturtevant and Beachy, 1993; Perlak *et al.*, 1991; Hilder *et al.*, 1987). Recently, potato plants expressing a gene encoding T4 lysozyme have been shown to possess increased resistance to a bacterial pathogen (Düring *et al.*, 1993).

Protection of crop plants against weeds has been achieved by the introduction of herbicide resistance through the expression of heterologous proteins. For instance, resistance to glyphosate, which inhibits the enzyme enolpyruvylshikimate-3-phosphate synthetase (EPSP synthase) and so blocks aromatic amino acid synthesis, has been obtained by transformation with a *Salmonella typhimurium* gene that encodes a mutated, glyphosate-insensitive EPSP synthase (Shah *et al.*, 1986). Such strategies are attractive to agrochemical companies, since they tie the customer to both specific herbicide and resistant plants. They are not so attractive to environmentalists, since they are perceived as being likely to encourage increased use of herbicides.

It seems certain that there will be many successful applications of the largely speculative heterologous protein-mediated strategies outlined above. Realization of the full potential of the approach will require further information on the factors that control heterologous protein production in plants. We have elected to centre our discussion of these factors around antibodies and their derivatives in order to provide a coherent exploration of the subject which is nevertheless relevant to the expression of any heterologous protein in plants. Antibodies, and their derivatives, are prime candidates for exploitation in heterologous protein farming and are also involved in a wide range of strategies for phenotypic modification. In addition, antibodies are complex multimeric glycosylated proteins with measurable *in vitro* function. Thus the expression of antibodies in plants provides a paradigm covering all aspects of heterologous protein expression.

Factors controlling heterologous protein production in plants

WHOLE PLANT FACTORS

Economics of protein farming

One of the key factors in determining whether crop plants can represent a viable heterologous system for protein farming is the overall cost of foreign protein production in plants, relative to the economics of protein production in alternative systems. The economic viability of protein farming in crop plants will depend upon the demand for, and commercial value of, the recombinant protein being produced. Where a high-value protein is needed in large quantities (hundreds of kilograms), then the high capacity and flexibility of agricultural production offers advantages over other production systems, such as microbial fermenters. In terms of biomass production, the growing of crops in the field is extremely cost-effective and can compete with any other system (Pen *et al.*, 1993). Crops that are already harvested and processed for some other, non-proteinaceous, primary product are particularly attractive as

candidate sources of biomass for the production of heterologous proteins. Thus, crops such as starch potatoes and oilseed rape, which are grown for starch and oil, have been the focus of attention. Clearly, the use of a non-dedicated crop for protein production requires that the extraction of the primary product does not interfere with the extraction and downstream processing of the heterologous protein. The wet milling processes used in the extraction of starch from potatoes are considered to be compatible with the separation of proteins from the 'waste' liquid fraction. However, oilseed rape is less attractive, since the extraction of oil is performed at high temperatures that are only likely to be compatible with thermostable proteins.

The economic potential of developing plants as heterologous systems for protein production can be clearly demonstrated when, for example, the production of a heterologous protein in soybeans is considered. If soybeans could accumulate the protein to a level of approximately 1% of their total protein, then its production cost, prior to extraction from soybean meal, will be approximately $0.1 per gram. However, in the case of, say, pharmaceutical proteins, this low cost of production might be offset by the high cost of meeting the requirement of a very high level of purity. At present, the lack of established techniques and procedures for the downstream processing of heterologous proteins produced in crops makes plants less attractive than competitive systems, such as bacterial fermentation systems and mammalian cell cultures, for the production of high-value immunotherapeutic and pharmaceutical proteins.

Transformability of major crop plants

In order to produce recombinant proteins on a commercial scale, a number of factors have to be taken into consideration, one of which is the ability to transform a suitable host crop species. Considerable effort has been directed to developing reliable transformation systems for a number of different plant species, and it is now possible to routinely transform many of the major crop species such as potato, tomato, oilseed rape and sugarbeet. Consequently, it is likely that it will be one of these crops that will be the first to be used as a protein factory. However, it seems certain that when a routine and reliable method becomes available for transforming monocots, the major cereal crops will also be used for this purpose.

A number of different techniques are available for transforming plants based either on a vector or a vector-free method. The most frequently used vector is *Agrobacterium tumefaciens* (reviewed in Klee, Horsch and Rogers, 1987). However, *A. tumefaciens* does have a rather narrow host range, limiting its use to a small number of crop species. As there has only been a limited amount of success with the *Agrobacterium*-mediated transformation of monocot species, vector-free systems or direct gene transfer methods which are not restricted to a particular host range, have attracted a considerable degree of attention. There are a number of different vector-free techniques, such as the chemical stimulation of protoplasts (Hain *et al.*, 1985; Krens *et al.*, 1982), microinjection (Griesbach, 1983; Reich, Iyer and Miki, 1986), electroporation (Lindsey and Jones, 1987) and microprojectile bom-

bardment (Klein *et al.*, 1987), which have all shown some degree of success.

It is envisaged that since a number of major crop species can now be routinely transformed, and the routine transformation of the others is a realistic expectation, the choice of crop for recombinant protein production will largely depend on the ability to extract and process the recombinant protein economically from a suitable harvestable tissue.

Tissue specificity of protein accumulation

The site of protein accumulation will be an additional important factor in determining which plant species will be used as protein factories. Specific promoters can be used to drive transgene expression only in the harvested tissues and not in tissues that are by-products of the harvesting process. For example, in potatoes, the promoter from the gene encoding the major storage protein of the tuber, patatin, can be used to drive expression specifically in the tuber. Storage proteins, such as patatin and seed proteins, are normally accumulated to very high levels in a relatively pure form within the vacuole or protein body of specific harvestable tissues. Consequently, these sites of protein accumulation are attractive for targeting heterologous proteins. The production of bioactive peptides in the form of fusions to seed storage proteins represents an example of the exploitation of the seed protein body as a site that has evolved to accumulate and store protein (see below).

Containability, storage and harvesting

The containability of plant genetic material presents a unique problem in the development of plants as a heterologous system for recombinant protein production. Nevertheless, the problems of both physical containability and genetic containability are not insurmountable. Physical containability will rely upon secure systems for growing the crop, whereas genetic containability will probably necessitate the growing of male sterile lines and/or F1 hybrids.

