Review article

Preventing unintended proteolysis in plant protein biofactories

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Summary
Numerous reports have been published over the last decade assessing the potential of plants as useful hosts for the heterologous expression of clinically useful proteins. Significant progress has been made, in particular, in optimizing transgene transcription and translation in plants, and in elucidating the complex post-translational modifications of proteins typical of the plant cell machinery. In this article, we address the important issue of recombinant protein degradation in plant expression platforms, which directly impacts on the final yield, homogeneity and overall quality of the resulting protein product. Unlike several more stable and structurally less complex pharmaceuticals, recombinant proteins present a natural tendency to structural heterogeneity, resulting in part from the inherent instability of polypeptide chains expressed in heterologous environments. Proteolytic processing, notably, may dramatically alter the structural integrity and overall accumulation of recombinant proteins in plant expression systems, both in planta during expression and ex planta after extraction. In this article, we describe the current strategies proposed to minimize protein hydrolysis in plant protein factories, including organ-specific transgene expression, organelle-specific protein targeting, the grafting of stabilizing protein domains to labile proteins, protein secretion in natural fluids and the co-expression of companion protease inhibitors.

Keywords: plant molecular farming, protease inhibitors, proteases, protein stabilization, protein targeting, proteolytic processing, recombinant protein degradation.

Introduction
A number of heterologous expression systems have been devised over the last decade for the production of clinically useful recombinant proteins, involving, in most cases, eukaryotic hosts competent in performing complex post-translational modifications typical of mammalian proteins (Dyck et al., 2003; Ma et al., 2003; Gerngross, 2004; Wurm, 2004). Plant-based expression platforms, in particular, offer unique advantages over traditional expression hosts in terms of cost efficiency, product safety, available expertise and facilities for culture and storage, and scalability easily adaptable to production needs (Daniell et al., 2001; Ma et al., 2003; Twyman et al., 2003; Streitfield, 2007). To date, several proteins of medical interest have been expressed successfully in plants, including a variety of antibodies, vaccine antigens, protein allergens, enzymes and enzyme inhibitors, coagulation factors, cytokines and hormones (Hiatt et al., 1989; Mason et al., 1992; Ma et al., 1995, 2003, 2005a; Ruggiero et al., 2000; Kirk and Webb, 2005; Twyman et al., 2005; Floss et al., 2007; Lienard et al., 2007).

A significant challenge in most systems is to optimize the yield and quality of the recombinant protein product. Significant progress has been achieved over the last 15 years in the optimization of transgene transcription and translation in plants (Potenza et al., 2004; Streitfield, 2007) and the elucidation and modulation of the complex protein post-translational modifications characteristic of the plant cell machinery (Gomord and Faye, 2004; Faye et al., 2005). Despite these advances, ensuring satisfactory yield and
quality of recombinant proteins often remains a difficult task. Unlike several more stable (and structurally less complex) pharmaceuticals, proteins present a natural tendency to structural heterogeneity, often giving a complex mixture of variants differing in their primary or tertiary structure (Faye et al., 2005). This natural bias of proteins, which underlines important issues of product authenticity and heterogeneity, also emphasizes the need for a correct understanding of the various post-translational steps of the whole protein synthesis-recovery process, from the maturation and assembly of nascent protein backbones in transgenic host cells to the extraction and purification of the protein product after biomass harvesting.

Proteolytic degradation of recombinant proteins in plant systems

One factor strongly influencing recombinant protein quality and yield is the relative inherent stability of polypeptide chains expressed in a heterologous environment (Faye et al., 2005). Living cells are the theatre of numerous proteolytic processes, essential to ensure vital metabolic functions, but often a burden for the effective production of biologically active proteins (Doran, 2006; Goulet and Michaud, 2006). Proteolytic enzymes, or proteases, contribute to the overall control of metabolic and transduction pathways by directing the activation or hydrolysis of proteins implicated in key regulatory processes, or by contributing to the elimination of misfolded proteins and the selective recycling of amino acids from short-lived proteins (Vierstra, 2003; Schaller, 2004). In plants, these enzymes also initiate the general recycling of proteins in senescing organs and the mobilization of amino acid constituents of seed or tuber storage proteins during germination (Müntz, 2007). Hundreds of genes code for proteins involved in proteolytic processes in plants (Rawlings et al., 2008), with, for instance, an estimate of 1900 genes in Arabidopsis directly or indirectly implicated in the hydrolysis of peptide bonds (Schaller, 2004; Smalle and Vierstra, 2004).

From a practical viewpoint, the ubiquitous nature of proteolytic processes (Schaller, 2004) and the diversity of possible protease forms in the plant genome (Beers et al., 2004) pose a significant challenge to the efficient production of several recombinant proteins. Although some proteins accumulate at high levels in plant systems, other proteins apparently undergo extensive hydrolysis, with a strong negative impact on the final yield despite easily detectable mRNA transcripts. Accumulation levels below 0.01% of total soluble protein (TSP) in plant tissues have been observed for several proteins of therapeutic value, including, for instance, human serum protein C, interferon β, erythropoietin and epidermal growth factor (Daniell et al., 2001). Low accumulation rates of intact protein products have also been observed for other recombinant proteins of practical interest, including immunoglobulins, antibodies and enzyme inhibitors (e.g. Stevens et al., 2000; Sharp and Doran, 2001; Ma et al., 2003; Ouitchkouroff et al., 2003; Schillberg et al., 2005), again suggesting the key influence of proteolysis on the overall efficiency of plant-based protein factories.

