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Review

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Phytofarming-Approaches to Foreign Protein Expression in Higher Plants

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Introduction

Molecular farming comprises the developing and use of genetically modified eukaryotic organisms for the synthesis of high value products. This technology has emerged a few years ago and has entered a commercially very attractive and fast growing market.

The products of special interest may be biochemical compounds, technical enzymes, nutrients and pharmaceuticals. To indicate the latter case the wording "molecular pharming" is used. In a narrower sense molecular farming does not include molecular engineering instead focuses on the production of peptides or proteins in a host animal or plant. Plants are of special interest for the production of proteins of high value. When plants are exploited as bioreactors molecular farming is generally termed phytofarming (or phytopharming to indicate the production of pharmaceuticals). There are several reasons which render plants attractive hosts for heterologous protein production. First of all, nowadays nearly all commercially interesting crops can be genetically modified, and with the help of well characterised promoters high expression levels in distinct organs can be achieved. Since plants are eukaryotic organisms they perform post-translational modifications of the expressed proteins such as glycosylation and phosphorylation. However, these modifications are not identical to those found in animals. This represents one of the biggest problems in phytofarming, especially in phytopharming, since glycosylation patterns often determine the function of pharmaceutical proteins. Moreover, foreign glycosylation patterns may serve as potent immunogenic epitopes promoting allergenic reactions. On the other hand, the use of plants instead of animals minimises the risk of contamination with human viruses or other disease promoting organisms. The most important reasons for using plants as hosts of heterologous protein expression, is the very low cost production of biomass and the facility to easily scale-up production. Another advantage is that foreign proteins are often stable for years in plant seeds. This allows easy storage circumventing the need for synchronisation of biomass production and industrial purification of the desired protein. Given the ease by which seeds are harvested and the fact that proteins are concentrated in seeds due to about 95% loss of water during maturation, this plant organ is the preferred target for heterologous protein production in plants.

Of course, high expression levels of the respective protein are desired or even necessary. In addition, a good expression system requires that the protein stays functional and is recovered at high yields. The extraction and purification of the protein is the most cost intensive step (called downstream production costs) in phytofarming often exceeding 90% of total costs. Thus, for exploiting plants as bioreactors the optimisation of expression levels of functional proteins as well as the development of cost effective methods for purification are required.

In this review we will focus on recent techniques which may become attractive tools for high level production and purification of proteins from plants. Advantages and disadvantages of the respective methods will be discussed.

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Approaches to obtain high levels of heterologous gene expression

Until recently, most approaches to express foreign proteins in plants made use of nuclear transformation methods to insert the transgene stable into the plant genome. Especially the choice of different promoters and untranslated regions allows to alter expression levels in a developmental and tissue-specific manner. Here, we will concentrate on two more recent systems, which have been described to yield high levels of protein expression: Proteins expressed (i) in transgenic plastids and (ii) by viral vectors.

Production of heterologous proteins in plastids of higher plants

Quite recently, plastids have become amenable to transformation. The first plastids to be transformed were those of tobacco (for example: Staub and Maliga, 1992; Zoubenko et al., 1994; Koop et al., 1996; Daniell et al., 1998; Kanevski et al., 1999; Khan and Maliga, 1999). By now, there have been reports on plastid transformation of *Arabidopsis* (Sikdar et al., 1998), potato (Sidorov et al., 1999) and rice (Khan and Maliga, 1999).

Plastidic genomes, termed plastomes, are believed to reach up to 100 copies per plastid. Since plastids may contain up to 100 organelles per cell there are several thousand copies of the plastome present in a single mesophyll cell (Bendich, 1987). Thus, by genetically engineering the plastome a very high copy number of the gene encoding for a heterologous protein of interest can be achieved. This high gene dosis may lead to high expression levels. Despite the high copy number of plastidic DNA gene silencing effects have never been observed in transplastomic plants. On the other hand the high copy number of plastidic DNA brings up the requirement for a time consuming selection procedure because all plastomes of any plastid and all plastids of any cell have to be uniformly altered.

A distinct feature of plastid transformation is the process of DNA integration into the plastome. Instead of random integration as observed in the nucleus of plants the creation of transplastomic plants occurs via homologous recombination. This property implies that there are no differences in a transplastomic population as opposed to nuclear transformants where position effects often influence the transcription level of the transgene. Moreover, the number of integrated gene copies is not randomly determined as in nuclear transformation events but is limited to one integration at the targeted region of the plastome.