One of the main advantages of developing plants as a heterologous system for recombinant protein production is that the methods of harvesting and storing plant material are already well established and consequently will require very little refinement. It is envisaged that the crops synthesizing recombinant proteins will be harvested in conventional manners and either removed for the immediate extraction of the recombinant protein or placed in storage. Methods for the long-term storage of many crops are well established, such as for cereal grains and potato tubers. The ability to store plant material uner conditions that require very little maintenance and supervision is an additional advantage in developing plants as a heterologous protein expression system. Additionally, the transportation of harvested plant material is also well established and in general is relatively simple and inexpensive. Consequently, crop production and processing facilities need not be located at a single site.

Downstream processing

The problems associated with the large-scale downstream processing of plant material for protein extraction have not previously been addressed. Consequently, the economic factors have only been investigated in hypothetical cases. The costs of manufacturing recombinant proteins in a few transgenic crops have been estimated and the calculations would indicate that, at the currently obtainable levels of transgene product accumulation, plants would make an ideal heterologous system for the large-scale production of recombinant proteins that do not require a high level of purity (Pen *et al.*, 1993). As techniques for increasing the level of transgenic protein accumulation and for protein purification improve, the manufacturing costs of high-grade pharmaceutical proteins will become less of an economic barrier.

Probably the most significant economic outlay in the development of plants as a source of recombinant protein will be in the development of downstream processing. It seems likely that the development of fusion proteins will be of importance in the improvement of methods for the downstream processing of recombinant proteins from plants. Protein fusions can increase protein stability and protect the recombinant protein from degradation. Purification tags (proteins, ligands, etc) for the affinity-purification of recombinant proteins are the most frequently used method in the downstream processing of proteins (Lowe, 1984; Narayanan and Cranen, 1990). Ideally, the fusion partner should be small and the fusion tag should be capable of being cleaved from the target protein following purification. A number of expression vectors which possess these characteristics are available for producing recombinant proteins in *Escherichia coli*, and similar systems could easily be developed for plants.

MOLECULAR AND CELLULAR FACTORS

Promoters and promoter activity

A key factor determining the level of heterologous protein in transgenic plants is the level of mRNA transcription. Attempts to increase transgene transcription have formed the basis for most strategies aimed at increasing heterologous protein yield. In particular, considerable effort has been invested in the development of strong promoters to drive the expression of heterologous genes in transgenic plants. The promoter most commonly used for this purpose is the cauliflower mosaic virus (CaMV) 35S promoter (Harpster *et al.*, 1988; Kay *et al.*, 1987; Odell *et al.*, 1988; Sanger, Daubert and Goodman, 1990; Teeri *et al.*, 1989). The CaMV 35S promoter acts as a strong, constitutive promoter in most organs of transgenic plants (Jensen *et al.*, 1986). Numerous attempts have been made to modify and improve the CaMV 35S promoter. For instance, it has been reported that duplication of some elements of the CaMV 35S promoter has a strong (up to 100-fold) enhancing effect on the expression of a number of reporter transgenes in tobacco (Kay *et al.*, 1987). However, multiplication of elements of the CaMV

35S promoter has been shown to lead to no significant enhancement of the expression of an ovalbumin gene in the tissues of transgenic plants (Wandelt *et al.*, 1991). Similarly, Comai, Moran and Maslyar (1990) found that reiteration of a CaMV 35S upstream region (the 35S enhancer) did not have a major efect on the expression of the *gusA* reporter gene in either tobacco or tomato transformants. However, a hybrid promoter called Mac, in which a region of the mannopine synthase (*mas*) gene promoter, derived from the TR-DNA of an *Agrobacterium tumefaciens* octopine Ti plasmid, was joined to the enhancer region of the CaMV 35S promoter, was observed to drive higher *gus* expression than the double CaMV 35S promoter. The expression of *gus* driven by the Mac promoter was 3–5 times higher in leaves, and 10–15 times higher in roots and hypocotyls than expression driven by the double CaMV 35S promoter (Comai, Moran and Maslyar, 1990). One feature of the Mac promoter that is thought to contribute to the higher expression driven by this promoter is the 5′ transcribed untranslated region of *mas*. It is possible that this region may be an mRNA leader that favours translation initiation, as is the case for several viral RNA 5′ leaders (Gallie *et al.*, 1987).

In some situations, inducible expression of a transgene may be necessary or desirable. For instance, a gene whose product confers resistance to a pathogen need only be expressed when the plant is challenged with the pathogen. The constitutive expression of the transgene may be detrimental to the normal growth and development of the plant. There are a number of inducible plant promoters that could be useful in inducible expression vectors. These include the promoters of genes normally induced by wounding and/or pathogen attack (e.g. Williams *et al.*, 1992), the promoter of the anaerobically inducible alcohol dehydrogenase gene (*Adh*), as well as the promoters from a range of light-inducible genes and genes induced by plant growth regulators. A tightly regulated, inducible promoter has been described by Ainley and Key (1990), who constructed a heat-inducible expression cassette containing the promoter and all but five bases of the mRNA leader from soybean heat shock protein gene, *Gmhsp17.5E*, linked to the GUS coding sequence. In a transient expression system, at heat shock temperatures, this promoter–GUS construct was expressed at roughly 80-fold higher levels, per unit time, than a CaMV 35S–GUS construct.

There are many situations in which a heterologous protein needs to be synthesized in a particular organ or tissue. Thus, for protein farming, it is likely that the heterologous protein will need to be synthesized in a readily harvestable and storable tissue such as seed, tuber or fruit (see above). While promoters that direct transgene expression in a wide range of organs, tissues and cell types have been identified and analysed, relatively few have been assessed for their use in protein farming. The promoter regions of seed storage proteins, together with the amino acid signals that direct seed storage proteins to storage organelles, have been employed in the production of some bioactive peptides (see below).

DNA methylation and transgene silencing

DNA methylation has been implicated in the control of endogenous gene expression in animal cells, where an inverse correlation exists between gene methylation and expression. Methylation also appears to be involved in the regulation of plant gene expression and similar inverse correlations between methylation and expression of endogenous genes have been reported (e.g. Bianchi and Viotti, 1988). In addition, methylation seems to be a mechanism for the inactivation of chimaeric transgenes in plants (e.g. Hobbs, Kpodar and Delong, 1990; Matzke *et al.*, 1989), clearly undesirable in a heterologous protein expression system. It has been suggested that, at least in some cases, transgene methylation and hence inactivation occurs in *Agrobacterium tumefaciens* prior to transfer to the cell (Palmgren, Mattson and Okkels, 1993). It is known that *in vitro* methylation of transgenes is maintained in plant cells with attendant reduced expression (e.g. Weber, Ziechmann and Graessmann, 1990), and that *Agrobacterium* DNA is methylated. In support of their view, Palmgren, Mattson and Okkels (1993) have shown that treatment of *Agrobacterium* with the demethylating agent 5-azacytidine leads to increased transient and stable expression of a reporter gene. Not all transgenes are subject to silencing and appropriate selection of suitable transformants will circumvent problems from this source.

