

Review article

Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants

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Summary

For the past two decades, therapeutic and industrially important proteins have been expressed in plants with varying levels of success. The two major challenges hindering the economical production of plant-made recombinant proteins include inadequate accumulation levels and the lack of efficient purification methods. To address these limitations, several fusion protein strategies have been recently developed to significantly enhance the production yield of plant-made recombinant proteins, while simultaneously assisting in their subsequent purification. Elastin-like polypeptides are thermally responsive biopolymers composed of a repeating pentapeptide 'VPGXG' sequence that are valuable for the purification of recombinant proteins. Hydrophobins are small fungal proteins capable of altering the hydrophobicity of their respective fusion partner, thus enabling efficient purification by surfactant-based aqueous two-phase systems. Zera, a domain of the maize seed storage protein γ -zein, can induce the formation of protein storage bodies, thus facilitating the recovery of fused proteins using density-based separation methods. These three novel protein fusion systems have also been shown to enhance the accumulation of a range of different recombinant proteins, while concurrently inducing the formation of protein bodies. The packing of these fusion proteins into protein bodies may exclude the recombinant protein from normal physiological turnover. Furthermore, these systems allow for quick, simple and inexpensive nonchromatographic purification of the recombinant protein, which can be scaled up to industrial levels of protein production. This review will focus on the similarities and differences of these artificial storage organelles, their biogenesis and their implication for the production of recombinant proteins in plants and their subsequent purification.

Keywords: molecular farming, recombinant protein purification, elastin-like polypeptides, hydrophobins, zein, protein bodies.

Introduction

The demand for recombinant proteins for medical and industrial use is expanding rapidly and transgenic plants are now recognized as a safe, efficient and inexpensive means of their production. Plants also offer other advantages over conventional expression systems such as microbial and yeast fermentation or insect and mammalian cell cultures (Ma *et al.*, 2003). These advantages include rapid

scalability, the absence of human pathogens, the ability to correctly fold and assemble complex multimeric proteins, and the potential for direct oral administration of unprocessed or partially processed plant material (Fischer *et al.*, 2004). Transgenic plants have shown promise over the past 20 years as bioreactors for the large-scale production of various recombinant proteins, such as vaccines, antibodies, biopharmaceuticals and industrial enzymes (Giddings *et al.*, 2000; Ma *et al.*, 2005).

Although a wide range of plant host systems have been developed, tobacco has the most established history for the production of recombinant proteins because it is readily amenable to genetic engineering and has many desirable agronomic attributes, such as high biomass yields (more than 100 000 kg/hectare) and high soluble protein levels (Sheen, 1983). Furthermore, the tobacco expression platform is based on leaves, removing the need for flowering and thus significantly reducing the possibility of gene leakage into the environment through pollen or seed dispersal (Rymerson *et al.*, 2002; Twyman *et al.*, 2003). Most importantly, tobacco is a nonfood, nonfeed crop, which minimizes regulatory barriers by eliminating the risk of plant-made recombinant proteins entering the food supply (Menassa *et al.*, 2001).

However, two major challenges still limiting the economical production of plant-made recombinant proteins include inadequate accumulation levels and the lack of efficient purification methods. The low-production yield of many recombinant proteins continues to be the most challenging problem limiting the commercial exploitation of transgenic plant expression systems (Doran, 2006). The inherent instability of foreign proteins expressed in a heterologous environment and their increased susceptibility to intracellular degradation processes are probably the most important factors responsible for the low accumulation of certain recombinant proteins in tobacco. In particular, proteolytic degradation is a major problem in the aqueous environment of leafy crops (Enfors, 1992; Benchabane *et al.*, 2008). As well, one disadvantage of using tobacco as an expression host is the presence of phenolics and toxic alkaloids, which may limit tobacco's therapeutic applications and preclude it from oral delivery. Thus, purification of the target protein may be required prior to administration (Menkhaus *et al.*, 2004) to eliminate any toxic components and to satisfy product consistency and formulation standards. However, the complex plant proteome and the typical low yield of plant-made recombinant proteins in stable transgenic plants, usually <1% of total soluble protein (TSP) (Joensuu *et al.*, 2008), complicate the purification scheme, which can contribute to >80% of the product cost (Kusnadi *et al.*, 1997).

Fusion proteins have been developed with many diverse functions, but they are generally used to increase recombinant protein accumulation in heterologous expression systems or to assist in their subsequent purification. In the event that the fusion tag alters the biological activity of the target protein, removal of the tag may be required during downstream processing (Terpe, 2003). In plants,

several studies have shown that expressing recombinant proteins as fusions to protein-stabilizing partners can have a positive impact on their accumulation. For example, the use of fusion proteins, such as ubiquitin (Garbarino *et al.*, 1995; Hondred *et al.*, 1999; Mishra *et al.*, 2006), β -glucuronidase (Gil *et al.*, 2001; Dus Santos *et al.*, 2002), cholera toxin B subunit (Arakawa *et al.*, 2001; Kim *et al.*, 2004; Molina *et al.*, 2004), viral coat proteins (Canizares *et al.*, 2005) and human immunoglobulin (IgG) α -chains (Obregon *et al.*, 2006), are common approaches for enhancing recombinant protein accumulation in plants. To simplify purification, recombinant proteins are also often fused translationally to small affinity tags or proteins with defined binding characteristics (Streatfield, 2007). A non-exhaustive list of fusions commonly used for the purification of recombinant proteins include: Arg-tag, His-tag, FLAG-tag, c-myc-tag, glutathione S-transferase-tag, calmodulin-binding peptide, maltose-binding protein, and the cellulose-binding domain (Terpe, 2003; Lichty *et al.*, 2005; Rubio *et al.*, 2005). More recently, an eight-amino acid StrepII epitope tag was developed (Skerra and Schmidt, 2000) and shown to be an easy and fast means of purifying recombinant proteins from plants (Witte *et al.*, 2004), while providing an acceptable compromise of excellent purification with good yields at moderate cost (Lichty *et al.*, 2005). However, all of these tags have been developed to facilitate the purification of recombinant proteins using affinity chromatography techniques, which are costly and difficult to scale-up to industrial levels of protein purification (Menkhaus *et al.*, 2004; Waugh, 2005).