So far, levels of foreign proteins have been determined in only a few cases (Table 1). An extreme light dependency of plastidic gene expression may be one of the limitations for the use of plastid transformation in plants. It should be kept in mind that the highest expression levels of plastidic genes are obtained in chloroplasts and thus dominate in leaves. For example, levels of GFP in transplastomic potato plants reached about 5% of total soluble protein (tsp) in potato leaves, but only about 0.05% tsp in micro-tubers (Sidorov et al., 1999). The so far only described attempt to overcome this problem was developed by McBride et al. (1994). They expressed a viral single subunit polymerase (T7) in the nucleus, targeted this enzyme to the plastid where it activated transcription from a viral promoter (T7 gene 10) upstream of a transgene (GUS) integrated into the plastome (Figure 1). Expressing the viral RNA polymerase under control of the constitutive CaMV

Table 1. Protein expression levels in transplastomic plants

Protein	P/L/T	Plant	Tissue	Level(tsp)	References
GUS	P*psb*A/T*psb*A	Tobacco	Leaf	2.5%	Staub and Maliga, 1993
NPT	P*rrn*/T*rps*16	Tobacco	Leaf	1%	Carrer et al., 1993
GUS	P*g*10/L*g*10/T*psb*A	Tobacco	Leaf	20-30%	McBride et al., 1994
CryIA(c)	P*rrn*/L*rbc*L/T*rps*16	Tobacco	Leaf	3-5%	McBride et al., 1995
GFP	P*rrn*/T*rps*16	Potato	Leaf	5%	Sidorov et al., 1999
			Tuber	0.05%	Sidorov et al., 1999
FLARE-S	P*rrn*/L*rbc*L/T*psb*A	Tobacco	Leaf	8-18%	Khan and Maliga, 1999

CryIA(c), crystal protein class I; FLARE-S, fluorescent antibiotic resistance enzyme - spectinomycin and streptomycin; *g*10, T7 gene 10 dependent on T7 polymerase; GFP, green fluorescent protein; GUS, β-glucuronidase; L, leader, 5'-untranslated region; NPT, neomycin phosphotransferase; P, promoter; *psb*A, encodes photosystem II D1 protein; *rbc*L, encodes ribulose bisphosphate carboxylase/oxygenase large subunit; *rps*16, encodes ribosomal protein S16; *rrn*, ribosomal 16S rRNA gene; T, terminator, 3'-untranslated region; tsp, total soluble protein

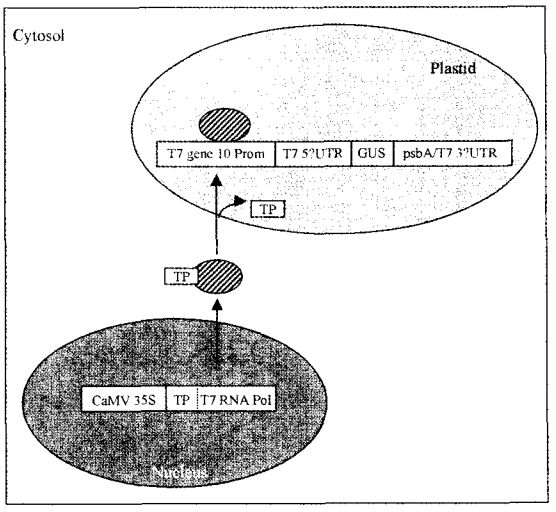

Figure 1. Principle of the T7-based plastidic expression system as described by McBride et al. (1995). The viral T7 RNA polymerase is expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in the nucleus. A transit peptide (TP) fused to the T7 RNA polymerase mediates import into plastids. In plastids T7 polymerase drives expression of the GUS (β-glucuronidase) gene behind the T7 gene10 promoter (UTR, untranslated region; *psb*A, encodes D1 protein).

(Cauliflower mosaic virus) 35S promoter resulted in GUS levels up to 30% of tsp in leaves (Table1). The T7 expression system offers the additional advantage of allowing a tissue-specific and inducible expression of the plastidic transgene just by modulating the transcription of the viral polymerase in the nucleus.

Besides developing plastid transformation techniques for other plant species much current research concentrates on understanding plastid gene expression. Despite their endosymbiotic origin there are many differences in the mechanisms of gene expression between plastids and living eubacteria like *Escherichia coli*. In contrast to operons in *E. coli* polycistronic mRNAs of plastidic gene clusters are typically processed into monocistronic transcripts. The extensive processing of transcripts seems to influence gene expression, probably by altering mRNA stability and translational efficiency (for review: Sugita and Sugiura, 1996). Since post-transcriptional events, especially translation, primarily effect plastidic gene expression (reviewed by Danon, 1997; Sugiura et al., 1998; Bruick and Mayfield, 1999), the choice of untranslated regions (UTRs), which encompass the foreign open reading frame (ORF), is of special importance in order to increase protein expression in plastids. For this reason recent research intends to quantify gene expression as a function of the chosen UTRs. Eibl et al. (1999) demonstrated that the *psb*A 5'-UTR in conjunction with its natural 3'-UTR confers the highest activity of the GUS reporter as compared to various combinations with 5'- and 3'UTRs of *rbc*L and *rpl*32, respectively. However, high expression mediated by the *psb*A 5'-UTR is due to light-dependent translation (Staub and Maliga, 1993, 1994; Eibl et al., 1999; Kim and Mullet, 1994), thus limiting the use of this UTR for high gene expression to green tissues.