Transcript processing

Post-transcriptional modification processes are extremely important to gene expression, and can therefore influence the yield of heterologous protein in transgenic plants. The presence of introns in both plant and animal genes can affect the level of transcription of those genes. The methanism by which this occurs is not fully understood. Enhancer sequences in the introns are thought to increase expression levels; however, the efficiency of splicing and other processing aspects of mRNA stability may also be involved.

In plants, a number of examples exist where the presence of introns has been shown to increase expression of a gene. Normal expression of the maize alcohol dehydrogenase 1 (*Adh 1*) gene is dependent upon the presence of at least one of the nine introns normally present in the gene (Callis, Fromm and Walbot, 1987). Intron 2 or 6 of the *Adh 1* gene have been shown to enhance chloramphenicol acetyl transferase gene expression in electroporated maize protoplasts by up to 20-fold (Mascarenhas *et al.*, 1990). McElroy *et al.* (1990) have demonstrated that the intron from the rice actin gene can enhance expression of the *gus* reporter gene in transformed rice. Similarly, Tanaka *et al.* (1990) have reported that the first intron of the castor bean catalase gene (*cat-1*) also enhances the expression of the *gusA* gene in transformed rice, but not in transgenic tobacco.

The mechanism by which these introns increase expression levels in plants is unknown, but it appears to be correlated to splicing efficiency. Where splicing is efficient, gene expression is increased, whereas limited splicing results in little or no enhancement (Simpson *et al.*, 1992).

Translation initiation and codon usage

It is well established that not all eukaryotic RNAs are translated with equal efficiency *in vivo* or *in vitro*. The RNAs of several plant viruses are known to be well translated and highly competitive and the 5′-untranslated leader sequences of these are known to act in *cis* to enhance the translation of foreign mRNAs both *in vitro* and *in vivo* (Gallie *et al.*, 1987; Sleat *et al.*, 1987). Numerous plant transformation vectors have been designed that incorporate viral translational enhancers. Guerineau, Lucy and Mullineaux (1992) have investigated the influence of sequences around the translation initiation codon on the expression of the *gus* reporter gene in tobacco. Sequences corresponding to the vertebrate consensus sequence, CCAC-CATGG (Kozak, 1987) and the plant consensus sequence AACAATGG (Lutcke *et al.*, 1987), were fused with the GUS-coding region and transient expression, driven by a duplicated CaMV 35S promoter, was determined in tabacco mesophyll protoplasts. The constructs containing translational fusions between the consensus translation initiation sequences gave over three times more GUS activity than a control construct containing a transcriptional fusion. There was no significant difference between the GUS activity seen for the two consensus sequences.

There is evidence that efficiency of translation of transgenic mRNAs in plants can be increased by modification of the coding sequence of the transgene. Genes that encode the insecticidal δ-endotoxins of *Bacillus thuringiensis*, such as *crylA*, have been expressed in transgenic plants and insect resistance has been reported (e.g. Perlak *et al.*, 1990). However, the level of expression of these insect control proteins in plants are typically very low, although expression of a truncated gene encoding only the *N*-terminal half of the protein does result in a modest increase in transgenic protein levels (Vaeck *et al.*, 1987). Substantial increases, up to 100-fold, in insect control protein accumulation have been observed following modification of the coding regions of both the *crylA(b)* and *crylA(c)* genes (Perlak *et al.*, 1991). Partial modification of the coding sequences, involving removal of a subset of potential polyadenylation signals and ATTTA sequences, increased the number of transformants displaying insect control, as well as increasing protein accumulation. Full modification of the genes, involving elimination of all potential polyadenylation sequences, all ATTTA sequences and increasing the G + G content led to even greater accumulation of the insect control proteins (Perlak *et al.*, 1991). Since more protein was produced for a given amount of the fully modified mRNA, compared with the partially modified mRNA, the presence of predominantly plant-preferred codons in the former may have increased translational efficiency.

Protein stability

In general, yields of transgene products in plants are low, although a very few heterologous proteins have been reported to accumulate to levels of 1% of total soluble plant protein. Considerable effort has been invested in devising

strategies to increase transgene transcription and transcript processing and translation (see above), yet relatively little is known about the factors that determine the stability of proteins in plants. Clearly, if the half-life of a transgenic protein could be increased, then an increase in yield of that protein could be achieved without the need for additional energetic expenditure by the plant. The molecular determinants of protein stability in eukaryotes are beginning to be identified, with particular progress being made in defining the steps that lead to degradation of short-lived cytoplasmic proteins (Jentsch and Bachmair, 1992). Plants are only rarely employed in these types of studies (Bachmair, Becher and Schell, 1993).

There are indications from several studies on transgenic protein accumulation that cellular factors, specifically cellular location, can have a profound effect on protein stability and hence accumulation. Starch potatoes have been assessed as a target crop for the agricultural production of human serum albumin (HSA). A number of modified forms of the HSA gene were transformed into potato and tobacco plants using an *Agrobacterium* binary vector system, in which transgene expression was driven by a modified CaMV 35S promoter and which included an alfalfa mosaic virus leader sequence. In plants transformed with the coding region of mature HSA fused to the coding region of a signal sequence derived from a secreted tobacco protein (PR-S, a pathogenesis-related protein), correctly processed HSA protein was found to accumulate up to 0.02% of the total protein (Sijmons *et al.*, 1990; Pen *et al.*, 1993). Similarly, potato and tobacco plants transformed with the gene sequence encoding the native pre-pro-HSA also accumulated a processed HSA protein. In both cases, the transgenic HSA was localized in the extracellular fluid, indicating that both the native and the tobacco signal sequences had directed secretion of the HSA via the endoplasmic reticulum. However, in tobacco plants transformed with gene sequences encoding the mature HSA without a signal or pro-sequence, no accumulation of the protein could be detected. The HSA mRNA was readily detectable in these transformed plants. Thus, the presence of the signal sequence was necessary for detectable transgene product accumulation. While it is possible that the presence of the signal sequence coding region influences translatability of the HSA mRNA, these observations suggest that non-secreted HSA is not stable in the cytoplasm of tobacco cells. Similar increased transgene product accumulation as a result of secretion has been reported for transgenic antibody and antibody fragments from tobacco. In the case of multimeric, intact immunoglobulins, however, secretion via the endoplasmic reticulum is probably also required for correct assembly of the protein (see below). Also, in a recent study on the production of the *Trichosanthes kirilowii* ribosome inactivating protein, α-trichosanthin, in tobacco, it was speculated that the high level accumulation of the transgene product (at least 2% of total soluble protein) was partly due to the native α-trichosanthin signal peptide targeting the protein to the extracellular space (Kumagai *et al.*, 1993).