Proteases may affect the integrity of recombinant proteins in different ways, both in planta during protein expression and ex planta during extraction and subsequent downstream processing (Michaud et al., 1998; Rivard et al., 2006). Depending on the number of ‘susceptible’ cleavage sites accessible to endogenous proteases for peptide bond hydrolysis, the protein may undergo complete hydrolysis directly impacting on its final yield or partial trimming, altering the activity or homogeneity of the final protein product. Although interesting yields may be obtained in terms of net protein levels, the final product may show altered integrity, structural heterogeneity and/or deficient biological activity, potentially altering its value for commercialization (Faye et al., 2005). Several strategies have been considered recently to minimize proteolysis in plant protein factories. This article summarizes these strategies, taking into account proteolysis observed in planta before harvesting, and unwanted proteolytic degradation observed ex planta during protein recovery from plant tissues and cells.

Stabilizing recombinant proteins in planta

In theory, the easiest way to minimize recombinant protein hydrolysis in planta is to use mutant lines deficient in protease(s) active against the protein of interest. Protease-deficient strains of simple expression hosts, such as Escherichia coli (Liang et al., 2002; Rozkov and Enfors, 2004) and yeasts (Sreekrishna et al., 1997; Cereghino and Cregg, 2000; Macauley-Patrick et al., 2005), were devised several years ago to minimize recombinant protein loss in vivo, and such strains are now available commercially and used routinely in most protein biochemistry laboratories. This approach, however, may not be applicable to complex, multicellular organisms such as higher plants, given the central role of proteolysis in growth and development (Schaller, 2004), the expected diversity and variability of protease forms in plant cells and tissues (Beers et al., 2004), and the large number of protease–protein interactions possibly taking place in vivo, which will depend on both the structural characteristics of each protein to be expressed and
the metabolic status of each cell compartment or tissue targeted for protein deposition. At this stage, a case-by-case empirical assessment involving the testing of different expression strategies remains the most straightforward way to optimize the production yield and structural integrity of a given protein. Common strategies to overcome unwanted proteolysis in planta involve the targeting of transgene expression or protein accumulation to specific tissues or cellular organelles. Approaches involving the grafting of protein-stabilizing fusion domains to recombinant proteins or the co-expression of companion protease inhibitors interfering with endogenous proteases have also been proposed recently.

Tissue-specific transgene expression

For several reasons, the specific tissue or organ selected for recombinant protein production has a strong influence on the final yield and product quality. Cellular proteases, notably, differ from one tissue to another in terms of quantity and quality (or overall substrate specificity) (Schaller, 2004), with a possible differential impact on the integrity of proteins. Historically, recombinant proteins in plants have often been expressed under the control of strong constitutive promoters, such as the cauliflower mosaic virus 35S promoter or the maize ubiquitin 1 promoter, but several tissue-/organ-specific (e.g. seed-specific) promoters have been isolated and are now used to express transgenes in selected tissues and organs (Potenza et al., 2004). In practice, an effective scheme for recombinant protein production should not only provide effective transcription of the transgene sequence, but also involve an accumulation site with low levels of overall proteolytic activity or with endogenous protease species showing no (or little) specific activity against accessible peptide bonds in the protein of interest.

To date, green leaves have been the destination of choice for several recombinant proteins, given their rapid growth rate, the possibility in some platforms to harvest leaf material more than once over the growing season, and the availability of numerous regulatory sequences well adapted to transgene expression in the leaf cell environment (Daniell et al., 2001). However, the highly active metabolism of leaf tissues, which typically exhibit high protein synthesis and turnover rates, may represent a significant hurdle to protein accumulation in vivo. In particular, increased protease levels in senescing leaves (Kato et al., 2004, 2005; Lin and Wu, 2004; Otegui et al., 2005; Parrott et al., 2005) represent a potential drawback in leaf-based production systems (Stevens et al., 2000; Birch-Machin et al., 2004), both in vivo before protein recovery when senescence-associated amino acid recycling is initiated, and in vitro during extraction once cellular proteases have been released into the extraction medium together with the protein(s) of interest. The abundance of poorly specific proteases and phenolic compounds in green tissues (Michaud and Asselin, 1995) also leads to the problem of post-harvest protein degradation and denaturation in leaves, which can make it necessary to process leaf biomass shortly after harvest, or to store this material at low temperatures until protein extraction (Schillberg et al., 2005).

By contrast with leaf tissues, the seeds and storage organs of several crops appear, in general, to be well suited for the efficient deposition and post-harvest storage of recombinant proteins (Stoger et al., 2005). Proteins of clinical interest have been successfully expressed in seeds of several species, including major cereal crops (e.g. maize, rice, barley and wheat), soybean and safflower, under the control of either constitutive or seed-specific promoters. Recombinant proteins in seeds often accumulate in storage organelles, mainly in protein bodies and protein storage vacuoles (Streatfield et al., 2003; Yang et al., 2005; Drakakaki et al., 2006). The desiccated nature of mature seeds, together with the low abundance of active proteases in seed tissues during dormancy, prevent extensive proteolysis and promote long-term stability of proteins in planta (Fiedler and Conrad, 1995; Stoger et al., 2000). In a similar manner, the tubers and storage roots of some species exhibit reduced metabolic activity, and may represent an interesting solution for recombinant protein storage. Artsaenko et al. (1998), for instance, documented the long-term stability of a single-chain Fv antibody in potato tubers after storage for more than a year. From a technological viewpoint, storage organs also present anatomical features favourable to protein stability, such as the presence of protein and oil bodies in seeds, that may sequester proteins and minimize their exposure to proteases during extraction (Stoger et al., 2005).