To our knowledge there has been only one report on the use of transgenic plastids in phytofarming. McBride et al. (1995) described the expression of an insecticidal protoxin (Cry1) of *Bacillus thuringiensis*. In contrast to plants, which expressed this protein from a nuclear transgene, transplastomic tobacco plants expressing Cry1 reached levels of 3-5% (tsp) of the protoxin in leaves of tobacco and thus guaranteed a high mortality on feeding insects. In general, expression of prokaryotic proteins in plastids appears to be very promising, because of a similar AT content, codon usage and no requirement for post-translational modifications.

Another striking advantage of plastid transformation in phytofarming is the maternal inheritance of plastids in nearly all higher plants. This feature provides biological safety by preventing spread of transgenes through pollen. Table 2 summarises the outlined advantages and disadvantages of the use of plastid transformation. In future it will be necessary to get a more detailed insight into the regulation of transcription and translation in plastids in order to develop several controllable plastid expression systems also for non-green tissues. Nevertheless, limitations of transgenic plastids as a tool in phytofarming will remain. It may be useful to reach high protein levels and to avoid spread of the transgene. However, proteins can exclusively be localised in the plastid itself and plastids will not perform post-translational modifications due to their inherent prokaryotic nature.

Use of viral vectors

Although viruses store information encoded on a nucleic acid molecule encompassed by a protein body they do not fulfil all criteria of life. Viruses infect living cells, reprogram their metabolism and cause the host cell to proliferate viruses by synthesising virus encoded proteins and by replicating viral RNA or DNA. Once entered a host cell, several plant viruses are able to rapidly spread all over the plant from the initial site of infection. An infected host plant wastes most of its resources for amplification of the virus, leading to economical losses in agriculture. Nevertheless, there are some reports, which point to benefits of plant viruses in phytofarming.

In the past, especially tobacco mosaic virus (TMV) and potato virus X (PVX) were extensively studied

and reprogrammed in order to transiently express heterologous proteins in plants (for example, Chapman et al., 1992; Turpen et al., 1995; McCormick et al., 1999; for review: Yusibov et al., 1999). TMV was the first virus identified and is probably the best understood viral pathogen (for review, Creager et al., 1999). Evolution of viral gene expression did not only aim at reducing genetic material but also at developing mechanisms for high protein expression. These mechanisms are due to transcriptional and translational controls, respectively (reviewed by Ftterer and Hohn, 1996; Gallie, 1996; Maia et al., 1996). In order to study the influence of several viral elements on transient gene expression, Shivprasad et al. (1999) constructed different vectors and compared accumulation of GFP in infected leaves of *Nicotiana benthamiana*. By empirical combining different cis-acting elements of various viruses they found levels of GFP as high as 10% of the total soluble protein. Although some translational mechanisms could be shown to be responsible for different expression levels, major effects were contributed to transcriptional mechanisms (Shivprasad et al., 1999). Table 3 summarises a few important examples of expression levels of heterologous proteins transiently expressed from viral vectors in plants.

There are two strategies to use viruses for foreign gene expression: (i) a virally encoded gene may be substituted by a heterologous gene or (ii) a foreign gene is added to the viral genome either as a fusion to a viral protein or independently. Brisson et al. (1984) described for the first time a recombinant virus vector based on the cauliflower mosaic virus (CaMV) for transient expression of a foreign gene in plants. However, the use of CaMV constructs seems to be limited by the size of the inserted DNA (up to ~250bp), which may be due to packing requirements of the virus (Gronenborn et al., 1981). TMV appears to accept larger DNA inserts although inserted sequences under the control of a duplicated viral subgenomic promoter may also be excised from the recombinant viral vector probably by homologous recombination (Dawson et al., 1989). In contrast, at least for some genes deletions can be omitted by the use of subgenomic promoters derived from a heterologous virus as shown for the first time by Donson et al. (1991). On the other hand it is possible to insert the heterologous gene in frame to a viral protein. The latter strategy was realised by Reinl and Turpen and resulted in yields of about 300mg of a TMV coat protein indolicin-fusion per kilogram of leaves (communicated by Yusibov et al., 1999). Moreover, Turpen et al. (1995) transiently expressed malarial epitopes fused to the TMV coat protein, which may serve as a highly immunogenic carrier, in tobacco. As result of a strategy involving a leaky stop signal with 5% readthrough the malaria epitope was predicted to be present at 0.3% of the virion weight. Since the recombinant virus could be recovered at 1.2mg/g (Turpen et al., 1995) this corresponds to about 3.6g of the epitope per gram fresh weight.