The observation that transgene products that are secreted to the extracellular space show increased accumulation is not general. As part of a study aimed at evaluating a model system based on the transient synthesis of

heterologous proteins in tobacco protoplasts, it has been reported that the presence of signal sequences correlates with a reduction in the level of the encoded gene products (Denecke, Bottermann and Deblaere, 1990). Thus, whereas the levels of neomycin phosphotransferase II mRNA were essentially unaffected by fusion of the neomycin phosphotransferase II gene to the coding regions of either the tobacco PR1 signal sequence or the *Hyalophora cecropia* cecropin B signal sequence, the levels of neomycin phosphotransferase II were always 5- to 10-fold lower for the secretory constructs. This observation is similar to that reported for the synthesis of wheat α-amylase in transgenic yeast, in which deletion of the α-amylase signal sequence coding region was found to increase protein level by about 10-fold (Rothstein *et al.*, 1984). A decrease in protein stability or translation efficiency are the most plausible explanations for these observations. That the protein : mRNA ratio of some transgene products is increased by signal sequence-mediated secretion of the transgene protein, while in other cases it is decreased, suggests that the effect may be gene-dependent.

Recently, a method for identifying transgenic plants with a high level of expression of the introduced gene, that relies upon secretion of the gene product, has been described (Pen *et al.*, 1992). In this case, tobacco protoplasts were transformed with an expression construct containing a translational fusion between mature *Bacillus licheniformis* α-amylase and the signal sequence of the tobacco PR-S protein. The transformed protoplasts were cultured to microcalli, then transplanted to solid media containing starch. Following a suitable period of incubation, the starch plates were stained with iodine solution and the individual calli scored for halo size. The levels of α-amylase protein in plants regenerated from the calli were found to be related to halo size. This method, which could be applied to a wide range of extracellular proteins by, say, scoring the formation of antibody–antigen complexes in solid media, could provide a very convenient early selection for high-level transgene expression.

Further evidence that transgene product accumulation can be significantly increased by targeting the protein to a different cellular compartment, thereby increasing its stability, has been provided by a recent study of the expression of the pea seed storage protein, vicilin, in transgenic tobacco and alfalfa plants (Wandelt *et al.*, 1992). Seed storage proteins normally accumulate to high levels in protein storage vacuoles, protein bodies, and are stable over long periods of time. The genes encoding some seed storage proteins, such as the cowpea trypsin inhibitor, can be modified to replace the upstream regions that confer seed specificity of expression, allowing the transgenic storage proteins to accumulate in the leaves of transgenic host plants (Hilder *et al.*, 1987). However, for some storage protein genes, expression in the leaves of transgenic host plants leads to very low accumulation of protein (Higgins and Spencer, 1991). It has been shown that the protein body targeting information contained within seed storage proteins leads to transport of transgenic storage proteins to the protease-rich vacuoles of host plant leaves (Chrispeels, 1991). The failure to detect the soybean storage protein, conglycinin, in the soluble (vacuolar) fraction isolated from transgenic

tobacco leaves is consistent with the notion that this storage protein is unstable in the leaf vacuole (Lawton *et al.*, 1987). To overcome the problem of storage protein instability in leaf vacuoles, Wandelt *et al.* (1992) have described the redirection of transgenic vicilin to the endoplasmic reticulum of host plants using the tetrapeptide, KDEL, endoplasmic reticulum lumen retention signal. The presence of the KDEL sequence at the C-terminus of transgenic vicilin was found to increase accumulation of the protein by up to 20- to 100-fold in the leaves of transgenic alfalfa and tobacco plants. The increased accumulation of vicilin-KDEL protein compared with native vicilin was not accompanied by a detectable change in vicilin mRNA level or translatability. Rather, the increased levels of vicilin-KDEL reflected a significant reduction in the degradation of newly synthesized vicilin-KDEL (half-life 48 h) compared with vicilin (half-life 4.5 h). Tobacco leaves expressing the vicilin-KDEL gene under control of the CaMV 35S promoter were found to accumulate vicilin at up to 3.7% of total leaf protein.

From the available evidence it appears that, for some proteins at least, there may be scope for increasing heterologous protein stability and, hence, accumulation through targeting of the protein to a more suitable cellular compartment. At present, this is supported by studies on a small number of heterologous proteins in relation to only three cellular compartments: the cytoplasm, the apoplast (extracellular space) and the lumen of the endoplasmic reticulum. It has been demonstrated that heterologous proteins can be targeted to other plant cell organelles, for instance the chloroplast (Schreier *et al.*, 1985). Indeed, many crop improvement based applications of heterologous protein production require that the foreign protein be resident in a cellular compartment other than the cytoplasm. However, the consequences, in terms of protein stability and yield, of targeting heterologous proteins to these locations have yet to be determined.

In bacterial expression systems, the expression of a foreign protein as fusion protein has been found to increase its stability both *in vivo* and *in vitro* (e.g. Hellebust, Veide and Enfors, 1988). Similar strategies may be effective for the production of some heterologous proteins in plants. For example, Hightower *et al.* (1991) produced transgenic tobacco and tomato plants which express the *afa3* gene, a synthetic gene encoding an analogue of the winter flounder anti-freeze protein, under control of the CaMV 35S promoter. Although the *afa3* gene was expressed to high steady-state mRNA levels in leaves of transformed plants, anti-freeze protein activity could not be detected. On the other hand, transformation of tomato with a chimaeric gene encoding a fusion protein between truncated *Staphylococcal* protein A and the *afa5* anti-freeze protein gene resulted in anti-freeze protein activity in tissue extracts. It is possible that the size and nature of the protein A portion of the fusion protein protects the small anti-freeze protein from proteolysis in plant cells.