Organelle-specific protein targeting

Protein sequestration in, or targeting to, specific cell compartments has also been readily recognized as a key factor determining the overall stability and yield of recombinant proteins in planta (Wandelt et al., 1992; Schouten et al., 1996; Gomord et al., 1997). Organelles play specific, complementary functions in the cell and thus harbour their own metabolic machinery, including a protease complement well adapted to their particular enzymatic and physicochemical environment (Callis, 1995). Not surprisingly, the targeting of foreign proteins to different organelles using appropriate peptide
signals has a strong impact on their accumulation rate (Table 1). In biochemical terms, the stability of a given protein in vivo will depend on the relative steric accessibility of peptides or peptide strings on the protein chain susceptible to the proteases present. In practice, the choice of a suitable cellular destination will also depend on the structural characteristics of the recombinant protein, which will often dictate specific co- or post-translational modifications essential for adequate activity, stability and/or homogeneity (Faye et al., 2005).

Eventual metabolic interference effects on the host plant actively expressing the recombinant protein should also be assessed, given the possible onset of organelle-dependent effects of biologically active proteins in planta. Cytosolic or apoplastic accumulation of the human growth factor, for instance, had toxic effects in leaves of Nicotiana benthamiana, whereas, in contrast, no negative effects were observed for the same protein targeted to the chloroplast (Gils et al., 2005). Likewise, the bovine protease inhibitor aprotinin was accumulated at high specific levels when retained in the endoplasmic reticulum (ER) of potato leaf cells, but this positive effect of ER retention was counterbalanced by a general decrease in total protein content in leaves, presumably as a result of the exogenous inhibitor affecting key steps of protein biosynthesis in vivo (Badri, 2006). Considering the complexity of protein maturation processes in plant cells and the often unpredictable nature of pleiotropic effects in transgenic host plants, the most appropriate way to select a suitable cellular destination at this stage involves the empirical testing of different possible destinations, taking into account

Table 1 Impact of subcellular targeting on recombinant protein yield in transgenic plant systems – selected examples

| Protein | Transformed species | Plant organ | Cytosol | Endoplasmic reticulum | Vacuole | Apoplasma | Plastid | Nucleus | Reference |
|---------|---------------------|-------------|---------|-----------------------|---------|-----------|---------|---------|-----------|
| Antibodies |                     |             |         |                       |         |           |         |         |           |
| Scfv anti-cutinase | Nicotiana tabacum | Leaf | 0 | 100 | 1 | Schouten et al. (1996) |
| Scfv anti-oxazolone | N. tabacum | Leaf | 10–20 | 1 | Fielder et al. (1997) |
| Scfv anti-oxazolone | Solarum tuberosum | Tuber | 5, 22 | 1 | |
| Scfv anti-dihydroflavonol 4-reductase | Petunia hybrida** | Petal | 1 | 2, 30 | De Jaeger et al. (1999) |
| BiscFv 2429 | N. tabacum | Leaf | 1 | 20, 60 | |
| FAB MAK33 | Arabidopsis thaliana | Leaf/seed | Traces | 10 | Fischer et al. (1999) |
| scFv anti-carciinoembryonic | N. tabacum | Leaf | 25 | 1 | Peeters et al. (2001) |
| Ab anti-carciinoembryonic | N. tabacum** | Leaf | 2–6 | 1 | Stoger et al. (2002) |
| Ab 1409 κ chain | N. tabacum | Leaf | 8 | 1 | Vaquero et al. (2002) |
| Ab 1409 γ chain | N. tabacum | Leaf | 4 | 1 | Petruccelli et al. (2006) |
| Vaccines |                     |             |         |                       |         |           |         |         |           |
| Escherichia coli heat-labile enterotoxin B | Zea mays | Seed | 1 | 100 | 20 000 | 3300 | 7 | 21 | Streitfeld et al. (2003) |
| Hepatitis B surface antigen | N. tabacum | BY-2 cells | 1.4 | 1.8 | |
| Japanese cedar pollen allergens | Oryza sativa | Seed | 0 | 4–6 | 1 | Takagi et al. (2005) |
| Medical proteins |                     |             |         |                       |         |           |         |         |           |
| Human epidermal growth factor | N. tabacum | Leaf | 1 | 10 000 | | Wirth et al. (2004) |
| Human growth hormone | N. benthamiana† | Leaf | 1 | 1000 | 10 | Gil et al. (2005) |
| Agronomic/industrial proteins |                     |             |         |                       |         |           |         |         |           |
| Vicilin | N. tabacum | Leaf | 100 | 1 | Wandelt et al. (1992) |
| Equistatin | Medicago sativa | Leaf | 20 | 1 | |
| Silk-like protein | A. thaliana | Leaf | 1 | 13 | 0 | 5 | Yang et al. (2005) |
| Seed | 1 | 7.8 | 5.4 | 0 | |

*Relative yield, compared with the yield in ‘control’ compartment (value of unity).
†Transient expression.
current knowledge on the protein being expressed and on the physicochemical and enzymatic microenvironment of the different organelles available for protein accumulation. Several subcellular compartments have been considered as possible destinations for recombinant proteins in plant cells, including the cytosol, the chloroplast and different subcompartments of the cell secretory pathway (Ma et al., 2003; Daniell, 2006; Goulet and Michaud, 2006).