Coat proteins have also been exchanged with recombinant genes, since they show highest expression levels of viral proteins. However, in many cases coat proteins are responsible for or are at least involved in viral movement within the vascular tissue of infected plants (for review, Seron and Haenni, 1996). In general, the replacement of any viral gene leads to reduced infection and subsequently to reduced expression of the heterologous protein (for example, French et al., 1986; Dawson et al., 1989; Joshi et al., 1990). For this reason insertion of the heterologous gene into the viral genome is favoured as long as the recombinant viral vector is sufficiently stable.

Since viruses use the eukaryotic translational machinery in the host cytoplasm for gene expression all known co- and post-translational localisations and

Table 2. Advantages and disadvantages of the use of transplastomic plants in phytofarming.

Advantages	Disadvantages
Maternal inheritance	Protein is localised exclusively in plastids
Homologous recombination	High expression levels are limited to green tissues
allows knock-out	No eukaryotic protein modifications
enables replacment of endogenous genes by mutated allele	Time consuming regeneration procedure in order to achieve uniformly altered plastids
avoids position effects	
No gene silencing	Low transformation efficiency, decreases with length of the introduced DNA
Use of operons	
Asexual inheritance	

modificatiens are conceivable. Kumagai et al. (1998) expressed a capsanthin-capsorubin synthase by use of a TMV/ToMV (tomato mosaic virus)-hybrid vector in *N. benthamiana* which resulted in the accumulation of high levels of capsanthin in chromoplasts. Thus, the enzyme seems to be correctly targeted into plastids by its transit peptide allowing metabolic engineering in the desired subcellular compartment. The same result was observed for viral expression of plastidic phytoene synthase (Kumagai et al., 1995). In addition, extracellular localisation of proteins expressed by infection of the host plant with a recombinant viral vector has been shown. For example, single-chain variable fragment (scFv) antibodies were expressed as secretory proteins with the help of PVX and hybrid-TMV derived vectors in *N. benthamiana* and tobacco, respectively (Franconi et al., 1999; McCormick et al., 1999). Franconi et al. (1999) expressed a monoclonal scFv antibody against tospoviruses with the help of a PVX expression system in *N. benthamiana*. The protein was either localised in the cytosol or directed to the apoplast in another approach. Only the latter approach resulted in functional expression of the antibody. Correct antibody assembly and folding appears to be facilitated in the secretory pathway (for review, Conrad and Fiedler, 1998).

In Table 4 advantages and disadvantages for using viral expression in phytofarming are listed. The most attractive feature of viral expression systems is the extremely short time from cloning the foreign gene until its expression in a plant. Moreover, in general high expression levels can be anticipated and there is no need for a plant regeneration system. The potential of targeting the proteins to distinct compartments within the plant allows eukaryotic modification patterns and metabolic engineering of biochemical pathways localised in different organelles. However, especially metabolic engineering seems to be restricted, since expression of several genes from one viral vector is not possible due to packing requirements of the viral particles. In addition, viral infections cause severe alterations in metabolism which may possibly superimpose the desired biochemical outcome (Herbers et al., submitted, and references therein).

Infection of the plant by a viral pathogen as carrier of a foreign gene may also lead to the accumulation of pathogenesis-related proteins, phytoalexins and other secondary metabolites involved in plant defence. Defence responses are very pronounced in incompatible plant-pathogen interactions. A general defence mechanism of plants against infecting viruses seems to be post-transcriptional gene silencing (for example, Ratcliff et al., 1999; for recent reviews, Baulcombe, 1999b, 1999c; Kooter et al., 1999; Smyth, 1999). On the other hand, this so-called virus induced gene silencing (VIGS) can be overcome by virus encoded suppressors (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau et al., 1998). These few examples demonstrate the subtle relationship between viral vectors and their hosts. Thus, for unopposed spread of the virus and high expression levels the plant host should be fully susceptible. This in turn indicates that viral vectors are limited in their use to their specific hosts. Due to lack of sufficient understanding of many plant-virus interactions and insufficient access to genetical manipulation of most plant viruses, TMV/*N. benthamiana* and PVX/*N. benthamiana* are probably the most widely used plant viral expression systems so far. In future, it will be necessary to get a deeper insight into the regulation of viral expression mechanisms and plant defence responses as well as to establish systems based on other crops each optimised for high foreign protein expression.

Table 3. Protein expression levels in plants infected by recombinant viral expression vectors.