A fusion protein strategy has also been adopted for the production of bioactive peptides in transgenic plants (Krebbers and Vanderkerckhove, 1990; Krebbers, van Rompaey and Vanderkerckhove, 1993). Sequences encoding leu-enkephalin (a neuropeptide) or magainin (an antibacterial

peptide) have been introduced into the coding regions of the 2S albumin seed storage protein from both *Arabidopsis* and *Brassica napus*. Expression of the fusion protein-encoding genes, driven by the *Arabidopsis* 2S1 promoter, in either transgenic *Arabidopsis* or transgenic *B. napus*, led to accumulation of the peptides at levels up to 100–200 nmol of peptide per gram of seed. The use of the seed-specific 2S1 promoter, together with exploitation of the protein body targeting signals present on the 2S albumin fusion partner, ensures that the peptides are produced as stable, stored fusions in the seeds of the transformed plants. At present, little is known about the downstream processing of these types of fusion proteins, but it is anticipated that incorporation of sites recognized by restriction proteases will allow specific cleavage of fusion proteins and release of the peptide.

Production of antibodies and antibody fragments in plants

As a case study to illustrate the complexity and problems associated with heterologous proteins in plants, whether from the point of view of factory-scale production or for crop improvement, the expression of antibody-encoding genes, or fragments of such genes, can be used to illustrate most of the points raised above.

USES OF PLANT-PRODUCED ANTIBODIES

Broadly speaking, there are three reasons for producing antibodies in transgenic plants: (1) the large-scale production of commercial antibodies, i.e. antibody farming; (2) the antibody-mediated modification of antigen activity *in planta*; and (3) the study of the plant cell protein targeting and assembly machinery.

Antibody farming

Monoclonal antibodies are produced commercially on a very large scale for a wide range of uses. For instance, therapeutic agents that consist of a cell type-specific monoclonal antibody, which may or may not be conjugated to a drug, isotope or toxin, are used in the treatment of cancer and in the diagnosis of cardiovascular diseases. In addition, monoclonal antibodies are used as vaccines in passive immunization. It has been estimated that the value of therapeutic antibodies will be about $1000 million in 1994. Outside of therapeutics, the exquisite binding specificities of monoclonal antibodies are also employed in a range of affinity separations and in a plethora of diagnostic kits.

The production of reagent monoclonal antibodies usually involves either complex and expensive procedures for hybridoma cell growth and antibody purification or the isolation of antibody from ascites fluid produced in animals. There has been considerable interest in the development of more convenient and cheaper methods for the production of antibodies. Crop plant-produced antibodies may prove to be significantly less expensive than

antibodies from other sources. This may be especially true where existing agricultural infrastructure can be employed for protein farming. Also, it has been speculated that transgenic crops may provide a convenient means of producing and delivering antibodies for use in passive immunization (Hiatt and Ma, 1992). In particular, it has been suggested that therapeutic monoclonal antibodies that are effective following oral delivery, such as those used to prevent the establishment of *Streptococcus mutans*, the agent that causes dental caries, could be administered directly in edible plant tissues.

Modification of antigen activity in planta

Antibody gene expression in heterologous cells provides a means of stably modifying the activity of antigen molecules present within the expressing cells. The feasibility of this approach was first demonstrated by Carlson (1988), who showed that transgenic yeast expressing gene sequences encoding truncated immunoglobulin heavy and light chains (signal sequences removed), derived from an anti-alcohol dehydrogenase antibody, displayed reduced activity of the target enzyme. Also, a gene encoding a recombinant antibody fragment displaying chorismate mutase activity has been shown to complement a chorismate mutase-deficient mutant of yeast (Tang, Hicks and Hilvert, 1991). More recently, Owen *et al.* (1992a) have shown that transgenic tobacco plants expressing a synthetic gene encoding an antibody fragment reactive with the plant regulatory photoreceptor, phytochrome, display aberrant phytochrome physiology. This latter demonstration opens the way to numerous possible applications of antibody gene expression for crop improvement, as well as for the study of plant metabolism (Hiatt, 1990; Owen *et al.*, 1992a, b). It has been speculated that the *in planta* synthesis of antibodies that bind to antigens necessary for pathogenesis may provide a means to enhance plant resistance to pathogens. For example, Saunal, Witz and van Regenmortel (1993) have identified a monoclonal antibody that interferes with the co-translational disassembly of tobacco mosaic virus (TMV) particles, and have suggested that expression of genes encoding this antibody in tobacco may provide resistance to the virus. Similarly, a strategy for engineering resistance to the potato cyst nematode, *Globodera rostochiensis*, has been described, which involves *in planta* expression of antibodies that bind to, and inactivate, the saliva proteins of the nematode (Schots *et al.*, 1992).

In addition to plant protection, the antibody-mediated modulation of plant antigen activity could be applied to the manipulation of plant growth and development through, for example, the *in situ* binding and inactivation of plant hormones or other regulatory molecules. It is envisaged that antibody synthesis may be complementary to antisense approaches for the creation of loss-of-function mutations. Also, plants synthesizing antibodies that bind to or detoxify environmental pollutants could be used in environmental clean-up and could be particularly appropriate for use with large bodies of water containing persistent pollutants such as polychlorinated biphenyls (PCBs) at low concentrations. A key determinant of the success of these approaches will

be establishing conditions that allow the synthesis of functional antibodies in the appropriate subcellular compartment of the plant cell.

The study of protein targeting, assembly and modification

Intact immunoglobulins are multimeric proteins, the product of two different genes, that undergo post-translational modification and require a specialized environment for assembly. In this regard, they represent an ideal model for studying the synthesis of complex heterologous proteins in plant cells. Also, the ease with which two plants expressing different genes encoding individual monomers of a multimeric protein can be sexually crossed to produce progeny that express both genes is very convenient in the study of complex protein assembly.

ANTIBODY ENGINEERING

The advent of recombinant DNA technology has led to the development of methods for the expression of immunoglobulin-encoding genes in a wide range of heterologous organisms, including non-lymphoid mammalian cells, insect cells, yeast, bacteria and plants (Wright, Shin and Morrison, 1992). The isolation of antibody-encoding genes from hybridoma cells and their expression in these heterologous hosts led directly to a revolution in monoclonal antibody technology in which recombinant DNA techniques have been used to alter the structure, properties, specificity, function and immunogenicity of antibodies. These developments in antibody engineering have extended so far that now it is possible to express the entire immune repertoire such that the antibodies are displayed on the surface of bacteriophage (Chiswell and McCafferty, 1992). Once displayed on phage surfaces, antibodies can be subjected to molecular evolution, thus extending the natural immune repertoire (Marks *et al.*, 1992).