An alternative method specific to plants is the oleosin fusion where the recombinant protein accumulates in seed oil bodies and can be purified using a simple extraction and centrifugation procedure followed by release of the target protein by proteolytic cleavage (Parmenter *et al.*, 1995; van Rooijen and Moloney, 1995; Boothe *et al.*, 2010). However, oleosins accumulate only in seed and their expression levels are still not high enough for economical production. In addition, leafy crops can not be used with this technology. More recently, the oleosin technology has been adapted for the affinity capture of recombinant antibodies through the expression of oleosin-protein A fusions on the surface of oil bodies (Moloney *et al.*, 2008). Thus, the oleosin technology seems to be poised to serve for the purification of specific proteins, rather than a tool for high-level expression of recombinant proteins. Therefore, there is still a need for a unique strategy allowing further improvements for both the accumulation of recombinant proteins and their purification from plants.

This review focuses on three proteins that have been used as fusion partners and have resulted in the accumulation of the recombinant protein in novel protein bodies. The three proteins are of very distinct origins: zein is a plant protein, elastin is an animal protein, and hydrophobin is a fungal protein. However, all three proteins share several physico-chemical characteristics which likely cause this unique phenotype and allow for a significant increase in recombinant protein accumulation, while also assisting in the purification of the target protein.

Zera/zeolin fusions

Seeds provide an attractive alternative to conventional large-scale recombinant protein expression systems because they can produce relatively high heterologous protein yields in a stable, compact environment for long periods of time, assisting in storage, handling and transport of the transgenic product (Stoger *et al.*, 2005). Compared with other eukaryotes, plants are unique in their ability to naturally store large reservoirs of protein in specialized endoplasmic reticulum (ER)-derived compartments in developing seeds (Galili, 2004). Prolamins are the most predominant class of seed storage proteins found in most cereals, such as maize, rice and wheat (Arcalis *et al.*, 2004). In developing maize endosperm cells, zeins (α -, β - and γ -zeins) are imported and retained in the ER despite the absence of an H/KDEL ER localization signal (Geli *et al.*, 1994; Kogan *et al.*, 2002). Although the sequestration mechanisms are not well understood, maize prolamin seed storage proteins are synthesized on the rough ER and deposited as large, dense accretions known as protein bodies (PBs) (Larkins *et al.*, 1979; Torrent *et al.*, 1986; Bagga *et al.*, 1997; Pompa and Vitale, 2006). In general, prolamins contain proline-rich domains and are alcohol-soluble, reflecting their general hydrophobic nature (Herman and Larkins, 1999). γ -Zein, a prolamin and the major constituent of maize storage proteins, is a sulphur-rich prolamin that is soluble in aqueous solutions in the presence of a reducing agent. γ -Zein is able to induce the formation of ER-derived PBs in seed and in vegetative tissues of transgenic dicots in the absence of other zein subunits (Geli *et al.*, 1994; Coleman *et al.*, 1996). Two domains within γ -zein confer its ability to be retained in the ER and to assemble into PBs: a highly repetitive proline-rich sequence (PPPVHL)₈ and a Pro-X motif. The repetitive proline-rich sequence adopts an amphipathic helical conformation, which is able to self-assemble and may be

responsible for this protein's ability to be retained in the ER (Geli *et al.*, 1994; Kogan *et al.*, 2001). More recently, a detailed study of the N-terminal γ -zein domains showed that the two N-terminal Cys residues are critical for oligomerization, the first step towards PB formation in *Nicotiana benthamiana* (Llop-Tous *et al.*, 2010).

While zeins (rich in sulphur amino acids, poor in lysine and tryptophan) accumulate naturally in ER-derived PBs in maize endosperm, phaseolin (poor in sulphur amino acids, rich in lysine) accumulates in storage vacuoles of legume seeds (Chrispeels, 1983). Zeolin, a chimeric protein derived from the N-terminal half of maize γ -zein and the whole bean vacuolar phaseolin seed storage protein, contains 6 cysteine and 25 lysine residues, making it more nutritionally balanced than the parent molecules. Zeolin was expressed in tobacco leaves and was shown to accumulate in ER-derived PBs, similar to γ -zein, and to reach accumulation levels of 3.5% TSP (Mainieri *et al.*, 2004). Thus, the localization of the γ -zein moiety appears to be dominant over that of phaseolin. In addition, disulphide bond formation in the γ -zein portion of the protein is essential for the formation of PBs (Pompa and Vitale, 2006).

The ability of γ -zein and zeolin to induce PB formation in leaf cells was extended in various studies as a strategy for increasing the accumulation of high-value recombinant proteins in plants. In those studies, the components responsible for stable seed protein storage in PBs were combined with the inherently biosafe and high biomass-yielding leaf-based tobacco expression platform. Fusing the N-terminal domain of γ -zein (Zera[®]; developed by ERA Biotech, Barcelona, Spain) to other proteins resulted in the formation of PBs in several other eukaryotic systems (termed StorPro organelles <http://www.erabiotech.com>; (Ludevid Mugica *et al.*, 2007; Ludevid Mugica *et al.*, 2009; Saito *et al.*, 2009; Torrent *et al.*, 2009a). Zera-fused enhanced cyan fluorescent protein (eCFP), calcitonin (Ct), human growth hormone (hGH) and epidermal growth factor (EGF) formed PB-like structures in the leaves of both transiently and stably transformed tobacco plants, with accumulation levels of the fused recombinant proteins increasing by about 100-fold for EGF [up to 0.5 g/kg fresh weight (FW)] and 13-fold for hGH (up to 3.2 g/kg FW) (Torrent *et al.*, 2009a). Similarly, the *Yersinia pestis* F1-V antigen fused to Zera accumulated in PBs up to fivefold higher in *N. benthamiana* leaves, alfalfa leaves and NT1 tobacco cell suspensions than when expressed alone, up to 0.9% TSP in NT1 calli (Alvarez *et al.*, 2010). As well, both γ -zein and zeolin fusions were compared when expressing the highly unstable human immunodeficiency

