Hydrophobin Fusions for High-Level Transient Protein Expression and Purification in
Nicotiana benthamiana[1][W][OA]

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Insufficient accumulation levels of recombinant proteins in plants and the lack of efficient purification methods for recovering these valuable proteins have hindered the development of plant biotechnology applications. Hydrophobins are small and surface-active proteins derived from filamentous fungi that can be easily purified by a surfactant-based aqueous two-phase system. In this study, the hydrophobin HFBI sequence from Trichoderma reesei was fused to green fluorescent protein (GFP) and transiently expressed in Nicotiana benthamiana plants by Agrobacterium tumefaciens infiltration. The HFBI fusion significantly enhanced the accumulation of GFP, with the concentration of the fusion protein reaching 51% of total soluble protein, while also delaying necrosis of the infiltrated leaves. Furthermore, the endoplasmic reticulum-targeted GFP-HFBI fusion induced the formation of large novel protein bodies. A simple and scalable surfactant-based aqueous two-phase system was optimized to recover the HFBI fusion proteins from leaf extracts. The single-step phase separation was able to selectively recover up to 91% of the GFP-HFBI up to concentrations of 10 mg mL⁻¹. HFBI fusions increased the expression levels of plant-made recombinant proteins while also providing a simple means for their subsequent purification. This hydrophobin fusion technology, when combined with the speed and posttranslational modification capabilities of plants, enhances the value of transient plant-based expression systems.

As the amount of plant genome and proteome information increases, the need has arisen to develop technologies to rapidly overexpress these genes and to characterize the proteins at the structural and functional levels. Based on two decades of research, plant expression platforms are now recognized as a safe, effective, and inexpensive means of producing heterologous recombinant proteins (Ma et al., 2003).

Agroinfiltration in Nicotiana benthamiana leaves (Kapila et al., 1997; Yang et al., 2000), when combined with the coexpression of a suppressor of gene silencing (Silhavy et al., 2002; Voinnet et al., 2003), has established itself as the most utilized transient expression system in plants. Agroinfiltration is a fast and convenient technique, producing recombinant protein within 2 to 5 d. This transient expression system is also flexible, as it allows for the expression of multiple genes simultaneously (Johansen and Carrington, 2001) and the transfer of relatively large genes (greater than 2 kb), which are genetically unstable in viral vectors (Porta and Lomonossoff, 1996). Although typically used for preliminary laboratory-scale analyses, agroinfiltration is now being scaled up for the rapid production of gram quantities of recombinant proteins in plants (Vézina et al., 2009).

Despite the success of plant expression systems, two major challenges still limiting the economical production of plant-made recombinant proteins include inadequate accumulation levels and the lack of efficient purification methods. Thus, several protein fusion strategies have been developed to address these issues (Terpe, 2003). For example, the use of protein-stabilizing fusion partners, 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 IgG α-chains (Obregon et al., 2006), are common approaches for enhancing recombinant protein accumulation in plants. To simplify purification, recombinant proteins
are often fused translationally to small affinity tags or proteins with defined binding characteristics, such as the StrepII tag, Arg tag, His tag, FLAG tag, c-myc tag, glutathione S-transferase tag, calmodulin-binding peptide, maltose-binding protein, and cellulose-binding domain (Terpe, 2003; Witte et al., 2004; Lichty et al., 2005; Rubio et al., 2005; Streitfeld 2007). However, these affinity chromatography methods are often ineffective when purifying proteins from the complex plant proteome and are costly and difficult to scale up for industrial applications (Waugh, 2005).