Protein	Viral vector	Host	Localisation	Level (tsp)	References
α-gal A	Tobamovirus	Not given	Apoplast	2%	Turpen and Pogue, communicated in Yusibov et al., 1999
ScFV	TMV	*N. benthamiana*	Apoplast	1-2%	McCormick et al., 1999
Interferon	Tobamovirus	Not given	Not given	1%	Pogue and Hanley, communicated in Yusibov et al., 1999
GFP	TMV	*N. benthamiana*	Cytosol	>10%	Shivprasad et al., 1999
α-amylase	TMV/ToMV	*N. benthamiana*	Apoplast	5%	Della-Cioppa et al., 1999
CTL-TMVCP	TMV	*N. tabacum*	Cytosol	3 g/g fw	Della-Cioppa et al., 1999; Turpen et al., 1995

α-gal A, α-galactosidase A; CTL, ocytotoxic T lymphocyte; GFP, green fluorescent protein; scFv, single chain variable fragment; TMV, tobacco mosaic virus; TMVCP, tobacco mosaic virus coat protein; ToMV, tomato mosaic virus; tsp, total soluble protein

The transient nature is a characteristic feature of the viral expression system. It may allow for expressing toxic proteins since inoculated plants are fully grown and do not necessary have to survive after protein production. Moreover, the transient expression of foreign proteins supports biological safety, since most plant viruses are not submitted through the germline. Thus, an outcross or inheritance of recombinant genes is prohibited. However, this characteristic also implies that seeds, which are attractive target organs in phytofarming, are not modified by these systems. Another feature contributes to the safety of recombinant viral vectors. They do not seem to be sufficiently stable so that the transgene does not survive in nature since each construct reverts to the wildtype-like virus (reviewed for tobamovirus derived vectors by Yusibov et al., 1999). On the other hand, the surviving wildtype viruses may lead to dramatic economical losses in neighbouring fields or in cultured plants of the same field in following seasons.

In addition to phytofarming, viral expression vectors can be used in functional genomics in order to validate and identify gene function (forward and reverse genetics, Baulcombe, 1999 a, c). Furthermore, they serve as useful tools in rapid expression of foreign proteins to determine, for example, their function and stability in different cellular compartments (see below). In conclusion, viral systems may provide useful tools in phytofarming under controlled greenhouse conditions.

Protein stability

The two expression systems described above particularly make use of high gene dosis, efficient transcription and translation in order to obtain high expression levels. The accumulation of a protein, however, does not solely depend on its rate of synthesis but also on its turnover. Thus, other approaches try to stabilise foreign proteins in the host cell. The possibility of targeting the expressed protein to different compartments may have impacts on protein yield, since many heterologous proteins show different amounts of accumulation in different compartments (Sonnewald et al., 1991; Fiedler and Conrad, 1998; Herbers and Sonnewald, unpublished data). This may be due to distinct proteases in various parts of a plant cell.

In another attempt proteolytic activities of the host plant cell may be inhibited during foreign gene expression. Plants possess endogenous proteinase inhibitors (PINs), which are induced in several defence responses and are especially directed against herbivores. The induction of PINs within a plant host depends on an accumulation of octadecanoid signalling molecules, especially jasmonic acid (JA; Farmer and Ryan, 1990, 1992; Howe et al., 1996; for review, Weiler et al., 1998). Since exogenously applied JA also leads to an accumulation of PINs, the application of this signal molecule may increase stability of foreign proteins by induction of PINs and subsequent inhibition of proteases (Della-Cioppa et al., 1999). Furthermore, a transgenic expression of PINs may lead to similar effects (Della-Cioppa et al., 1999).

Protein degradation may not only be decreased by inhibiting proteolytic activity but also by increasing protein stability. Hondred et al. (1999) showed that fusions between ubiquitin and foreign proteins increased protein stability. The ubiquitin part was quickly removed by an endogenous ubiquitin specific protease, thereby releasing the fusion partner in its natural form. The ubiquitin fusion, in addition, did not affect proper localisation to the apoplast and

Table 4. Advantages and disadvantages of the use of plant viral expression vectors in phytofarming.

Advantages	Disadvantages
Extremely rapid process, thus cost-effective	Risk of undesired viral infection of crops
No transgenic plant, no outcross of transgenes, no need for plant regeneration	Major expression is restricted to leaves Restricted to fully compatible host-virus interactions
Subcellular localisation possible Eukaryotic post-translational modifications	Allows only one or at least a few genes due to size limitations in vector construction
Offers possibilities for easy protein purification	Defense responses and metabolic perturbance may restrict use of viral system
Facility for forward genetics to identify gene function taking advantage of VIGS	
Ability to express phytotoxic proteins	
High expression levels	

VIGS, virus induced gene silencing

chloroplasts, respectively (Hondred et al., 1999). Since ubiquitinated proteins are normally subjected to the eukaryotic 26S proteasome for controlled proteolysis (for review, Voges et al., 1999) the mechanisms whereby ubiquitin moiety augment protein accumulation remains unclear (Hondred et al., 1999). A better understanding of the mechanisms of protein turnover in plants may be the basis for valuable systems for high expression levels by stabilising foreign proteins in future.