**Retention in the cytosol**

In practice, the absence of a targeting signal in the transgene sequence prevents migration of the recombinant protein out of the cytosol following mRNA translation (Figure 1). Recombinant proteins retained in the cytosol are usually detected at very low levels despite good transgene transcription rates, giving, in several cases, accumulation rates below 0.1% of TSP (Conrad and Fiedler, 1998). Cytosolic targeting of the tomato mosaic virus antibody ‘rAb29’ in tobacco leaf cells, for instance, resulted in very weak accumulation rates, whereas the same transgene including a signal peptide for extracellular secretion produced easily detectable amounts of this protein (Schillberg et al., 1999). Similarly, retaining human growth hormone in the cytosol of *N. benthamiana* leaf cells led to protein levels of about 0.01% of TSP, in contrast with concentrations reaching 10% of TSP for the same protein targeted to the apoplast (Gils et al., 2005). Several factors may explain the limited suitability of the cytosol as a destination for recombinant proteins: (i) the negative redox potential of the cytosolic milieu, unfavourable to the correct folding of proteins with disulphide bonds (Goulet and Michaud, 2006); (ii) the absence of important co- and post-translational modification processes, such as glycosylation, which may have a positive impact on the folding, assembly and/or structural stability of several nascent and mature proteins (Faye et al., 2005); and (iii) the effective housekeeping activity of the ubiquitin–proteasome proteolytic pathway in this cellular compartment (Vierstra, 1996, 2003), involved notably in the recognition and degradation of incorrectly folded proteins. Although some recombinant proteins remain stable in the cytosol (e.g. Michaud et al., 1998; De Jaeger et al., 1999; Rajabi-Memari et al., 2006; Marusic et al., 2007), alternative destinations, such as the chloroplast, the ER or the apoplast, appear to be more appropriate for most proteins.

**Retention in the ER**

Targeting to the cell secretory pathway, in particular, has been proposed to improve the stability and yield of several proteins (Ma et al., 2003; Yoshida et al., 2004; Vitale and Pedrazzini, 2005). In practice, the addition of an N-terminal
signal peptide sequence to the protein-encoding transgene triggers a co-translational transfer of the nascent protein to the endomembrane system (Chrispeels and Faye, 1996) (Figure 1). Proteins bearing a signal peptide for cellular secretion first enter the ER via the ER protein translocation channel (Galili et al., 1998), and then migrate through this compartment and the Golgi apparatus until reaching the extracellular medium (default pathway) or the vacuole, if a vacuolar sorting signal is found in the primary sequence. Recombinant proteins entering the ER may also be retained in this compartment by simple apposition of the tetrapeptide ER retention signal (K/H)DEL (Michaud et al., 1998) or grafting of a γ-zein proline-rich domain (Mainieri et al., 2004) at the C-terminus. The (K/H)DEL motif is a common ER retrieval signal in eukaryotes, believed to redirect tagged proteins to the ER after their recognition by a (K/H)DEL receptor complex in the Golgi apparatus (Lee et al., 1993; Pagny et al., 2000). In contrast with most reticuloplasmins, cereal prolamins accumulate in the ER of seed cells without the involvement of the (K/H)DEL motif, forming high-density protein bodies in the ER compartment of plant cells, using, in most cases, a (K/H)DEL retention signal (Ma et al., 2003). The ER, which constitutes a natural reservoir for some storage proteins in seed cells (Shewry and Halford, 2002), can physically accommodate high levels of recombinant protein product in planta (Wandelt et al., 1992). At the biochemical level, the low abundance of proteolytic enzymes and the presence of molecular chaperones in the ER, together with an oxidizing status favouring disulphide bond formation, make this organelle a suitable destination for several proteins susceptible to rapid turnover or showing a complex folding pathway (Nuttall et al., 2002; Faye et al., 2005). Several reports have documented the positive impact of ER retention on the production of recombinant antibodies in terms of protein stability, quality or yield (Schouten et al., 1996; Conrad and Fiedler, 1998; Stoger et al., 2002; Gomord et al., 2004). Similar tendencies have been observed for several other proteins of medical or industrial interest, including, as recent examples, human interleukin-4 (Ma et al., 2005b), the SARS coronavirus S protein antigen (Pogrebnyak et al., 2005), the synthetic silk-like protein DR1B (Yang et al., 2005) and a recombinant phytase from Aspergillus niger (Peng et al., 2006).

Despite these promising developments, the ER cannot be considered as a suitable destination for all proteins. To be stable or active, a number of clinically useful proteins require late post-translational modifications, such as the formation of complex glycans, the addition of a lipid moiety or the proteolytic removal of a propeptide sequence, which may occur downstream of the ER along the secretory pathway, notably in the Golgi, vacuole or apoplast (Gomord and Faye, 2004; Faye et al., 2005). Other proteins may exhibit an altered integrity or structural heterogeneity in the ER, as a result of unintended proteolytic processing by ER-resident proteases (Faye et al., 2005). The ER lumen is generally considered to be a mild environment for labile, e.g. immature, proteins, but proteolytic processing events altering the structure of secreted proteins have been observed in this compartment (Bass et al., 2000; Schmitz and Herzog, 2004). For instance, the bovine plasma protein aprotinin expressed in leaves of transgenic potato showed structural heterogeneity when accumulated in the ER, presumably as a result of the sequential removal of specific amino acids at the N- and C-termini by endogenous peptidases (Badri et al., 2005). A potential solution to prevent unintended proteolytic processing in the ER, or later along the secretory pathway (Sharp and Doran, 2001), is to identify and mutate susceptible amino acid sites by site-directed mutagenesis, as suggested by Outchkourov et al. (2003) for the sea anemone protein equistatin, processed to several truncated forms in the secretory pathway of transgenic potato leaf cells by arginine/lysine- and asparagine-specific cysteine proteases.