Although there are several different classes of antibodies that differ in some important characteristics, the antibodies most commonly employed as reagents and as the starting point for antibody engineering are members of the immunoglobulin G (IgG) family. These proteins are Y-shaped and are formed by four polypeptide chains – two identical glycosylated heavy chains and two identical non-glycosylated light chains. The heavy chains are joined to each other by disulphide bonds, and each of the light chains is joined to one of the heavy chains by a disulphide bond (*Figure 1*). The domains that form the tips of the arms of the Y, the Fv domains, are solely responsible for binding to the antigen and are formed by the variable region of the heavy chain (V_H) and the variable region of the light chain (V_L). Within each of the variable regions lie three hypervariable segments, the complementarity determining regions (CDRs), which form the antigen-binding site. Proteolytically derived fragments of immunoglobulins, such as the $F(ab)_2$ fragment, that possess the Fv region, show antigen-binding activity that is essentially the same as that of the intact antibody molecule. The constant, Fc, region of the

immunoglobulin molecule is involved in activation of the complement system leading to elimination of antibody–antigen complexes.

The domain structure of the immunoglobulin molecule makes antibodies particularly suitable for protein engineering (Winter and Milstein, 1991). Thus, gene sequences that encode fragments carrying the Fv regions of immunoglobulins, for instance Fab and Fv fragments, have been expressed in heterologous hosts to yield active antigen-binding immunoglobulin fragments (see Wright, Shin and Morrison, 1992). These small, recombinant antibody fragments have attracted much interest in applications where the Fc region of an intact antibody is unnecessary or undesirable. For example, a major drawback of using murine antibodies for human therapy is their slow clearance times and their immunogenicity leading to the induction of the HAMA (human anti-mouse antibody) response. These problems are reduced by reducing antibody size. Further improvements in the design of recom-

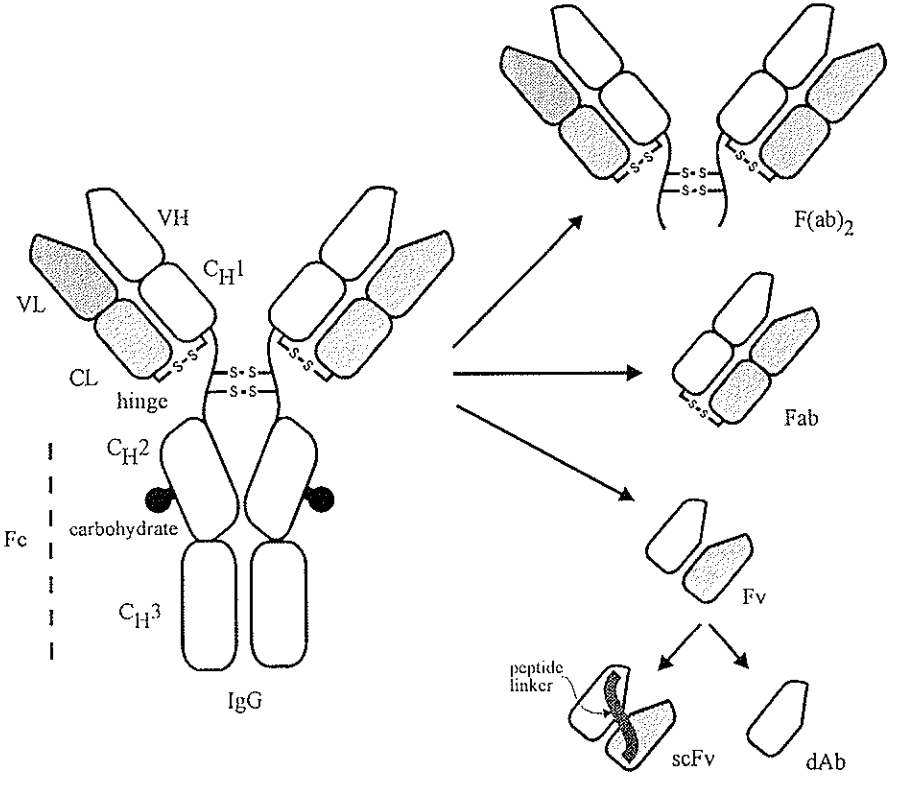

Figure 1. Schematic diagram of the domain structure of an antibody (IgG) molecule and of active antibody fragments. V_H and V_L are the variable domains of the heavy and light chains, respectively. C_H1-C_H3 are constants domains 1–3 of the heavy chain and C_L is the constant domain of the light chain. Fc is the constant fragment. The active fragments $F(ab)_2$ and Fab can be obtained either by proteolytic cleavage of an IgG or by recombinant DNA technology. The smaller active fragments, the Fv, the single-chain Fv (scFv) and the single domain antibody (dAb) are obtained by recombinant DNA technology.

binant immunoreagents for human therapy have been achieved by humanization of murine antibodies by CDR-grafting. In this technique, the hypervariable antigen-binding loops of a murine antibody with the desired specificity are transplanted to the variable region framework of a human antibody or antibody fragment (Winter and Milstein, 1991). The Fv, a small recombinant antibody fragment that retains a complete antigen-binding site, is formed by the association of two separate polypeptides and is not especially stable. The single-chain Fv (scFv), in which the V_H and V_L domains of an immunoglobulin are linked together by a peptide (*Figure 1*), is a more stable reagent and is the product of a single synthetic gene. The scFv is the focus of much activity in the design of new therapeutic, diagnostic and affinity reagents.

Recombinant, antigen-binding fragments, such as the scFv, can be endowed with new properties by creating fusions between the scFv gene and sequences encoding proteins with other functions. Thus, recombinant immunotoxins designed to target and destroy specific types of cancer cells, have been constructed by fusing an anti-cancer cell scFv gene to a cytotoxin-encoding gene (e.g. Kreitman *et al.*, 1990). Active immunotoxin has been recovered following expression of these gene fusions in *Escherichia coli*.

SYNTHESIS AND ASSEMBLY OF INTACT IMMUNOGLOBULINS IN PLANTS

The production of a recombinant antibody in plants was first described by Hiatt, Cafferkey and Bowdish (1989). The catalytic IgG1 antibody 6D4, which recognizes a synthetic phosphonate ester, P3, and which can catalyse the hydrolysis of certain carboxylic esters, was chosen for study. A two-step strategy was adopted in order to produce transgenic tobacco plants capable of synthesizing intact immunoglobulin. In the first step, cDNAs encoding the γ-heavy chain and its signal sequence or the κ-light chain and its signal sequence, were placed behind the CaMV 35S promoter and used for *Agrobacterium*-mediated tobacco transformation. In addition, transformations were performed with truncated heavy- or light-chain encoding cDNAs in which the signal sequence coding regions were removed. The second step of the strategy involved the sexual crossing of individual plants expressing either heavy- or light-chain cDNA, in order to produce progeny that expressed cDNAs encoding both chains.