Approaches to express several proteins at the same time

For metabolic engineering in transgenic plants, it is often necessary to express several proteins at the same time. The most obvious approach is to clone all required genes with respective promoters between two T-DNA borders of a binary vector and deliver them to the plant via *Agrobacterium* mediated transformation. To facilitate such a strategy de Majnik et al. (1997) developed a set of vectors with many unique restriction sites for simple cloning. Both the cloning as well as the transformation efficiency of binary vectors seem to decrease with increasing length of the inserted DNA. Yet, on the other hand there are several reports which describe an efficient, stable and complete transfer of artificial chromosomes including 70-100kbp of cloned DNA. These attempts made use of the *Agrobacterium* mediated (Hamilton et al., 1996; Hamilton, 1997; Liu et al., 1999) or biolistic (Adam et al., 1997) transformation systems. All these strategies aimed at complementing plant mutants and at accelerating positional gene cloning (for review, Sawahel and Fukui, 1995). Thus, in principle large DNA fragments can be inserted into plant chromosomes. However, the described literature exclusively contains examples in which plant genomic DNA fragments have been transformed. It may be that the intrinsic chromatid structure facilitates the insertion of DNA into plant chromosomes.

There are also efforts to develop plant artificial chromosomes (PACs) which autonomously replicate in the plant nucleus. Noutoshi et al. (1997, 1998) characterised structural properties of chromosomes such as elements for replication, maintenance and segregation of the uni-cellular green algae *Chlorella vulgaris*. However, to our knowledge there is no report on successfully maintaining PACs in plant cells so far.

Another possibility to introduce several genes into a single transgenic plant makes use of crossing lines which express a subset of the desired proteins (for example, Lloyd et al., 1992; Nawrath et al., 1994; Ma et al., 1995). However, such a strategy is time consuming and thus not acceptable in commercial approaches. Equivalent approaches would be co-transformation (for example, Lyznik et al., 1989; Barcelo et al., 1994; Chen et al., 1998) and re-transforming transgenic plants (for example, Matzke et al., 1989; Fujiwara et al., 1993) with different sets of genes. Yet, for efficient plant selection these approaches require the use of several selection markers which is under intense public debate.

For modifying or introducing biochemical pathways it might be desirable to co-ordinately express all proteins needed in equimolar amounts. However, the use of the same promoter and UTRs for controlling expression of the desired proteins may result in (post-)transcriptional gene silencing ([P]TGS; for review, Vaucheret et al., 1998; Kooter et al., 1999). Therefore, several regulating regions with similar characteristics are required still leaving the possibility that expression levels vary due to insertion into different locations within the genome.

To avoid these positional effects, it might be possible to introduce several genes as an operon in plastids (see chapter on plastid transformation). However, in case of metabolic engineering this approach is only applicable to biochemical pathways of plastids. Although polycistronic mRNAs do not naturally occur in eukaryotes due to the nature of the translational machinery, viruses have developed a strategy to translate several peptides from a single mRNA. They often encode precursor polyproteins which are subsequently cleaved into individual proteins in the cytoplasm of plant cells. A short linker serving as protease recognition site generally divides the individual proteins. The cleavage of the precursor can occur both co- and post-translationally, and it can happen in cis and in *trans*, respectively (for example, Thole and Hull, 1998; Yang et al., 1998; for review, Maia et al., 1996).

This viral strategy has been transferred to plants in order to express co-ordinate levels of several proteins at the same time (Marcos and Beachy, 1994, 1997; Dasgupta et al., 1998; Urwin et al., 1998; Halpin et al., 1999; Table 5). Protein accumulation should only be a function of their inherent stability but not of transcription and translation, respectively. These methods also allow different subcellular localisation of the cleaved products as demonstrated by Dasgupta et al. (1998). They inserted the tobacco vein mottling virus (TVMV) Nla recognition site between acetate kinase and chloramphenicol acetyltransferase and targeted both of these enzymes into plastids. The proteolytic activity to process the precursor polyprotein can be (i) expressed from a recombinant gene in cis or trans (Dasgupta et al., 1998),

(ii) endogenously provided by the plant (Urwin et al., 1998) or can be (iii) an intrinsic function of the linker between the individual polypeptides as has been shown for the 2A peptide (Halpin et al., 1999). In the latter case the size of the vector construct is reduced and no cellular factors other than eukaryotic ribosomes are required (Halpin et al., 1999). The underlying mechanism of the co-translational proteolytic process mediated by the 2A peptide region of foot-and-mouth disease virus (FMDV) is not yet fully understood. One possibility is that the peptide interferes with the translational process and inhibits peptide bond formation (Ryan and Drew, 1994).

Beck von Bodman et al. (1995) used a polyprotein approach to introduce a new biosynthetic pathway in tobacco. They expressed enzymes associated with the mannityl opine biosynthetic pathway of *Agrobacterium* in conjunction with Nla protease derived from TVMV. All described strategies for proteins expressed from an ancestral polyprotein have the disadvantage that they contain additional amino acids arising from the proteolytic recognition sites. These may interfere with protein function and structure.