**Targeting to the apoplast**

Simply targeting the protein to an alternative compartment, even downstream in the cell secretory pathway, may also help to prevent unintended processing. For instance, recombinant bovine aprotinin targeted to the apoplast of potato leaf cells can be detected as a homogeneous, unprocessed form, similar in size to the native protein purified from bovine pancreas (Badri et al., 2008), in contrast with the proteolysis-related heterogeneity mentioned above for the same protein retained in the ER. The extracellular medium typically exhibits a high proteolytic content (Callis, 1995; Hellwig et al., 2004; Schiermeyer et al., 2005), but several recombinant proteins tagged for extracellular secretion have been produced successfully in planta or plant cell platforms over the last few
Targeting to the vacuole

Similar to the apoplastic medium, the vacuole represents a suitable accumulation site for several recombinant proteins, especially for production platforms relying on seed tissues (Stoger et al., 2005). The vacuole plays several important roles in planta, including the control of cell turgor, the turnover of macromolecules, the sequestration of toxic secondary metabolites and the storage of high-energy compounds in seeds or vegetative storage tissues (Marty, 1999). Recent evidence in the literature has suggested the occurrence of two distinct types of vacuole in plant cells: lytic (or vegetative) vacuoles, which present an acidic environment rich in hydrolytic enzymes; and protein storage vacuoles, which show a slightly acidic or neutral pH well adapted to protein storage (Robinson et al., 2005). Targeting to the vacuole, although not yet fully understood, is determined by small stretches of amino acids within the protein primary sequence acting as sorting signals to direct the maturing protein towards the vacuole (Neuhaus and Rogers, 1998; Mackenzie, 2005; Vitale and Hinz, 2005). In general, lytic vacuoles are not considered as a suitable destination for recombinant proteins in planta, owing to their high proteolytic content (Goulet and Michaud, 2006). By contrast, protein storage vacuoles present a milder environment compatible with protein accumulation (Stoger et al., 2005), especially in seeds, where they are most abundant (Müntz, 1998; Park et al., 2004).

Good accumulation levels have been reported for a number of recombinant proteins targeted to the vacuole (Table 1), including, for instance, the synthetic analogue of spider dragline silk protein DP1B (Yang et al., 2005), the heat-labile enterotoxin B from E. coli (Struefield et al., 2003), the toxic biotin-binding proteins avidin and streptavidin (Murray et al., 2002) and a thermostable β-glucanase of bacterial origin (Horvath et al., 2000). In practice, correct in situ localization of recombinant proteins bearing a sorting sequence for vacuolar targeting should be undertaken on a systematic basis, considering the species- or tissue-dependent functionality of some sorting signals (Vitale and Hinz, 2005). A good example of this phenomenon has been provided for a fungal phytase expressed in rice, which was readily detected in the apoplastic environment of leaf tissues, but retained in ER protein bodies and protein storage vacuoles in the seed endosperm (Drakakaki et al., 2006). As illustrated with the silk-like protein DP1B expressed in Arabidopsis (Yang et al., 2005), the impact of vacuolar targeting on the stability and yield of recombinant proteins is also tissue dependent. Although this protein was found at levels reaching 8% of TSP in seed storage vacuoles, no detectable levels of the same protein could be observed in leaf cell vacuoles. In a similar manner, targeting DP1B to the apoplast provided good yields in leaves, but poor yields in seeds (Yang et al., 2005), again stressing the need for an empirical case-by-case assessment of different tissue and cellular destinations for each protein expressed.

Targeting to the chloroplast

The chloroplast, peroxisome and nucleus have been proposed as other cellular destinations for protein production in plant platforms (Daniell et al., 2002; Hyunjong et al., 2006). In practice, recombinant proteins may be sent to these organelles by the inclusion of an appropriate targeting peptide (or localization signal) in the transgene sequence (Figure 1). For instance, the heat-labile toxin Lt-B from enterotoxigenic E. coli exhibited increased levels in corn grains when diverted from the cytosol to the nucleus by the addition of a nuclear localization signal from the simian virus 40 large T-antigen in the C-terminal position (Struefield et al., 2003). In a similar manner, a fungal xylanase useful in environment-related technologies showed high accumulation levels in Arabidopsis leaf tissues when sent to chloroplasts using the ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activase transit peptide, or sent to peroxisomes using the tripeptide targeting motif SKL (serine-lysine-leucine) grafted at the C-terminus (Hyunjong et al., 2006). Another example was the improved production of human growth hormone in N. benthamiana leaf cells when transferred from the cytosol to the chloroplast using the chloroplast transit peptide of rubisco small subunit (Gils et al., 2005).

Efficient procedures have also been devised to insert transgenes in the chloroplast genome, and then to regenerate transplastomic plant lines accumulating high levels of recombinant protein directly in the chloroplast stroma (Daniell et al., 2001; Maliga, 2002). Chloroplast transformation offers several advantages over nuclear transformation, including uniform transgene expression rates, multiple copies of the transgene in each cell, co-expression of multiple genes from the same construct, minimal gene silencing and minimal transgene escape in the environment owing to the maternal...
inclusion of chloroplast DNA in several species (Daniell et al., 2002). The chloroplast stroma supports protein post-translational modifications, such as multimerization and disulphide bridge formation (Daniell, 2006), making it a suitable environment for the expression of proteins not relying on complex modifications, such as glycosylation, typical of the cell secretory pathway. Several transplastomic plant lines have been engineered over the last 10 years for recombinant protein expression, providing very high yields for a number of useful proteins of prokaryotic and eukaryotic origin, including somatotropin, serum albumin, anthrax protective antigen, cholera toxin B subunit and tetanus toxin fragment C (Daniell et al., 2001, 2005; Tregoning et al., 2003).