virus antigen Nef. Although the γ -zein fusion was degraded by ER quality control, the zeolin fusion formed small PBs (<0.5 μ m in diameter) and allowed the accumulation of zeolin-Nef to reach 1.5% TSP in stably transformed tobacco plants (de Virgilio *et al.*, 2008). It was hypothesized that the reason for the failure of γ -zein-Nef to accumulate into PBs might be attributable to the inability of γ -zein to divert a structurally defective polypeptide from degradation, while the zeolin-Nef fusion was not recognized as a defective protein (de Virgilio *et al.*, 2008). It is also possible that the phaseolin portion of zeolin may contribute to PB formation in a manner complementary to γ -zein.

Because Zera and zeolin accumulate in dense PBs, simple isopycnic sucrose density centrifugation can be used to enrich the fusion protein up to 85% in a single step. Both Zera and zeolin PBs settle at a density around 1.25 g/mL (Mainieri *et al.*, 2004; Torrent *et al.*, 2009a,b). However, this technique is difficult to scale-up, and the company ERA Biotech has developed a low-speed direct centrifugation method for substantially concentrating the PBs prior to further purification of Zera fusions (Torrent *et al.*, 2009a).

Thus far, the biological properties of Zera- or zeolin-fused proteins have not been reported, but it is anticipated that proteins not requiring complex glycosylation typically acquired in the Golgi or other post-translational modifications not occurring in the ER should be functional.

Elastin-like polypeptides

Elastin-like polypeptides (ELPs) are synthetic biopolymers composed of the repeating pentapeptide sequence 'VPGXG', where the guest residue X can be any amino acid except proline (Urry, 1988). These repetitive sequences occur in all mammalian elastin proteins (Raju and Anwar, 1987). In an aqueous solution, ELPs undergo a reversible inverse phase transition from soluble protein into insoluble hydrophobic aggregates that form β -spiral structures when heated above their transition temperature (T_t) (Urry, 1997). This thermally responsive property of ELP is also transferred to fusion partners, enabling a simple, rapid, scalable and inexpensive nonchromatographic method for protein purification called 'inverse transition cycling' (ITC) (Meyer and Chilkoti, 1999). The T_t of an ELP fusion varies with the sequence, size and concentration of ELP, as well as the surface hydrophobicity of the fusion partner and the ionic strength of the aqueous solution

(Urry *et al.*, 1991; Meyer and Chilkoti, 2004; Trabbic-Carlson *et al.*, 2004b).

Although ITC has been used to purify cytokines (Lin *et al.*, 2006), antibodies (Floss *et al.*, 2009; Joensuu *et al.*, 2009), and spider silk proteins (Scheller *et al.*, 2004) from transgenic plants, very little work had been done to optimize ELP tag size for protein yield and recovery. More recently, various sizes of ELP tags were fused to green fluorescent protein (GFP), interleukin-10 (IL-10), erythropoietin and a single chain antibody fragment (scFv) and then transiently expressed in tobacco leaves. In this study, ELP size was shown to have a significant impact on the purification efficiency of ELP fusion proteins (Conley *et al.*, 2009a). Regardless of the recombinant protein fusion partner, a minimum number of 20 pentapeptide repeats was generally necessary for the ELP tag to selectively enrich for the protein of interest in the resolubilized ITC pellet, relative to the original soluble leaf extract. In general, relatively large ELP tags (i.e. 80–160 pentapeptides) resulted in the highest recovery rate of target protein following ITC (Conley *et al.*, 2009a).

Under optimized ITC purification conditions, recovery rates of 70%–95% have been obtained with bacterial expression systems (Shimazu *et al.*, 2003; Trabbic-Carlson *et al.*, 2004a; Ge and Filipe, 2006), whereas recovery rates of 30%–60% are typically observed for plant expression systems (Conley *et al.*, 2009a; Joensuu *et al.*, 2009). The reduced recovery rates obtained for plant-made recombinant proteins is attributable partly to their much lower expression levels in stable transgenic plants (i.e. 0.01%–0.8% TSP) relative to bacteria (i.e. 0.1–1.6 g/L) (Meyer *et al.*, 2001; Trabbic-Carlson *et al.*, 2004b; Chow *et al.*, 2006), and partly to suboptimal conditions used in the reported purifications even at high levels (20% TSP) in transient expression experiments (Conley *et al.*, 2009a). Since the purity and recovery efficiency is rather low when using ITC for the purification of plant-made proteins that accumulate to low levels, expensive and tedious affinity chromatography steps may still be needed for these cases.

In addition to functioning as a method of purification, ELP fusions have also been shown to significantly enhance the accumulation of a range of different recombinant proteins in plants. For example, ELP fusions increase the concentration of human IL-10 and murine interleukin-4 (Patel *et al.*, 2007), spider silk proteins (Scheller *et al.*, 2004; Patel *et al.*, 2007), the full-size anti-human immunodeficiency virus type 1 antibodies 2F5 (Floss *et al.*, 2008) and 2G12 (Floss *et al.*, 2009) and a scFv antibody (Joensuu

et al., 2009) in tobacco leaves, and scFv antibodies in tobacco seeds (Scheller *et al.*, 2006). In general, ELP fusions increased the production yield of these target proteins by 2- to 100-fold. However, in some cases, the presence of an ELP fusion has been unable to positively affect the concentration of certain recombinant proteins in plants (Conley *et al.*, 2009a,c). Thus, the beneficial effect of ELP on recombinant protein accumulation is likely protein specific.