Approaches to improve purification of the heterologous protein

As mentioned in the introduction, downstream production costs account for more than 90% of total costs in phytofarming. Thus, in future these fixed charges have to be reduced. In this chapter we present a few strategies, which may become attractive solutions for the purification of foreign proteins expressed in plants. All systems described use peptide tags fused to the protein of interest, which allows developing universal purification procedures for the tag. The tags may be cleaved off and further purification steps can be performed if necessary.

Oleosins

An import approach exploits the hydrophobicity of oil bodies (also called fat lipid bodies, spherosomes or oleosomes). These organelles consist of drops of storage lipids, especially triacylglycerols (TAGs), enclosed by a phospholipid monolayer. Special proteins termed oleosins cover the surface of these oil bodies in seeds and anthers of plants (for review on oil bodies and oleosins the reader is referred to Huang, 1996; Napier et al., 1996; Staehelin, 1997; Murphy and Vance, 1998).

Since oleosins comprise the vast majority of the protein content of oil bodies in mature seeds and since oil bodies can be easily purified by floatation centrifugation, oleosins represent a useful tag for foreign protein purification (for review, Moloney, 1996; Moloney and Holbrook, 1997). Van Rooijen and Moloney (1995a) demonstrated that GUS fused to the C-terminus of an *Arabidopsis* oleosin was correctly targeted to oil bodies in transgenic *Brassica napus*. Moreover, about 1/10 of the oleosin proteins (equivalent to 0.2 to 1% of total seed protein) comprised the fusion protein which was easily enriched 13-fold by isolation of the oil bodies (van Rooijen and Moloney, 1995a).

Parmenter et al. (1995) fused hirudin, a pharmaceutical protein, to oleosin of *Arabidopsis* and reached levels of 1% of total seed protein in transgenic *Brassica* plants. However, the fusion protein did not show any hirudin activity. This could subsequently be restored by proteolytic release of hirudin from its oleosin tag using a factor Xa/clostripain

Table 5. Examples for proteins derived form a polyprotein.

Joined proteins	Linker peptide	Features of proteolytic cleavage	References
CAT-GUS	FMDV-2A region	Exclusive co-translational processing, intrinsic function of the linker, depends on eukaryotic ribosomes, 80-95% cleaved *in vitro*	Halpin et al., 1999 Ryan and Drew, 1994
ACK, CAT, Nla in different order	TVMV Nla rec.site	Depends on TVMV Nla proteinase in *cis* or *trans*	Dasgupta et al., 1998
TMVCP-Nla TMVCP-Nla-SMVCP SMVCP-Nla-TMVCP	TEV Nla rec.site	Depends on TEV Nla proteinase in *cis* (or *trans*, not shown)	Marcos and Beachy, 1994, 1997
OcIΔD86-CpTI	PsMTa rec.site	Depends on a unidentified plant proteolytic activity	Urwin et al., 1998

ACK, acetate kinase; CAT, chloramphenicol acetyltransferase; CpTI, cowpea trypsin inhibitor; FMDV, foot-and-mouth disease virus; GUS, β-glucuronidase; Nla, nuclear inclusion protein a; ocI, oryzacystatin; PsMTa, metallothionein-like protein; rec.site, recognition site; SMVCP, soybean mosaic virus coat protein; TEV, tobacco etch virus; TMVCP, tobacco mosaic virus coat protein; TVMV, tobacco vein mottling virus

cleavage site between these proteins. Two additional chromatography steps were necessary to further purify hirudin (Parmenter et al., 1995).

In addition to their ease of enrichment oleosins represent a useful natural tag for protein fusions because they accumulate up to 20% of total seed protein. High oleosin levels are found in plants with fats as their main storage form. Among these plants there are several species of agronomic importance such as rapeseed (*Brassica napus*) and sunflower (*Helianthus annus*). Yet, it has to be kept in mind that oil bodies may not be a favourable compartment for all proteins with respect to stability and post-translational modifications. Although oleosins are translated at the rough ER, they do not enter the ER lumen and therefore do not become glycosylated (van Rooijen and Moloney, 1995b).

Universal tags

A mechanistically equivalent approach to the described oleosin fusions makes use of viral expression vectors, in which the recombinant gene is fused to the viral coat protein. The heterologous protein can directly be enriched by isolation of virions by simple procedures. If the foreign protein is needed without viral portion, a protease cleavage site can be introduced between the viral and the heterologous protein. Thus, the protein of interest can be released from its 'carrier' after purification of the virion (for example, Turpen et al., 1995, see chapter on use of viral vectors and Table 3).