However, a number of endogenous proteases are present in the chloroplast (Adam and Clarke, 2002), which can impair the overall stability and accumulation of recombinant proteins. An interesting example has been provided for the rotavirus VP6 protein, which showed high accumulation rates in chloroplasts of young tobacco leaves, but negligible rates in older leaves, despite comparable levels of mRNA transcripts (Birch-Machin et al., 2004). A similar decline in older tissues was observed for a fungal xylanase (Hyunjong et al., 2006) and for the insecticidal Bt toxin Cry2Aa2 (De Cosa et al., 2001), again stressing the importance of a careful assessment of the stability of each recombinant protein in different physiological or environmental contexts. Recombinant protein degradation by chloroplast proteases might appear, however, to be a non-relevant issue in terms of net production yields for some proteins expressed at very high levels (Daniell, 2006). The proteolysis-labile protein human serum albumin, for instance, was found at levels reaching 11% of TSP in transplastomic tobacco lines developed using chloroplast untranslated regions in gene constructs, in sharp contrast with levels below 0.02% of TSP in lines developed using the commonly employed Shine–Delgarno regulatory sequence (Fernandez-San Millan et al., 2003). This dramatic increase, probably the result of an increased expression rate of the transgene, was also associated with the formation of large inclusion bodies in planta (Fernandez-San Millan et al., 2003), which presumably sequestered the recombinant protein and prevented its hydrolysis in planta, as described earlier for a number of proteins expressed in heterologous environments (Enfors, 1992).

Stabilizing fusion partners

Together with strategies based on tissue-specific transgene expression or organelle-specific protein targeting, protein engineering approaches involving site-directed mutagenesis and/or protein domain grafting have been proposed to prevent recombinant protein hydrolysis in planta. Site-directed mutagenesis may prove to be useful, in particular, in improving the intrinsic stability of labile proteins in plant cells, as proposed previously for recombinant proteins expressed in microbial systems (Rozkov and Enfors, 2004; Macauley-Patrick et al., 2005). Any recombinant protein, depending on its structural characteristics, potentially includes protease-susceptible amino acid sites sterically accessible to endogenous proteases in the transgenic host organism. In theory, the identification of such susceptible sites in the polypeptide chain, together with a correct knowledge of the host proteolytic machinery, can provide useful information for the design of protein variants with improved stability in vivo (Rozkov et al., 2000).

To our knowledge, this approach has not yet been applied to recombinant proteins expressed in plants, but the characterization of specific proteolytic events implicating recombinant proteins in transgenic plants (e.g. Outchkourov et al., 2003; Badri, 2006) should provide, in forthcoming years, a workable framework for the rational engineering of protein variants with improved resistance to plant proteases. The design of structurally stabilized, thermostable protein variants integrating one or several ‘consensus’ (conserved) amino acids (Lehmann et al., 2002) may also prove to be interesting in the future, considering the strong positive correlation between the improved thermostability of such mutant proteins and their resistance to proteolysis (Amin et al., 2004).

When structural constraints in the protein do not allow for an alteration of protease-susceptible sites, or when a restrictive regulatory framework for therapeutic proteins dictates maximal identity between the recombinant protein and its original counterpart, expression of the protein attached to a protein-stabilizing fusion partner may prove to be useful to enhance its stability and/or folding, whilst keeping intact its original primary structure. Early studies with staphylococcal protein A and streptococcal albumin-binding protein demonstrated the potential of fusion partners as promoting agents for recombinant protein accumulation in vivo (Nygren et al., 1994; Stahl et al., 1997). In plants, several studies have been published describing the positive impact of protein fusion partners on the accumulation of recombinant proteins. Examples have been provided for the marker proteins β-glucuronidase and luciferase and, more recently, for the cholera toxin B subunit, which were accumulated at higher rates in transgenic potato or tobacco leaves when the regulatory protein ubiquitin was fused and transiently present at the N-terminus (Garbino et al., 1995; Hondred et al., 1999; Mishra et al., 2006). More recently, the human immunodeficiency virus (HIV) p24 core antigen expressed in
tobacco leaves showed an accumulation level increased by more than 10-fold when heavy chains of a human immunoglobulin A were fused at the C-terminus (Obregon et al., 2006). In a similar manner, the accumulation rates of spider silk proteins, single-chain antibody fragments, gp130 polypeptides and mammalian interleukins in transgenic tobacco lines were significantly improved when elastin-like polypeptides, including repeats of the elastin motif VPGXG (valine-proline-glycine), were fused at the C-terminus (Scheller et al., 2004, 2006; Lin et al., 2006; Patel et al., 2007). Although work is still required to devise efficient procedures for the proteolytic removal of fusion partners and to understand the mechanisms by which the different fusion partners exert their stabilizing effect, these observations clearly suggest the potential of fusion protein technologies for the improvement of recombinant protein yields in plants.

Companion protease inhibitors

Another strategy proposed recently to increase recombinant protein production in planta involves the use of transgenic hosts with reduced proteolytic capacities. In theory, protease processes affecting recombinant protein accumulation could be contained using antisense or RNA silencing strategies implemented in transgenic host plants (Watson et al., 2005). Alternatively, recombinant protease inhibitors active against specific endogenous proteases could prove functional to modulate proteolytic activities in situ (Faye et al., 2005). Recent evidence in the literature has suggested that the ectopic expression of protease inhibitors may have a positive impact on protein levels in leaves, with negligible effects on growth and development. The broad-spectrum inhibitor tomato cathespin D inhibitor (CDI), for instance, has been shown to yield increased TSP levels (by 20%–35%) in leaves of transgenic potato lines accumulating this inhibitor in the cytosolic compartment (Michaud et al., 2005). Likewise, the rice cysteine protease inhibitor oryzacystatin I led to total protein levels higher than expected in tobacco leaf tissues expressing this inhibitor in the cytosol (van der Vyver et al., 2003). The design of transgenic plant lines deficient in specific protease activities could now represent a challenging but worthwhile task, which could provide mild cellular environments tailored for the expression and accumulation of fragile proteins. A convincing demonstration supporting the potential of this approach was provided by Komarnytsky et al. (2006), who successfully stabilized recombinant antibodies secreted by the roots of transgenic tobacco plants also expressing (and co-secreting) a Bowman–Birk trypsin inhibitor from soybean.