In a study evaluating the general utility of ELP fusions in various plant intracellular compartments, the presence of an ELP fusion tag had a negligible effect on the concentration of GFP in the cytoplasm and apoplast, whereas it decreased the accumulation of GFP in the chloroplasts. The ER was the only intracellular compartment in which an ELP tag was shown to significantly enhance recombinant protein accumulation (Conley *et al.*, 2009b).

As only two C-terminal ELP fusion tags (i.e. a 28- and a 100-pentapeptide tag) had been utilized in plants (Scheller *et al.*, 2004; Patel *et al.*, 2007), a recent study thoroughly examined the effect of fusion orientation and ELP size on the accumulation of four different recombinant proteins (Conley *et al.*, 2009a). The C-terminal orientation of ELP fusion tag produced twice as much target protein compared to the N-terminal ELP tag (Conley *et al.*, 2009a), which was also observed in a similar study investigating bacterial-produced ELP fusion proteins (Christensen *et al.*, 2009). Across all sizes of ELP tags, there was a dramatic effect on the level of recombinant protein accumulation observed, with the range of concentrations for GFP, IL10, erythropoietin and scFv varying by 60, 550, 50, and 450 times, respectively. The accumulation of recombinant protein is inversely proportional to the size of ELP tag with small ELP tags (i.e. 5–40 pentapeptides) providing the highest yield of recombinant proteins in plant leaves (Conley *et al.*, 2009a). In *E. coli*, Meyer *et al.* (2001) also showed that decreasing the ELP length from 90 to 20 pentapeptides increased the expression yield of a thioredoxin-ELP fusion protein by fourfold. Moreover, large ELP tags make up a greater proportion of the total recombinant protein fusion, thus reducing the amount of actual target protein attained.

Elastin-like polypeptides are thought to increase the stability of recombinant proteins because they are not susceptible to hydrolysis (Raucher and Chilkoti, 2001) or protease cleavage (Zhang *et al.*, 1996), thus reducing the level of protein degradation. ELP tags have also been shown to increase the solubility of target proteins by protecting against irreversible aggregation and denaturation

at high protein concentrations (Trabicc-Carlson *et al.*, 2004a). In addition, aggregated ELP fusion proteins are more stable relative to the soluble fusion protein, allowing for better long-term storage and application of the protein (Shimazu *et al.*, 2003; Shamji *et al.*, 2007). More recently, it was shown that ELP fusions accumulate in PBs in the leaves and seeds of tobacco (Conley *et al.*, 2009b; Floss *et al.*, 2009). It is hypothesized that PBs protect the protein from proteolytic degradation while protecting the cell from the toxic effects associated with high foreign protein accumulation.

Importantly, the effect of ELP fusion tags on the biological function of recombinant proteins has been investigated in numerous studies. In general, ELP fusions do not negatively impact the functionality of the target protein, which demonstrates the inert nature of these tags (Trabicc-Carlson *et al.*, 2004a; Scheller *et al.*, 2006; Shamji *et al.*, 2007; Floss *et al.*, 2008, 2009; Conley *et al.*, 2009c). As well, the bioactivity of target protein-ELP fusions is typically preserved after ITC purification (Meyer and Chilkoti, 1999; Shimazu *et al.*, 2003; Floss *et al.*, 2009). These are important steps towards demonstrating that ELP will not modify the functionality of recombinant proteins needed for clinical or industrial applications. On the other hand, other studies have shown that ELPs may slightly alter the activity of certain fusion proteins (Meyer and Chilkoti, 1999; Shimazu *et al.*, 2003; Joensuu *et al.*, 2009).

From the literature, it can be generally concluded that C-terminal ELP fusion tags are most advantageous for increasing the concentration of target recombinant proteins without affecting their biological activity. In addition, ELP tags with approximately 30 pentapeptide repeats provide the best compromise between the positive effects of small ELP tags ($n = 5\text{--}40$) on recombinant protein accumulation and the beneficial effects of larger ELP tags ($n = 80\text{--}160$) on recombinant protein recovery during ITC purification. ELP fusion technology provides a general method for enhancing the yield of recombinant proteins in plants, while also providing a simple and scalable means for their subsequent purification.

Hydrophobins

Hydrophobins are a class of small (ca. 10 kDa) surface-active proteins that are produced by filamentous fungi. In fungi, hydrophobins can be secreted out of the cell or can remain intracellular. Their biological function is involved in the adaptation of fungi to their environment by controlling interfacial forces. A distinct feature in the structure of

hydrophobins is that one part of the protein's surface is occupied by hydrophobic aliphatic side chains, forming an exposed 'hydrophobic patch' on one end of the protein. This is notable because hydrophobic side chains are usually buried in the core, which stabilizes the protein fold. The residues forming this 'hydrophobic patch' are conserved among hydrophobins indicating an important functional role. Instead of a core stabilized by hydrophobic interactions, hydrophobins share a characteristic pattern of eight conserved cysteine residues, forming four intramolecular disulphide bridges (Hakanpää *et al.*, 2004). The amphipathic appearance of hydrophobins closely resembles the common structure and behaviour of surface-active molecules with one hydrophobic and one hydrophilic part, only the size and structural detail being distinct from typical surfactants. Because of these properties, hydrophobins possess a propensity to self-assemble into an amphipathic protein membrane at hydrophilic–hydrophobic interfaces (Wösten and de Vocht, 2000; Paananen *et al.*, 2003; Wang *et al.*, 2005). Hydrophobins have numerous potential applications, which include the ability to interface proteins with nonbiological surfaces, to alter the wettability of different materials, to act as biosurfactants and oil stabilizers and to form medical and technical coatings. Also, the unique surface absorption and self-assembly of hydrophobins have potential uses in the field of nanotechnology (Wessels, 1997; Askolin *et al.*, 2001; Linder *et al.*, 2005; Valo *et al.*, 2010).