Analysis of transgenic plants from the first step revealed that only transformed plants expressing the intact, full-length heavy- or light-chain encoding cDNAs accumulated detectable immunoglobulin protein. In contrast, although transformants expressing the truncated heavy- or light-chain cDNAs contained readily detectable levels of appropriate transcripts, they did not accumulate significant amounts of the proteins. Thus, the native immunoglobulin signal sequences were necessary in order to observe protein accumulation. The F1 plants produced by crossing plants expressing individual, full-length cDNAs were found to contain significant amounts of assembled antibody that, upon extraction, bound P3 with the same affinity as the parental, hybridoma-derived antibody (Hiatt, Cafferkey and Bowdish,

1989; Hiatt and Mostov, 1993). The levels of intact immunoglobulin protein present in F1 plants expressing both heavy- and light-chain cDNAs was found to be significantly higher than the levels of individual chains produced in the parent plants. Furthermore, crossing of plants producing heavy and light chain at vastly different levels did not lead to the creation of F1 plants that accumulated an excess of one chain; only assembled antibodies were detected. Because co-expression of both chains increases the yield of each chain, it appears that assembly of the chains enhances their stability. The yield of 6D4 from F1 plants was in the range of 1% of the total protein, representing one of the highest reported yields for a heterologous protein produced in transgenic plants. It appears possible that with appropriate modifications to the immunoglobulin cDNA codons and with optimization of promoter and untranslated leader sequences, that even higher yields could be obtained.

Assembled antibodies were not detected in F1 plants expressing truncated cDNAs, suggesting that co-translational insertion into the endoplastic reticulum (ER) lumen is necessary for chain assembly and stability. In antibody-producing B cells, the assembly of heavy- and light-chain complexes occurs in the ER and is thought to be facilitated by (a) protein disulphide isomerase, which catalyses the formation of disulphide bonds both in and between nascent polypeptide chains, and (b) a heavy-chain binding protein (BiP), a chaperonin related in structure to hsp70 (Haas and Wabl, 1983). The ER lumen of plant cells contains protein disulphide isomerase and a BiP homologue (Fontes *et al.*, 1991). The absence of these proteins from the cytoplasm, together with the reducing nature of this compartment that would prevent disulphide bond formation, may lead to the conclusion that intact immunoglobulins could not be produced in the cytoplasm of plant, or other heterologous, cells. However, transient expression of truncated cDNAs encoding the μ-heavy chain and λ-light chain of an IgM antibody, following their microinjection into the nucleus of *Acetabularia*, has been shown to result in synthesis and assembly of antibody in the cytoplasm (Stieger *et al.*, 1991). Similarly, truncated cDNAs encoding the heavy and light chains of this, and other antibodies, have been expressed in cultured mammalian or yeast cells, and evidence for the synthesis of low levels of functional antibody in the cytoplasm has been presented (Carlson, 1988; Piccoli, *et al.*, 1991). Thus, for some antibodies at least, targeting to the Er is not an absolute requirement for synthesis, assembly and accumulation.

In F1 plants expressing both intact cDNAs, the assembled antibody protein is located in the intercellular spaces (Hein *et al.*, 1991), indicating that following targeting to the ER the polypeptides enter the default pathway and are secreted. In callus cell suspension cultures established from the F1 plants, functional, whole immunoglobulins are actively secreted to, and can be isolated from, the culture medium at concentrations up to 20 mg l^{-1} (Hein *et al.*, 1991). The native mouse immunoglobulin signal sequence coding regions of the 6D4 heavy- and light-chain cDNAs have been replaced with the coding region of the yeast α-mating factor pre-pro sequence, resulting in successful synthesis, assembly and secretion of functional immunoglobulin in transgenic

F1 plants (Hein *et al.*, 1991). Levels of antibody were similar to those observed when secretion was facilitated by the native signal sequences and assembled, secreted immunoglobulin was produced in F1 plants expressing heavy and light chains possessing different signal sequences (Hein *et al.*, 1991). In contrast to these observations, During *et al.* (1990) have reported that the expression in tobacco plants of chimaeric genes composed of the coding region of the barley α-amylase signal sequence fused to cDNAs encoding mature heavy and light chains of a monoclonal IgM antibody leads to assembly of a functional immunoglobulin that is not secreted from the cell. In this case, tobacco plants were transformed using an *Agrobacterium* vector containing both the μ-heavy chain and λ-light chain under the control of the pNOS and pT_R promoters. Assembled, functional antibody was detected in transformed plant tissues, with immuno-gold electron microscopy showing that this immunoglobulin protein was present in the ER and, surprisingly, the chloroplasts, but not the intercellular spaces. No feature of the engineered antibody protein was expected to lead to chloroplast targeting. The reason for the different targeting of this immunoglobulin, in which plant signal sequences are used to target the polypeptides to the ER, compared with the 6D4 antibody, in which mouse or yeast signal sequences have been used, is not known.

In the ER of B-cells, a pre-formed dolichol phosphate donor glycosylates the nascent immunoglobulin heavy chain. The added glycan moiety is of the endoglycosidase H-sensitive, high mannose type, containing *N*-acetylglucosamine and mannose, but lacking galactose and sialic acid. Following immunoglobulin assembly and passage to the golgi, the glycan undergoes modification, involving the addition of galactose and sialic acid producing a modified glycan that is resistant to endoglycosidase H (Tartakoff and Vasalli, 1979; Goding and Herzenberg, 1980). The γ-heavy chain of the 6D4 IgG antibody produced in tobacco plants was found to bind to the lectin concanavalin A (specific for mannose and glucose), indicating that it is glycosylated (Hein *et al.*, 1991). However, in contrast to the heavy chain from parental, hybridoma-produced 6D4, the plant-produced 6D4 heavy chain was not reactive with *Ricinus communis* agglutinin and wheat germ agglutinin, indicating an absence of galactose and sialic acid residues. Nevertheless, the glycan of the plant-produced γ-chain was found to be resistant to endoglycosidase H digestion, suggesting that it was of the modified type. Furthermore, the glycan of the plant-produced γ-chain was observed to bind to concanavalin A with the same affinity as that of the parental antibody, suggesting that both antibodies had undergone similar processing.

Although the difference in targeting of plant-produced IgG and IgM antibody awaits explanation, it does seem that plants are capable of producing significant amounts of processed, assembled, functional immunoglobulin virtually indistinguishable from antibodies produced in animal cells. At present, intact immunoglobulins have only been stably produced in tobacco plants and no attempts have been made to express of the heavy- and light-chain cDNAs in storage tissues. Also, little is known about whether the isolation of antibodies is compatible with current crop-processing practices.