More recently, elastin-like polypeptide (ELP) and Zera protein fusions have been shown to significantly enhance recombinant protein accumulation in the leaves of plants (Patel et al., 2007; Floss et al., 2008; Conley et al., 2009c; Torrent et al., 2009) while also providing a means for their purification. ELPs are thermally responsive synthetic biopolymers composed of a repeating pentapeptide (VPGXG) sequence (Urry, 1988) that are valuable for the simple nonchromatographic “inverse transition cycling” bioseparation of recombinant proteins (Meyer and Chilkoti, 1999; Lin et al., 2006). However, the purity and recovery efficiency are rather low when using inverse transition cycling for the purification of plant-made proteins that accumulate to low levels, so expensive and tedious affinity chromatography steps are still needed in these cases (Conley et al., 2009a; Joensuu et al., 2009). Alternatively, Zera, the Pro-rich domain derived from the maize (Zea mays) seed storage protein γ-zein, can facilitate the recovery and purification of fused recombinant proteins by density-based separation methods, but this technique is difficult to scale up (Torrent et al., 2009). Interestingly, both of these protein fusions, derived from taxonomically distinct kingdoms, have been shown to induce the formation of novel endoplasmic reticulum (ER)-derived protein bodies (PBs; Conley et al., 2009b; Torrent et al., 2009). These PBs are physiologically inert and allow for the stable storage of large amounts of recombinant protein within the cell. To overcome the current limitations of the ELP and Zera purification schemes, we chose to investigate hydrophobins as fusion partners for the expression and purification of plant-made recombinant proteins, since they share many interesting physicochemical properties with ELP and Zera.

Hydrophobins are small surface-active fungal proteins that have a characteristic pattern of eight conserved Cys residues, which form four intramolecular disulfide bridges and are responsible for stabilizing the protein’s structure (Hakanpaa et al., 2004). In nature, hydrophobins contribute to surface hydrophobicity and function to coat various fungal structures important for growth and development (Linder, 2009). Hydrophobins have 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). Because of these unique properties, hydrophobins have numerous potential applications, including 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 (Wessels, 1997; Askolin et al., 2001; Linder et al., 2005; Linder, 2009).

Hydrophobins are also capable of altering the hydrophobicity of their respective fusion partners, thus enabling efficient purification using a surfactant-based aqueous two-phase system (ATPS; Linder et al., 2004). The ATPS concentrates the hydrophobin fusions inside micellar structures and partitions them toward the surfactant phase (Lahtinen et al., 2008). ATPSs 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, the one-step ATPS purification 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).

Here, we used agroinfiltration to study the effect of a hydrophobin fusion on the accumulation of GFP and the commercially valuable enzyme Glc oxidase (GOx). We also determined the capability of hydrophobins for purifying recombinant proteins from leaf extracts using an ATPS. The hydrophobin fusion partner significantly enhanced the production yield of GFP while also providing a simple, efficient, and inexpensive approach for the purification of recombinant proteins from plants.

RESULTS

Effects of the Gene Terminator and T-DNA Size on the Accumulation of GFP and GFP-HFBI

To enhance Agrobacterium tumefaciens-mediated transient gene expression in N. benthamiana, two strategies were tested to optimize the expression cassette of our standard plant transformation vector pCaMterX (Fig. 1). First, the Agrobacterium nopaline synthase (nos) gene terminator (Bevan et al., 1983) was replaced with the soybean (Glycine max) vegetative storage protein B (vspB) gene terminator (Mason et al., 1988). Second, the expression cassette for the nptII selectable marker was removed to determine whether minimizing the size of the transferable T-DNA region would have an effect on protein accumulation. A genetic construct encoding for an ER-targeted GFP was then cloned into these expression vectors. Similarly, the gene encoding for the hydrophobin HFBI (approximately 7.5 kD) from Trichoderma reesei was fused to the C terminus of GFP to examine the effect of a hydrophobin tag on the accumulation and purification of GFP. For all expression analyses, the p19 suppressor of posttranscriptional gene silencing (Silhavy et al., 2002) was coinfiltrated with the various GFP constructs to coinfiltrate with the various GFP constructs to...
whereas minimizing the T-DNA size by selectable marker gene deletion did not affect the accumulation of GFP or GFP-HFBI (Fig. 2A).