As a result of their unique surface-active properties, hydrophobins are also capable of altering the hydrophobicity of their respective fusion partner, thus enabling efficient purification using a surfactant-based aqueous two-phase system (ATPS) (Linder *et al.*, 2004). In an ATPS, a surfactant is added to crude protein extracts which concentrates the hydrophobin fusions inside micellar structures and separates them towards the surfactant phase while the majority of the proteins remain in the aqueous phase (Figure 1). The fusion protein can be then easily back-extracted from the surfactant phase with nondenaturing organic solvents, such as isobutanol (Linder *et al.*, 2001).

Aqueous two-phase systems offer several benefits since they are simple, rapid and inexpensive while providing volume reduction, high capacity, and fast separations (Persson *et al.*, 1999). Most importantly, a one-step ATPS is particularly attractive because it can be easily and effectively scaled up for industrial-scale protein purification (Linder *et al.*, 2004; Selber *et al.*, 2004). In general, proteins are not prone to denaturation in ATPSs, or in the following back-extraction processes. However, this may be a

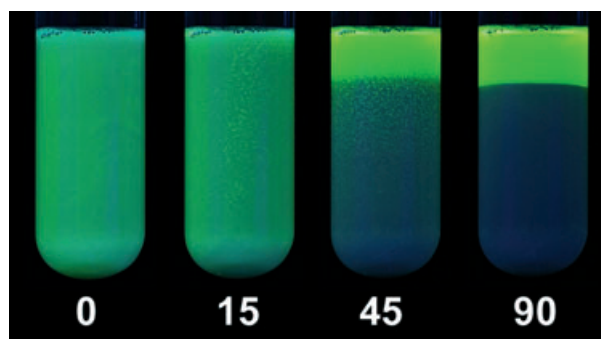


Figure 1 Purification of green fluorescent protein (GFP)-HFBI fusion protein from plant leaf extract by an aqueous two-phase system. Four consecutive images represent leaf extract supplemented with surfactant and imaged under ultraviolet light over the course of 90 min. The GFP-HFBI fusion separates from the protein extract and concentrates to the upper surfactant phase by micelle formation, while the majority of endogenous plant proteins remain in the lower aqueous phase.

concern and require optimization for some labile target proteins (Linder *et al.*, 2004).

Hydrophobin fusion technology was originally developed for purifying proteins from fungal culture supernatants having a simple composition and a high concentration of secreted proteins (Linder *et al.*, 2001, 2002, 2004; Selber *et al.*, 2004). However, the utility of this ATPS technology was also demonstrated for recovering hydrophobin fusions from insect and plant cell extracts (Lahtinen *et al.*, 2008; Joensuu *et al.*, 2010). Laborious and expensive purification of recombinant proteins from complex leaf extracts often hinders the feasibility of molecular farming applications. Recently, the hydrophobin I (HFBI) from *Trichoderma reesei* was expressed as a fusion with GFP to facilitate its recovery from plant leaf extracts (Joensuu *et al.*, 2010). This study demonstrated that an ATPS was able to selectively recover up to 91% of the GFP-HFBI, up to concentrations of 10 mg/mL. Furthermore, the functionality of this purification procedure was demonstrated when only small amounts (0.1% of TSP) of the fusion protein is present in the leaf extract, also making it feasible for the purification of hard-to-express, but high-value protein pharmaceuticals. Similarly, the HFBI fusion technology worked efficiently for the purification of the enzyme glucose oxidase (GOx; catalyses the oxidation of D-glucose to D-gluconolactone and hydrogen peroxide) from leaf extracts (Joensuu *et al.*, 2010).

It has been demonstrated that hydrophobin fusions can increase the accumulation of target proteins in plants and fungi (Linder *et al.*, 2004; Joensuu *et al.*, 2010). Transient expression of GFP in *N. benthamiana* as a fusion with HFBI

increased GFP accumulation by twofold from 18% to 38% TSP, which is equivalent to 3.7 mg of the target protein per gram of leaf FW. Similar high expression levels were also obtained with GOx (Joensuu *et al.*, 2010), an enzyme that has been challenging to overexpress in an active form in other more conventional expression systems (Bankar *et al.*, 2009).

Although targeting recombinant proteins to the ER often enhances their accumulation in plants (Schouten *et al.*, 1996; Fiedler *et al.*, 1997; Ramirez *et al.*, 2002; Conley *et al.*, 2009c), the overexpression of particular proteins in the ER tends to be harmful, especially with the transient expression systems, which often lead to higher accumulation of target proteins than in stable transgenic plants. For example, strong overexpression of GFP in agro-infiltrated *N. benthamiana* leaves leads to the formation of necrotic lesions, starting at 4 days postinfiltration (dpi). However, when GFP was expressed as a fusion with HFBI, the infiltrated leaves remained fairly healthy up to 10 dpi (Joensuu *et al.*, 2010). Therefore, GFP-HFBI accumulation continued to increase relative to the endogenous plant proteins, allowing for simpler downstream purification processes. This tissue-protective effect is probably due to the accumulation of the fusion protein in PBs, similar to those observed with ELP. This reduced toxicity still needs to be further studied with other model proteins, but it holds promise to solve one of the issues that has been hindering the development of transient expression platforms. The hydrophobin fusion technology might be especially suitable for hard-to-express and toxic protein candidates.

Although it needs to be tested on a case-by-case basis, the presence of a hydrophobin fusion partner does not generally appear to inhibit the function of the target protein. For example, an HFBI fusion did not hinder the enzymatic activity of endoglucanase I (Linder *et al.*, 2004), laccase (Kiiskinen *et al.*, 2004) or GOx (Joensuu *et al.*, 2010). Similarly, the HFBI fusion did not have a negative effect on the fluorescence properties of GFP. In addition, fungal hydrophobins are highly biocompatible because they are a common part of our daily dietary intake and can be used to immobilize target proteins to surfaces, or used as carriers to focus enzyme activities to interfaces. These facts are important towards demonstrating the feasibility of hydrophobin fusion technology for clinical or industrial applications.

The unusually strong and specific interaction between polymeric surfactants and highly soluble hydrophobins shows promise to revolutionize protein purification from

complex intracellular protein extracts. Furthermore, the interesting properties of hydrophobins also open avenues for new developments in interfacing proteins and non-biological materials for new applications using this technology.