Nevertheless, plants do have the potential to be exploited for the large-scale production of immunoglobulins.

SYNTHESIS OF ANTIBODY FRAGMENTS IN PLANTS

Tobacco plants have been shown to be capable of producing a functional scFv protein in both the cytoplasm and the extracellular space (Owen *et al.*, 1992a; Firek *et al.*, in press). A non-antigen-binding, smaller fragment of a rat IgG monoclonal antibody directed against the tachykinin neuropeptide, substance P, has been synthesized in transgenic tobacco (Benvenuto *et al.*, 1991). Gene sequences encoding a fusion between the leader sequence of the *Erwinia* pectate lyase, *pelB*, protein and the V_H domain of the anti-substance P antibody, under the control of the CaMV 35S promoter, were used to transform tobacco leaf discs. Although this isolated V_H domain displayed no antigen-binding activity, similar single domains from other monoclonal antibodies have been shown to bind antigen (Ward *et al.*, 1989). The V_H domain polypeptide was reported to accumulate in the transgenic tobacco plants to concentrations up to 1% of the soluble protein fraction, a similar level to that reported for intact, assembled immunoglobulins. It is not clear why the construct employed by Benvenuto *et al.* (1991) included the *pelB* leader sequence. This sequence is extensively employed in bacterial expression systems, where it directs transport to the periplasmic space (e.g. Ward *et al.*, 1989). The leader was reported to have been cleaved from the plant-produced V_H domain polypeptide, but the antibody fragment appeared to be predominantly located in the intracellular fraction of tobacco cells. It is possible that, as was reported for the assembled IgM antibody (Düring *et al.*, 1990), the V_H polypeptide was retained in the ER.

For functional scFv protein expression in transgenic tobacco, Owen *et al.* (1992a) used PCR amplification to isolate the V_H and V_L coding regions derived from cDNA prepared from a mouse hybridoma. The hybridoma line, AS32, secretes an IgG1 antibody directed against a conserved epitope on the plant regulatory photoreceptor, phytochrome A. The synthetic scFv gene, shown to encode an antigen-binding protein following its expression in *Escherichia coli*, was placed under the control of the CaMV 35S promoter and was used for *Agrobacterium*-mediated leaf disc transformation. More than 100 individual transformants were screened by Northern blot analysis and the plant displaying the highest expression levels selected. The accumulation of scFv protein in the homozygous progeny of this individual was low, amounting to a maximum of only about 0.06% of total soluble leaf protein. Many transformants that were clearly positive for scFv mRNA were found to accumulate undetectable amounts of scFv protein. The anti-phytochrome scFv protein produced in the high-expressing tobacco line was found to be functional following extraction from plant tissues. Thus, the scFv could be purified to near homogeneity by passage of crude protein extracts through a phytochrome A-Sepharose column (Owen *et al.*, 1992a). Evidence that the scFv protein was functional *in vivo* was obtained from analysis of phytochrome-mediated developmental responses of the transformed plants.

Phytochrome A has been implicated in the control of the de-etiolation responses of higher plant seedlings, including germination, growth and greening. Homozygous seeds expressing the anti-phytochrome A scFv gene were found to show poor phytochrome-dependent, light-mediated promotion of germination (Owen *et al.*, 1992a). In addition, other aspects of the photomorphogenesis of the transformed plants were perturbed, including the photocontrol of hypocotyl elongation, the photocontrol of cotyledon expansion and the photocontrol of greening (Owen *et al.*, unpublished observations). These observations provide validation of the use of *in planta* synthesized antibodies, or antibody fragments, for the modulation of plant antigens. Progress is being made on the *in planta* expression of gene sequences that encode antibodies reactive with a range of other plant cell antigens, including regulatory molecules and plant pathogens.

Since phytochrome A is cytoplasmic, the anti-phytochrome scFv gene was designed to lead to cytoplasmic synthesis of the encoded protein. The perturbation of phytochrome responses seen in the transformed seedlings is strongly suggestive that at least some of the scFv protein was present in the cytoplasm. In order to determine the consequences, in terms of yield and activity, of secretion of an scFv in plants, a translational fusion between the anti-phytochrome scFv and the signal sequence of the tobacco PR1 a protein was constructed and used for tobacco transformation (Firek *et al.*, in press). A processed, functional scFv protein was found to accumulate in the extracellular space of the transformed plants. Cultured callus cells derived from transformed plants were observed to secrete functional scFv protein into the culture medium, such that it accumulated to a concentration of about 50% of total medium protein. In plants expressing the secretion construct, scFv protein was readily detectable and was found to accumulate to levels up to 0.5–1.0% of total soluble leaf protein. The elevated levels of scFv protein, in plants expressing the secretion construct compared with those expressing the 'native' scFv gene, were observed against a background of lower scFv mRNA accumulation. Thus, the protein : mRNA ratio was very significantly higher for plants expressing the secretion construct. This suggests that the presence of the signal sequence coding region leads to increased stability of the scFv protein and/or to increased translatability of the scFv mRNA.

The behaviour of this scFv is therefore similar to the behaviour of the whole 6D4 immunoglobulin and of HSA, in which the addition of a signal sequence was found to be necessary for significant accumulation of the transgene product. In all three cases, the most likely explanation for this observation is an instability of the transgenic protein in the cytoplasm of plant cells. Recently, we have transformed tobacco plants with gene sequences that encode cytoplasmically located fusions between the anti-phytochrome scFv and the GUS reporter enzyme. Preliminary analyses of the plants suggests that fusion of the scFv to a stable enzyme have increased its level of accumulation in tobacco cell cytoplasm. This work also shows that plants may be appropriate hosts for the large-scale production of a range of immunoreagents that are based around the scFv.

Prospects

At the present time, the number of agronomically important species which can be reliably, routinely transformed with foreign genes is relatively limited and the major monocot crops rice, maize and wheat are not yet routinely transformable. However, there can be no doubt that this is a temporary problem, which is currently being solved through the more sophisticated use of *Agrobacterium*-based gene transfer methods and the introduction and optimization of new direct transfer technology. Vector systems based on viral genomes may also play a part in this process.

Transformation technology relies heavily on the ability to grow cells and tissues of the crop plant in question and to reliably regenerate whole plants from these cultures and the technology for this, too, is advancing rapidly.

It seems virtually certain that in the near future, essentially all major crops including the monocots will become fully accessible to transgenic technology and, therefore, that the expression of heterologous proteins will represent an important and many-faceted tool for the improvement of current crops, the development of novel crops and for the evolution of entirely novel strategies for the exploitation of the unique characteristics of plants.

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