A HFBI Fusion Enhances GFP Accumulation and Prevents the Necrosis of Infiltrated \textit{N. benthamiana} Leaves

When expressed as a fusion with HFBI, GFP accumulation increased by 2-fold from 18\% to 38\% of total soluble protein (TSP; Fig. 2A). To study the influence of the HFBI fusion on GFP accumulation more thoroughly, a new batch of \textit{N. benthamiana} plants was infiltrated with the constructs carrying the \textit{vspB} gene terminator and sampled every other day for 10 d post infiltration (dpi; Fig. 2B). In the absence of the HFBI fusion, GFP concentration peaked at 6 dpi (20\% of TSP) and then decreased. In comparison, the GFP-HFBI construct expressed more GFP throughout the entire time period studied, with the expression stabilizing at an average of 38\% of TSP from 6 to 10 dpi. When taking into account the HFBI portion of the fusion, this corresponds to 51\% GFP-HFBI of TSP. Pooled samples from the same plants were also analyzed by SDS-PAGE and GelCode staining, which confirmed the higher abundance of GFP-HFBI (37 kD) compared with GFP (29 kD; Fig. 2C).

Phenotypically, there was also an obvious difference between GFP-infiltrated leaves and leaves expressing GFP-HFBI. GFP infiltration induced the formation of lesions starting at 4 dpi, leading to almost complete necrosis of the leaf tissue by 10 dpi (Fig. 3A). Meanwhile, the areas infiltrated with GFP-HFBI remained nearly nonnecrotic and relatively healthy up to 10 dpi and only showed a pale green color in appearance (Fig. 3B), similar to control leaves agroinfiltrated with the p19 construct alone (data not shown). As expected, progressive necrosis decreased the amount of TSP present in the GFP-expressing leaves, whereas the massive amounts of GFP-HFBI protein being produced actually increased the amount of TSP in the GFP-HFBI leaves (Supplemental Fig. S1A). The amount of TSP in the GFP-HFBI leaves was 1.1, 1.3, 1.5, 1.5, and 1.6 times higher than in p19-infiltrated control leaves at 2, 4, 6, 8, and 10 dpi, respectively. Since the amount of TSP decreased for the GFP-
expressing plants while it increased for the GFP-HFBI-expressing plants, the absolute amounts of GFP produced between the two constructs was even more drastic than the GFP per TSP data suggest (Fig. 2B). The GFP-HFBI extracts contained 1.4 (2 dpi), 1.5 (4 dpi), 2.6 (6 dpi), 5.4 (8 dpi), and 8.3 (10 dpi) times more GFP than the GFP control extracts (Supplemental Fig. S1B).

The HFBI Fusion Enhances Recombinant Protein Accumulation in Leaves by Forming PBs

To understand the mechanism by which HFBI fusions increase GFP accumulation in plants, the leaves expressing GFP and GFP-HFBI were visualized by confocal and electron microscopy. For confocal microscopy, protoplasts were prepared from the N. benthamiana leaves at 4 dpi. The fluorescence in the protoplasts expressing ER-targeted GFP control protein resembled the characteristic reticulate pattern of the ER network (Fig. 4A). However, in the presence of a HFBI fusion partner, the distribution of GFP was observed as brightly fluorescing spherical structures distributed throughout the protoplasts (Fig. 4B; Supplemental Video S1). The spherical structures, which are presumed to be a novel type of PB, were similarly observed in intact leaf epidermal cells (Fig. 4C; Supplemental Video S2). These PBs were mobile and moved about the cell in a similar manner to ELP-induced PBs (Conley et al., 2009b). To further investigate the final destination of the PBs, the leaf tissues expressing GFP and GFP-HFBI were analyzed by electron microscopy and immunogold labeling. Electron micrographs showed the presence of electron-dense, spherical PB-like organelles in the cytosol of the GFP-HFBI-expressing cells (Fig. 4D, i and ii). The size of the PBs was heterogeneous, but they typically had a diameter of about 500 nm, confirming the size observed with the confocal microscope. PB-like structures were not found in nontransfected cells (data not shown). When observed at higher magnification, the PBs were surrounded by a distinct, ribosome-studded membrane that did not appear to be continuous with the ER network (Fig. 4D, iii), suggesting that the PBs are derived from the ER but are terminally stored in the cytosol. The presence of GFP-HFBI protein within the PBs was confirmed by examining leaf sections that were immunogold labeled with an anti-GFP antibody (Fig. 4D, iv). No significant labeling was observed in other cellular locations or from nontransfected leaves (data not shown).