Protein bodies

Recently, several studies have attempted to elucidate the mechanism of action by which prolamins storage proteins, ELPs and hydrophobins are able to increase recombinant protein accumulation in plants. Based on confocal and electron microscopy analyses, it has been shown that these three fusion tags are capable of inducing the formation of novel PBs (Figure 2) (Mainieri *et al.*, 2004; Conley *et al.*, 2009b; Torrent *et al.*, 2009a; Joensuu *et al.*, 2010), which may be responsible for their positive effect on recombinant protein accumulation by excluding the heterologous protein from normal physiological turnover. For the purposes of this article, we will group prolamins, ELPs and hydrophobins together and refer to them as protein body-inducing fusions (PBIFs), as they all seem to have the same effect of increasing recombinant protein yield and inducing the formation of protein bodies.

Although expressed in the leaves of plants, these artificially induced PBs appear similar in size and morphology to the PBs found naturally in the seed of maize (Lending *et al.*, 1988; Washida *et al.*, 2004), rice (Muench *et al.*, 2000; Nicholson *et al.*, 2005; Takaiwa *et al.*, 2007; Saito *et al.*, 2009), wheat (Levanony *et al.*, 1992; Arcalis *et al.*, 2004) and soybeans (Schmidt and Herman, 2008). Seed PBs are ER-derived organelles that stably accumulate large amounts of storage proteins. In nature, PBs generally form directly within the lumen of the ER where they can remain permanently stored, as demonstrated with maize and rice (Coleman *et al.*, 1996). After formation, PBs can alternatively bud off from the ER as discrete spherical organelles, where they can either reside in the cytosol or be sequestered into protein storage vacuoles by autophagy as shown for wheat, barley and oats (Levanony *et al.*, 1992; Herman and Larkins, 1999; Galili, 2004).

In a similar fashion to natural PBs, the current evidence suggests that the PBIFs are synthesized on ribosomes associated with the rough ER and then transported into the ER lumen, where they accumulate and assemble into PBs by some unknown mechanism (Galili, 2004; Joensuu *et al.*, 2010). The ER-derived PBs are then thought to disconnect and bud out from the cisternal ER into the cytoplasm where they remain surrounded by ER membranes and are

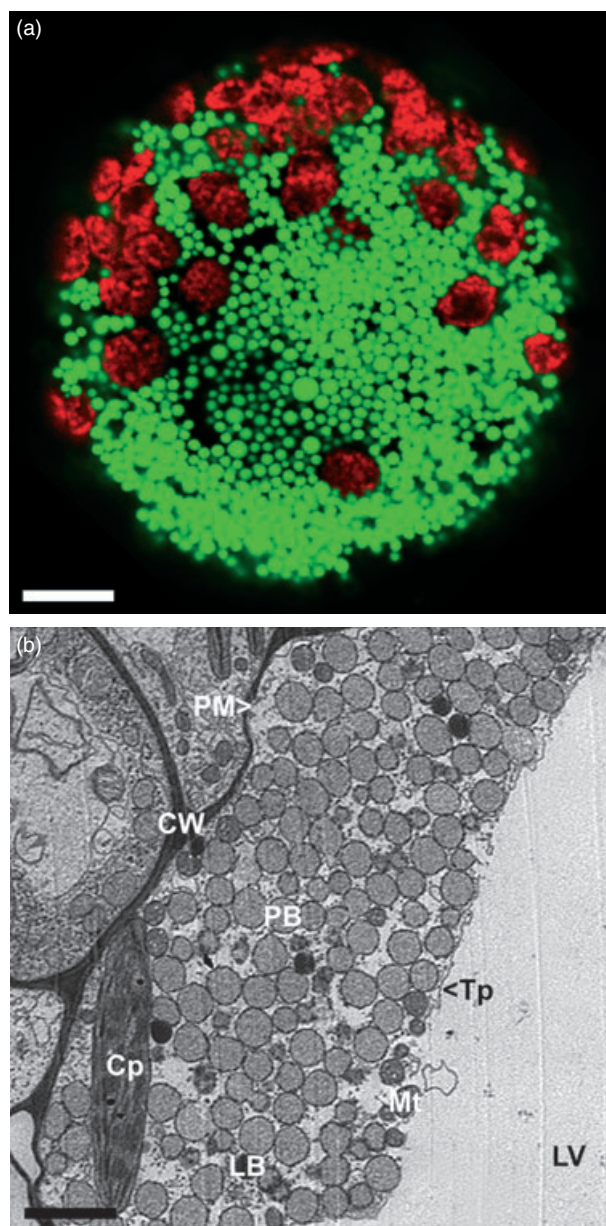


Figure 2 Overexpression of endoplasmic reticulum-targeted GFP-HFBI and GFP-ELP fusions induce protein body (PB) formation in *Nicotiana benthamiana* leaf cells. (a) A confocal image of a mesophyll protoplast expressing GFP-HFBI. The PBs are shown in green and the chloroplasts are shown in red. (b) An electron microscopy image of a mesophyll cell expressing GFP-ELP. The PBs occupy the space between the tonoplast (Tp) and plasma membrane (PM). The different cell compartments are labeled: CW; cell wall, Cp; chloroplast, LB; lipid body, LV; lytic vacuole, Mt; mitochondria. Bar, 10 μm (a); 2 μm (b).

terminally stored as cytoplasmic organelles (Bagga *et al.*, 1995; Conley *et al.*, 2009b). Although the origin and contents of PBs still remain to be elucidated, evidence supporting the working model of ER origin indicates that the novel PBs: (i) contain proteins with ER-specific glycans; (ii)

contain an ER-resident BiP protein; and (iii) are surrounded by a distinct membrane studded with ribosomes (Conley *et al.*, 2009b; Torrent *et al.*, 2009a; Joensuu *et al.*, 2010). Although the distribution pattern of the PBs is highly variable, they are most often found clustered together within the cells of the leaf and have a typical observable diameter of between 0.5 and 1.0 μm (Mainieri *et al.*, 2004; Torrent *et al.*, 2009a; Joensuu *et al.*, 2010). However, the size of the induced PBs is fairly heterogeneous, with some of the PBs having diameters of 8.0 μm , approaching the size of the cell's nucleus (Conley *et al.*, 2009b).