There are other proteins or peptides known which can serve as affinity tags. These proteins or peptides can be purified following standardised affinity chromatography. Widely used tags include *Schistosoma* glutathione S-transferase (GST), *Escherichia coli* maltose-binding protein (MBP), *Staphylococcus* protein A, strep-tag / *in vivo* biotinylated peptide, chitin-binding domain or polyhistidine stretches. These tags bind to glutathione-agarose, amylose resin, IgG, streptavidin, chitin and nickel-chelate, respectively. Up to now all outlined systems have often been used in *E. coli*, yeast or insect cells but there are only few examples using these tags in order to purify proteins from plants. For instance, polyhistidine tags were fused to the coding regions of the helper component of tobacco etch virus and of the capsid protein of beet yellows closterovirus, respectively, in order to purify these proteins from crude extract of infected plants with the help of nickel-chelate columns (Dolja et al., 1998; Blanc et al., 1999).

As stated before, the protein fusion can be proteolytically cleaved if desired (for review, Ford et al., 1991; LaVallie and McCoy, 1995; Sheibani, 1999). As proteases are not always specific and because the proteolytic cleavage reaction may require unfavourably temperatures affecting protein stability, the protease has to be chosen considering the conditions under which it will be functional. Further drawbacks of using proteases concern the facts that they may leave additional amino acid residues at the purified protein and that they will be co-eluted together with the protein of interest. To circumvent these problems new approaches take advantage of the intrinsic splicing activities of inteins.

Inteins excise themselves in a self-splicing reaction from a precursor protein while the flanking regions, the exteins, become joined to restore host gene function (for review, Perler et al., 1997). By removing the internal domain of the intein, usually comprising an endonucleolytic activity, the resulting mini-intein retains its splicing activity. Several scientists have engineered inteins to enhance splicing efficiency or to allow cleavage solely at one intein border (Chong et al., 1996; Southworth et al., 1999; for review, Amitai and Pietrokovski, 1999). Moreover, inteins were mutated in order to develop inducible cleavage activity. Thus, cleavage can be initiated by addition of thiol reagents (Chong et al., 1997), by pH change (Wood et al., 1999) or by a decrease in temperature (Chong et al., 1998). In order to use these systems for protein purification well-characterised tags are fused to an intein with inducible cleavage activity and to the protein of interest. This hybrid protein can be easily purified by affinity chromatography. By inducing the intein cleaving activity the foreign protein becomes released and can be eluted in an appropriate buffer while the tag and intein portion remains bound to the affinity matrix (Figure 2; for example, Chong et al., 1997, 1998; Wood et al., 1999; for review, Amitai and Pietrokovski, 1999). Thus, systems based on mini-inteins do avoid the use of cost-intensive proteases and do not lead to contamination of the desired protein with a proteolytic activity.

Since the cleavage activity of inteins is affected by flanking residues it would be useful to obtain several intein-based purification vectors. For the future it is planned that an appropriate intein can be selected in dependence of the amino acid composition of the protein to be combined to the intein-tag fusion in order to achieve high efficiency of cleavage (Southworth et al., 1999). Again, up to now the intein based systems have not been adjusted for use in plant systems.

In principle, all known tags and purification methods will be equally useful in downstream processes of phytofarming. However, the utilised tags need to be chosen carefully since the tag should

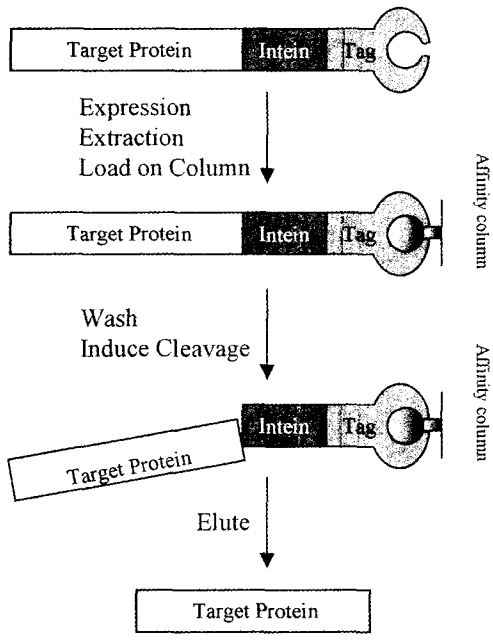

Figure 2. Purification of target proteins with the help of an affinity tag intein fusion (according to Southworth et al., 1999).

not decrease protein stability and the corresponding affinity matrices should not bind endogenous plant proteins.

Conclusions

In the last years several tools have been developed which may become very attractive in phytofarming. However, none of these tools can be universally applied. Many aspects including (i) conditions and climate for growth of the plant species, (ii) harvesting and storage of the organ where the protein of interest will be expressed, (iii) protein stability and protein modifications in different cellular compartments as well as (iv) requirements for protein purification procedures have to be taken into account when proteins of economic interest are expressed in plants. These factors will help to decide which plant, organ and organelle is the preferred host for the foreign protein. In future a better understanding of the tools described and the development of new methods in phytofarming will simplify choosing the right system in order to express heterologous proteins in plants at an appropriate level, with proper modifications and with the opportunity for simple purification.

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