Purification of GFP-HFBI by an ATPS

To develop a simple and scalable method for recovering recombinant proteins from the complex plant proteome, GFP-HFBI was transiently expressed in N. benthamiana plants. Although hydrophobins have been used as purification tags in ATPS (Linder et al., 2004; Lahtinen et al., 2008), they have not been previously used to express or purify plant-made proteins. Therefore, we optimized the ATPS separation of GFP and GFP-HFBI leaf extracts using three different concentrations (2%, 4%, and 8% [w/v]) of the surfactant Agrimul NRE 1205. We conducted fluorometric analysis on both upper and lower phases of the ATPS and found that the GFP-HFBI fusion protein was concentrated in the upper phase while the unfused GFP protein remained equally distributed among the two phases (Fig. 5A). This result was confirmed by visual observation under visible and UV light (Fig. 5B). The volume of the ATPS upper phases recovered from the GFP-HFBI extracts differed with the amount of surfactant used (2%, 4%, and 8%) and was 10%, 21%, and 43% of the original leaf extract volume, respectively. The 2% surfactant concentration resulted in the smallest upper phase volume and contained the highest concentration of GFP at 7.4 ± 0.5 mg mL⁻¹ (mean ± SD, n = 3), which corresponds to 10.0 mg mL⁻¹ of the GFP-HFBI fusion protein. The GFP concentrations recovered with 4% and 8% surfactant were 3.0 ± 0.3 and 1.5 ± 0.2 mg mL⁻¹, respectively. As a result, GFP concentration was enriched 13.5, 5.5, and 2.8 times in the ATPS relative to the original leaf extract. To track...
the behavior of GFP-HFBI in the ATPS, the phase separation was monitored under UV light, and time-lapse imaging of the ATPS purification demonstrated that the GFP-HFBI fusion was transferred to the upper surfactant phase by micelle formation (Supplemental Video S3).

To assess the purity of the GFP-HFBI following ATPS purification, the leaf extract, lower phase, and upper phase were analyzed by SDS-PAGE (Fig. 5C). The gel analysis confirmed the high abundance of GFP-HFBI protein (37 kD) in the upper phase. In addition, the ATPS selectively recovered GFP-HFBI,
since no major contaminating plant proteins were observed on the gel. The 74- and 111-kD bands visible on the gel were confirmed to be GFP-HFBI dimers and trimers by HFBI immunoblotting (data not shown).

To estimate the efficiency of the ATPS purification, the amount of GFP present in the leaf extract and ATPS phases was measured by fluorometry (Fig. 5D). The highest recovery (91% ± 4%) was obtained when using the highest surfactant concentration (8%). The 2% and 4% surfactant concentrations recovered 54% ± 4% and 71% ± 4% of the GFP originally present in the TSP. SDS-PAGE analysis also confirmed these results, since the appearance of the GFP-HFBI band in the lower ATPS phase diminished with an increasing concentration of surfactant (Fig. 5C). To be able to compare the efficiency of ATPS purification with affinity chromatography purification, a StrepII tag (Schmidt et al., 1996) was fused to the C terminus of the GFP-HFBI protein. The StrepII affinity tag has been shown to be efficient in capturing proteins from leaf extracts (Witte et al., 2004). Following the StrepII capture, the eluted fractions contained 90% ± 7% of the initial amount of GFP-HFBI, which is very comparable to the 91% recovery obtained with ATPS purification using 8% surfactant (Fig. 5D).

Since the concentration of GFP-HFBI fusion protein in the agroinfiltrated leaf extract was very high, accounting for 51% of TSP, we wanted to establish the utility of the ATPS for purifying proteins that accumulate to lower levels. Therefore, total soluble proteins were extracted from noninfiltrated wild-type N. benthamiana leaves and spiked with different concentrations (5%, 1%, and 0.1% TSP) of purified GFP-HFBI protein. The leaf extracts and ATPS phases were then analyzed by SDS-PAGE followed by GelCode staining (Fig. 6A). Again, the ATPS showed very selective recovery of the hydrophobin fusion protein, and the GFP-HFBI protein was clearly visible in the upper phases derived from all spiked TSPs, while the endogenous plant proteins remained in the lower phases. Fluorometric quantification showed effective recoveries of 81% ± 11%, 80% ± 14%, and 64% ± 7% for the 5%, 1%, and 0.1% GFP-HFBI-spiked extracts, respectively (Fig. 6B).
Transient Expression and Purification of GOx in *N. benthamiana*