The induced PBs are highly mobile organelles, exhibiting various dynamic patterns of movement throughout the cells, which are dependent on intact actin microfilaments and a functional actomyosin motility system (Conley *et al.*, 2009b). The movement of the PBs is very reminiscent of Golgi stack trafficking because they both move in a stop-and-go manner, alternating between periods of rapid vectorial movement and periods of relatively static, nondirected oscillation. The PBs generally move throughout the cell in a sporadic, saltatory fashion, but they can also be rapidly transported in a unidirectional manner via cytoplasmic streaming (Figure S1; Batoko *et al.*, 2000; Boevink *et al.*, 1998; Nebenfuhr *et al.*, 1999). Thus far, the significance of incessant PB movement in the plant cells is unclear, as they appear to move continuously about the cell without a final destination.

The biochemical properties of the PBIFs share many similarities. For example, these fusion tags are generally amphipathic or hydrophobic with the ability to self-assemble and form metastable supramolecular secondary structures consisting of helices and spirals as a result of their sequences (Linder *et al.*, 2001; Kogan *et al.*, 2002; Miao *et al.*, 2003). Thus, to remain soluble, it is thought that the PBIFs aggregate and self-assemble into stable PBs in the cell as a means of reducing the hydrophobic effect experienced in the lumen of the ER by directing the hydrophobic patches away from the aqueous environment (Tanford, 1978). Although it was originally thought that PBIFs aggregated in a nonspecific manner within the ER lumen, additional studies showed that additional cellular factors are necessary for their accretion into ER-derived PBs (Coleman *et al.*, 1996; Herman and Larkins, 1999; Saito *et al.*, 2009). Thus, the special intrinsic biophysical properties shared by the PBIFs is at least partly responsible for the generation of novel PBs in plants. Interestingly, these PBIFs are derived from three taxonomically distinct kingdoms (i.e. plants, fungi and animals), which suggests that the ER possesses an intrinsic ability to form PB-like accretions in

eukaryotic cells when overexpressing particular proteins with special physicochemical properties.

Protein bodies function in the cells of seeds to store high concentrations of particular proteins in a localized stable intracellular environment. Presumably, encapsulation of large amounts of recombinant protein into physiologically inert storage organelles excludes them from normal physiological turnover in the plant secretory pathway by ER-associated proteolytic degradation (ERAD), which is a component of the protein quality control system (McCracken and Brodsky, 2003; Di Cola *et al.*, 2005). As a result of induced PB formation, Torrent *et al.* (2009a) demonstrated that recombinant protein accumulation in leaf material remained stable when dried at 37 °C and stored for 5 months at room temperature, which are conditions usually responsible for extensive proteolysis. Furthermore, a transgenic rice seed-based vaccine expressing cholera toxin B subunit was resistant to the harsh environmental conditions of the gastrointestinal tract when administered orally and maintained immunogenicity as a result of its accumulation in stable PBs (Nochi *et al.*, 2007). Another study showed that the induction of PBs greatly decreased the rate of proteolysis of a thioredoxin-ELP fusion protein containing a thrombin cleavable linker as the proteolytic enzyme (in this case thrombin) was excluded from the phase where the protein targeted for proteolysis was present. By excluding thrombin from the PBs, the cleavage reaction can only happen at the interface of the two phases, thereby dramatically reducing the rate of reaction (Ge *et al.*, 2009). In addition, because the recombinant protein is constantly shunted away from the ER via PBs containing high concentrations of the target protein, the synthesis/degradation equilibrium within the ER will shift towards increased synthesis without excessive buildup becoming fatally toxic to the cell. Thus, a continual renewal of ER production capacity may exist, which may be responsible for allowing subsequently higher levels of heterologous protein to accumulate inside the cell.

In summary, PB formation enables high local concentrations of heterologous proteins to exist within the limited space of the cell, while insulating the protein from normal cellular protein degradation mechanisms, and without subjecting the ER to an intolerable level of stress (Galili, 2004; Vitale and Ceriotti, 2004; Barbante *et al.*, 2008). Therefore, a PBIF approach provides an effective strategy for enhancing the production yield of recombinant proteins in plant leaves via accumulation in stable PB-like organelles. Prolamins, ELPs and hydrophobins significantly enhance the production yield of recombinant proteins in plants,

while also providing simple, efficient and inexpensive approaches for their subsequent purification.

Intellectual property status of PBIF proteins

All three PBIF are covered by active patents that we have compiled in Table 1. Abandoned or expired patents were not included, neither were patents relating to ELP for medical applications or hydrophobin patents for surface coating.

For ELPs, patent US6,852,834 covers expression of ELP fusion proteins in prokaryotes and eukaryotes, including *E. coli*, *Caulobacter*, yeast, *Pichia*, mammalian systems, but not plants. This patent describes ITC for purification of fusion proteins, and the beneficial effect of smaller ELP tags on expression of recombinant proteins in *E. coli*. Patents US7,364,859 and US7,429,458 are divisionals of patent US6,582,926 and are focused on binding compounds and delivery to specific locations within the animal body. Patent US7,709,227 describes the incorporation of cysteines in the ELP tag, thus allowing it to form disulphide bonds. This approach produces multimeric ELP spider protein complexes that enhance proteolytic resistance of a protein or peptide moiety in a fusion construct. A further improvement on the ELP technology as a purification method involves the use of self-cleaving inteins for easy removal of the ELP tag (US/2006/0263855). The only patent application still active for the use of ELP fusions in plants is US/2010/0278775 for the fusion of ELP to interleukin-24 (IL-24) and describes the increase in accumulation levels of IL-24.