Stabilizing recombinant proteins ex planta

Together with strategies aimed at preserving the integrity of recombinant proteins in planta, basic strategies have been devised over the last 10 years to protect recombinant proteins ex planta, at the time of extraction from plant tissues. While the avoidance of unwanted proteolysis in planta is essential to provide sufficient protein levels before harvesting, proteolysis control ex planta is essential to prevent protein loss in crude preparations. Protein extraction is often considered as a simple preliminary step for subsequent purification procedures, but it has a dramatic influence on the overall stability and quality of extracted proteins, and hence on the nature and abundance of proteins available for subsequent analysis, enrichment and purification (Michaud and Asselin, 1995). Simple basic precautions may be taken in the laboratory to prevent excessive proteolysis and to preserve recombinant protein integrity in plant samples and protein extracts (Michaud, 1998). Molecular and cellular approaches have also been proposed in recent years to minimize degradation ex planta, relying on the co-expression of protein-stabilizing protease inhibitors or the continuous secretion of recombinant protein products.

Basic precautions during protein extraction

From a practical viewpoint, plant cells are often seen as a ‘recalcitrant’ source of material for protein extraction, owing to their particular architecture and content (Saravanan and Rose, 2004; Carpentier et al., 2005). Compared with animal or microbial cells, plant cells from metabolically active tissues typically show a low protein content, and the vacuole(s), which account(s) for most of the cell volume, contain(s) highly reactive compounds potentially deleterious to proteins (Michaud and Asselin, 1995). During extraction, at least some protein loss is to be expected, given the occurrence of poorly soluble material interacting non-specifically with proteins in crude preparations, and the systematic release of protein denaturing agents, including phenolics and proteases, after cell disruption (Menkhaus et al., 2004; Saravanan and Rose, 2004). Although the onset of protein–protease interactions specific to each protein and expression platform will generally dictate the empirical development of adapted protection strategies during protein recovery, general guidelines have been proposed over the years to prevent protein loss in plant biomass and protein extracts (Jervis and Pierpoint, 1989; Michaud and Asselin, 1995; Michaud, 1998; Menkhaus et al., 2004; Schillberg et al., 2005). In summary, these guidelines recommend: (i) the processing of biomass shortly
after harvest for plant material retrieved from metabolically active tissues; and (ii) the adoption of laboratory practices preventing or at least minimizing protein loss and denaturation in crude extracts, including the processing of sample tissues at low temperatures, the addition of chelators and phenolic-neutralizing compounds in the extraction buffer, the use of low-molecular-weight protease inhibitors active against the host plant endogenous proteases and the use of pH conditions unfavourable to proteolytic activity in crude extracts (Michaud and Asselin, 1995). Protein extraction in acidic conditions, notably, may provide a simple method of preventing unwanted degradation of proteins extracted from several plant tissues, whilst also helping to avoid contamination of crude preparations with proteolytic fragments of larger proteins when low-molecular-weight proteins are to be isolated (Zhang et al., 2006). For instance, studies have illustrated the usefulness of trifluoroacetic acid for the effective isolation and enrichment of bioactive polypeptides from different sources in stable and active forms (Pearce et al., 2001; Huffaker et al., 2006; Yamaguchi et al., 2008).

Protein-stabilizing protease inhibitors

At the laboratory scale, protease inhibitors with complementary inhibitory functions may prove to be of particular interest for the rapid assessment of the inherent (in)stability of recombinant proteins challenged with the host plant’s resident proteases, or to retrieve the protein product in a recombinant proteins expressed in planta or green leaves (Figure 2).

Table 2 Low-molecular-weight protease inhibitors commonly used for protease characterization and protein stabilization in plant extracts*

| Inhibitor      | Molecular weight (Da) | Target protease          |
|----------------|-----------------------|--------------------------|
| AEBSF          | 240                   | Serine                   |
| Amastatin      | 475                   | Metallo-(aminopeptidases)|
| Antipain       | 605                   | Serine (trypsin-like), cysteine |
| APMSF          | 216                   | Serine (trypsin-like)    |
| Bestatin       | 308                   | Metallo-(aminopeptidases)|
| Chymostatin    | 605                   | Serine (chymotrypsin-like), cysteine |
| 3,4-DCI        | 215                   | Serine                   |
| Diprotin A     | 359                   | Metallo-(aminopeptidases)|
| Diprotin B     | 327                   | Metallo-(aminopeptidases)|
| E-64           | 357                   | Cysteine                 |
| EDTA           | 372                   | Metallo-(chelating agent)|
| Elastatininal   | 513                   | Serine (elastase-like)   |
| Iodoacetamide  | 185                   | Cysteine (may interfere with other enzymes) |
| Leupeptin      | 427                   | Serine (trypsin-like), cysteine |
| Pepstatin      | 686                   | Aspartate                |
| 1,10-Phenanthrolnine | 198         | Metallo-(chelating agent)|
| PMSF           | 174                   | Serine, cysteine in non-reducing conditions |
| TLCK           | 333                   | Serine (trypsin-like)    |
| TPCK           | 352                   | Serine (chymotrypsin-like) |
| Z-Phe-Ala-CHN₂ | 395                   | Cysteine                 |

*See Michaud et al. (1998) for details on stock solutions and working concentrations.

Figure 2 Stabilization of exogenous proteins in plant crude protein extracts by phenylmethylsulphonyl fluoride (PMSF) and chymostatin (CHY). The example is human fibronectin (hFb) challenged with alfalfa leaf proteases. hFb was first incubated for 10 min at 20 °C with major protease fractions of alfalfa leaf proteases (P1 or P2) in the presence or absence of 0.1 mM PMSF or 10 μM CHY, and then immunodetected using appropriate anti-hFb polyclonal antibodies. CTL, control extract with no inhibitor added.