In order to test the applicability of HFBI fusion technology further, the GFP portion of the GFP-HFBI fusion was replaced with the enzyme GOx, which catalyzes the oxidation of D-Glc to D-gluconolactone and hydrogen peroxide. GOx has several commercial applications in the food industry as a preservative, for improving the color and flavor and increasing the shelf life of food products, and in the biomedical industry as a biosensor. GOx is naturally produced in some fungi and insects, but the recombinant expression of enzymatically active GOx has remained challenging (Bankar et al., 2009). SDS-PAGE analysis followed by Coomassie Brilliant Blue staining confirmed a high accumulation of GOx-HFBI fusion (92 kD) in agroinfiltrated leaf extracts (Fig. 7A). Similar to the results obtained with GFP-HFBI, the ATPS showed efficient and selective recovery of the GOx-HFBI fusion protein in the upper phase (Fig. 7A). The enzymatic activity of the GOx-HFBI fusion was measured by a kinetic assay and showed a similar catalytic activity to a commercial *Aspergillus niger* GOx reference standard (Fig. 7B).

**Figure 6.** Two-phase separation with 8% surfactant of the wild-type *N. benthamiana* leaf extracts spiked with different concentrations of purified GFP-HFBI. The spiked GFP-HFBI was recovered efficiently from the leaf extracts with minimal background. A, Fractions (10 μL) of the spiked TSP, upper phase, and lower phase were analyzed by SDS-PAGE. B, Quantification of the GFP-HFBI recovery was determined by fluorometry (n = 3; sd is represented with error bars).

**Figure 7.** Expression and purification of an enzymatically active GOx-HFBI fusion protein in *N. benthamiana* leaves. A, Purification of the GOx-HFBI fusion by an ATPS. SDS-PAGE demonstrates a selective recovery of the GOx-HFBI (92 kD) in the upper phase with minimal background from the leaf extract. Equal volumes of the TSP, upper phase, and lower phase were loaded. A commercial GOx (80 kD) isolated from *A. niger* was used as a reference. B, A comparison of the molar GOx activity of the plant-derived GOx-HFBI fusion protein and commercial GOx.

**DISCUSSION**

Insufficient recombinant protein yields in transgenic plants and the lack of efficient purification methods for their recovery remain a major hindrance for the advancement of plant biology and biotechnology (Doran, 2006; Joensuu et al., 2008; Kaiser, 2008). Recent developments in transient plant expression systems based on *Agrobacterium* infiltration have shown great promise for boosting expression with the aid of plant viral vectors (Marillonnet et al., 2005; Giritch et al., 2006; Lindbo, 2007) and/or posttranscriptional gene-silencing suppressors (Silhavy et al., 2002; Voinnet et al., 2003; Sainsbury and Lomonossoff, 2008). Although transient plant expression systems can now offer good production speed and yield with suitable posttranslational modification capabilities, the recovery of recombinant proteins from the complex plant proteome has remained challenging. Thus, we evaluated the use of hydrophobin fusion technology for facilitating the expression and recovery of recombinant proteins in plants.

The GFP-HFBI fusion protein was transiently expressed in *N. benthamiana* leaves via agroinfiltration and accumulated to 51% of TSP, which corresponds to...
5.0 mg of the GFP-HFBI fusion protein per gram of leaf fresh weight or 3.7 mg g⁻¹ fresh weight when calculated for the GFP fraction alone. These exceptionally high recombinant protein yields were attained with a binary T-DNA vector coinfiltrated with the p19 suppressor of gene silencing without the aid of viral replication. These levels of expression are equivalent to the highest GFP yields reported with virus-based systems (Marillonnet et al., 2005; Lindbo, 2007) but are not restricted by the size of the insert that can be expressed, which is a limitation associated with viral RNA polymerases (Ahliquist et al., 2005; Castro et al., 2005).