With respect to hydrophobins, one patent describes the use of hydrophobin fusions for the purification of a partner protein by ATPS (US7,060,669) and its divisional (US 7,335,492), which claims fusions produced in a recombinant microorganism such as bacteria, yeasts or filamentous fungi. As well, patent US7,078,192 covers the use of the ability of hydrophobin fusions to spontaneously bind to a solid surface for diagnostic and biosensor applications.

The earliest patent describing the overexpression of zeins and the appearance of protein bodies in vegetative organs of plants is US5,990,384. This patent advocates the use of this technology to improve the content of sulphur-containing amino acids in the diet of foraging animals, and for expressing heterologous fusion proteins. More recently, several patents and patent applications held by ERA Biotech/Plantech describe the accumulation of zein fusion proteins in ER-derived protein bodies in plants (US7,575,898, US/2010/0083403, WO/2004/003207), and in other

Table 1 Active patents covering the three protein body-inducing fusions described in this review

Reference number	Title	Assignee	Issue/pub date	Inventors
ELP				
US6,582,926	Methods of using bioelastomers	Duke University	June 24, 2003	Chilkoti; Ashutosh
US6,852,834	Fusion peptides isolatable by phase transition	Chilkoti, Ashutosh	February 8, 2005	Chilkoti; Ashutosh
US/2006/0263855	Intein-mediated protein purification using <i>in vivo</i> expression of an elastin-like protein		November 23, 2006	Wood; David, Banki; Mahmoud
WO/2008/024311	Purification of low-abundant recombinant proteins from cell culture	Duke University	February 28, 2008	Chilkoti; Ashutosh, Carlson; Kimberly T., Christensen; Trine, Filipe; Carlos, Ge; Xin
US7,364,859	Methods of using bioelastomers	Duke University	April 29, 2008	Chilkoti; Ashutosh
US7,429,458	Methods of using bioelastomers	Duke University	September 30, 2008	Chilkoti; Ashutosh
US7,709,227	Multimeric ELP fusion constructs	PhaseBio Pharmaceuticals, Inc.	May 4, 2010	Dagher; Suzanne
US/2010/0278775	Plant bioreactor for the production of interleukin-24 cytokine	Agriculture and Agri-Food Canada	November 4, 2010	Menassa; Rima, Reid; Alexandra
Hydrophobin				
US7,060,669	Process for partitioning of proteins	Valtion Teknillinen Tutkimuskeskus (VTT)	June 13, 2006	Penttilä; Merja, Nakari-Setälä; Tiina, Fagerström; Richard, Selber; Klaus, Kula; Maria-Regina, Linder; Markus, Tjerneld; Folke
US7,078,192	Method for immobilization of polypeptides	Valtion Teknillinen Tutkimuskeskus (VTT)	July 18, 2006	Linder; Markus, Nakari-Setälä; Tiina, Penttilä; Merja, Soderlund; Hans
US7,335,492	Process for partitioning of proteins	Valtion Teknillinen Tutkimuskeskus (VTT)	February 26, 2008	Penttilä; Merja, Nakari-Setälä; Tiina, Fagerström; Richard, Selber; Klaus, Kula; Maria-Regina, Linder; Markus, Tjerneld; Folke
Zein				
US5,990,384	Co-expression of proteins	New Mexico State University	November 23, 1999	Bagga; Suman, Sengupta-Gopalan; Champa, Kemp; John D.
WO/2004/003207	Production of peptides and proteins by accumulation in plant endoplasmic reticulum-derived protein bodies	ERA Plantech	August 1, 2004	Ludevid Múgica; María Dolores, Torrent Quetglas, Margarita, Lasserre-Ramassamy; Sabine
WO/2006/056483	Production of proteins	ERA Biotech, SA	June 1, 2006	Ludevid Múgica; María Dolores, Bastida Virgili; Miriam, Llopart Royo; Blanca, Marzábal Luna; Pablo, Torrent Quetglas; Margarita
WO/2006/056484	Protein isolation and purification	ERA Biotech, SA	June 1, 2006	Ludevid Múgica; María Dolores, Bastida Virgili; Miriam, Llopart Royo; Blanca, Marzábal Luna; Pablo, Torrent Quetglas; Margarita
WO/2007/096192	Production of biologically active proteins	ERA Biotech, SA	August 30, 2007	Heifetz; Peter Bernard, Llopart Royo; Blanca, Marzábal Luna; Pablo, Bastida Virgili; Miriam, Ludevid Múgica; María Dolores, Torrent Quetglas; Margarita, O Connor; Kevin James, Pallisse Bergwerf; Roser, Llop; M ^a Inmaculada
US7,575,898	Production of peptides and proteins by accumulation in plant endoplasmic reticulum-derived protein bodies	ERA Plantech	August 18, 2009	Ludevid Múgica; María Dolores, Torrent Quetglas; Margarita, Ramassamy; Sabine

Table 1 Continued

Reference number	Title	Assignee	Issue/pub date	Inventors
US/2010/0083403	Production of peptides and proteins by accumulation in plant endoplasmic reticulum-derived protein bodies	ERA Plantech	April 1, 2010	Ludevid Múgica; María Dolores, Torrent Quetglas, Margarita, Lasserre-Ramassamy; Sabine
US7,732,569	Zein-based peptide tags for the expression and purification of bioactive peptides	E I Du Pont De Nemours And Company	June 8, 2010	Decarolis; Linda Jane, Fahnestock; Stephen, Wang; Hong

ELP, elastin-like polypeptides.

eukaryotic cells and organisms (WO/2006/056483). Other patent applications held by ERA include WO/2006/056484 for the purification of PBs from eukaryotic cells by density gradients, and WO/2007/096192 for the production of biologically active proteins as zein fusions in PBs for therapeutic, nutraceutical or industrial uses. Finally, patent US7,732,569 describes the use of zein fusions in microbial cells for the generation of insoluble fusion peptides.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Movement of the protein bodies within *Nicotiana benthamiana* leaf epidermal cells.

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