For large-scale set-ups, protein protease inhibitors co-expressed in planta with the recombinant protein of interest may represent an effective alternative to commercial low-molecular-weight inhibitors (Rivard et al., 2006). Protein
protection with exogenous protease inhibitors, which relies on expensive and often toxic compounds, appears to be useful for diagnostic purposes or small-scale production scenarios, but is not applicable to large-scale production schemes involving the extraction and downstream processing of large biomass samples. By contrast, in-built companion inhibitors co-expressed in the host plant are ‘included’ de facto in protein extracts after tissue grinding, and then act as ‘mouse traps’ against the proteases present to reproduce the protein-stabilizing effects of exogenous chemical inhibitors (Benchabane et al., 2008). Broad-spectrum protease inhibitors, such as the serine-type inhibitors tomato CDI and bovine aprotinin, appear to be well suited as such effective companion inhibitors, as illustrated by the strong stabilization of the marker protein neomycin phosphotransferase II released in leaf crude extracts of tomato CDI- and bovine aprotinin-expressing potato plants (Rivard et al., 2006). Inhibitors of cysteine proteases, such as cystatins (Girard et al., 2007), or hybrid fusion inhibitors integrating dual (or multiple) inhibitory functions active against cysteine, serine and/or aspartate proteases (e.g. Urwin et al., 1998; Brunelle et al., 2005) also represent interesting candidates, given the general occurrence of serine, cysteine and aspartate protease activities sensitive to such inhibitors in plant crude extracts (Rivard et al., 2006, 2007; Goulet et al., 2008).

Continuous secretion and recovery

An alternative to protease inhibitors for the avoidance of extraction-related protein degradation involves skipping this processing step by taking advantage of cellular and tissue secretion processes occurring naturally in plants. In practice, this strategy involves the targeting of recombinant proteins to the extracellular medium using appropriate signal peptides for extracellular secretion, followed by recovery of the extracellular proteins in secreted fluids or culture media. Rhizosecretion and leaf guttation represent two such processes allowing for the non-destructive secretion and extraction-free recovery of recombinant proteins (Komarnytsky et al., 2000; Gaume et al., 2003). Rhizosecretion, notably, has been exploited recently for the heterologous expression of several recombinant proteins, including human alkaline phosphatase (Gaume et al., 2003), hepatitis B surface antigen (Kumar et al., 2006) and immunoglobulin G antibodies (Komarnytsky et al., 2006). This approach is based on the ability of hairy roots to secrete proteins in hydroponic media, which can be recovered easily and used as simple source material for protein enrichment and purification (Komarnytsky et al., 2004). Interestingly, rhizosecretion, and hence recombinant protein release, can be significantly boosted by the initiation of hairy roots following treatment with Agrobacterium rhizogenes (Gaume et al., 2003), thus providing an efficient source of recombinant protein throughout the host plant’s life.

Plant cell cultures have also been proposed for the continuous secretion of recombinant proteins in culture media (Hellwig et al., 2004). Compared with transgenic plant lines, cultured cells offer a high level of containment, and represent a useful tool for designing simple recovery and purification strategies from source media containing low levels of contaminants. However, some recombinant proteins may undergo extensive hydrolysis in culture media, owing to the secretion of proteolytic enzymes by growing cells (Lee et al., 2002; Kwon et al., 2003; Schiermeyer et al., 2005; Ganapathi et al., 2007). The extracellular concentration of human granulocyte–macrophage colony-stimulating factor, for instance, reached a maximum in tobacco cell cultures at the mid-exponential phase, but then decreased rapidly in the culture medium, together with a dramatic increase in protease activity in the culture medium (Lee et al., 2002). In a similar manner, accumulation of the fibrinolytic protease α1 plasminogen activator from Desmodus rotundus was dramatically hampered in the culture medium of BY-2 tobacco cells, presumably as a result of extensive degradation by secreted metalloproteases (Schiermeyer et al., 2005). The addition of protein-stabilizing agents, such as low-molecular-weight protease inhibitors or polymers (e.g. gelatin, albumin, polyethylene glycol or polyvinylpyrrolidone), to the culture medium has been proposed to prevent protein loss in plant cell cultures (Bateman et al., 1997; LaCount et al., 1997; Tsoi and Doran, 2002; Schiermeyer et al., 2005; Soderquist and Lee, 2005; Ganapathi et al., 2007). The co-secretion of recombinant protease inhibitors hindering endogenous protease activities along the cell secretory pathway or in the culture medium (Komarnytsky et al., 2006; Kim et al., 2007), or the development of protease-deficient transgenic plant cells lacking specific, potentially damaging secreted proteases (Schiermeyer et al., 2005), could represent interesting alternatives in the future, allowing recombinant proteins secreted in culture media (or natural secretion fluids) to be stabilized in situ, without the need to use exogenous protein-stabilizing chemicals.

Conclusion

In summary, major advances have been made over the last decade to improve the post-translational stability and accumulation of recombinant proteins in plant systems. The use of tissue-specific promoters or the addition of subcellular targeting peptide signals to the nascent protein, in particular,
have been proven to be useful in increasing the stability of several proteins in planta. Complementary approaches relying on protein-stabilizing fusion partners, companion protease inhibitors or natural secretion processes have also been proposed recently to protect recombinant proteins, either in planta during the production stage or ex planta at the time of extraction (or secretion). Although a case-by-case exploratory assessment involving the testing of different strategies may always remain necessary given the individual characteristics of each protein being expressed or plant platform being exploited, the fine tuning of molecular tools and strategies for recombinant protein stabilization should be made easier in the near future, together with our growing understanding of proteolytic and protein maturation processes in plants. Rapid developments in structural biology and protein engineering should also contribute to the biotechnologist’s toolbox in forthcoming years, and help us to face the challenge of recalcitrant protein production.

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