Although it needs to be tested on a case-by-case basis, the presence of a HFBI fusion partner does not appear to affect the function of the GFP or GOx proteins. In the event that the HFBI has a deleterious effect on the activity of a particular protein, the HFBI may need to be cleaved off from the fusion protein. However, this is not a major problem, since a second round of ATPS purification can be done to segregate the HFBI tag to the surfactant phase, while the purified unfused target protein remains soluble in the buffer phase. This was demonstrated by adding a tobacco etch virus (TEV) protease cleavage site between the GFP and HFBI portions of the GFP-HFBI fusion. First, the ATPS-purified GFP-TEV-HFBI protein (38 kD) was cleaved with TEV protease. Then, a second round of ATPS was performed to separate the released GFP (29 kD, lower phase) from the uncleaved GFP-HFBI and cleaved HFBI tag (9 kD, upper phase; Supplemental Fig. S2).

Greater levels of GFP and GFP-HFBI expression were obtained with the vspB gene terminator than the nos terminator, which is similar to the results obtained previously when expressing the hepatitis B surface antigen in transgenic potato (Solanum tuberosum) plants (Richter et al., 2000). To enhance the efficiency of T-DNA transfer and to eliminate superfluous transcription, the size of the T-DNA was minimized by deleting the antibiotic resistance marker, which is unnecessary with transient expression systems. However, removal of the antibiotic resistance marker had no effect on the accumulation of GFP or GFP-HFBI, suggesting that T-DNA size does not play a major role in this expression platform.

We typically choose to target heterologous proteins into the oxidizing environment of the ER lumen because of the lack of proteases and the abundance of molecular chaperones in this subcellular compartment (Ma et al., 2003). Furthermore, the ER provides the most suitable environment for complex posttranslational modifications to occur, such as glycosylation and disulfide bond formation (Hwang et al., 1992). 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 result in the formation of necrotic lesions on the agroinfiltrated leaf extracts using a simple phosphate-buffered saline (PBS)-based extraction buffer without any detergents. The composition of the extraction buffer can have a major impact on the ATPS purification efficiency (Selber et al., 2001). Acidic sodium acetate buffers (pH 5.0–5.5) have been used successfully with ATPSs (Linder et al., 2004; Lahtinen et al., 2008), but these buffers offered a low recovery when attempting to extract GFP-HFBI from the N. benthamiana leaves after 4 dpi (J.J. Joensuu, unpublished data). In this particular case, overexpression of the GFP control protein was relatively harmful to the plant leaves, whereas the leaves infiltrated with the GFP-HFBI fusion protein remained fairly healthy up to 10 dpi. Thus, the accumulation of GFP-HFBI continued to increase relative to the endogenous plant proteins, allowing for simpler downstream purification processes.

Based on the confocal and electron microscopy analyses, the HFBI fusion is thought to enhance GFP accumulation by forming large PBs, which probably excludes the recombinant protein from normal physiological turnover. The electron micrographs suggest that the PBs are surrounded by an ER-derived membrane and are ultimately stored in the cell’s cytoplasm. These novel protein storage organelles are very similar in size, morphology, and mobility to the PBs induced when expressing ELP (Conley et al., 2009b) or Zera (Torrent et al., 2009) protein fusions. Because of their amphipathic nature, the dissolved state of hydrophobins is known to be metastable (Linder et al., 2001). Thus, to remain soluble, we hypothesize that hydrophobin fusions aggregate and self-assemble into stable PBs in the cell as a way of shielding their exterior hydrophobic patches from the aqueous environment. This mechanism of heterologous protein storage is analogous to the one proposed for ELP-induced PBs (Conley et al., 2009b), suggesting that the special biophysical properties shared by these fusion proteins are at least partly responsible for the generation of novel PBs in plants.

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). More recently, the utility of this ATPS technology was demonstrated for recovering hydrophobin fusions from insect cell extracts (Lahtinen et al., 2008). To our knowledge, fungal hydrophobins have not been previously expressed in plants, so we evaluated the effectiveness of HFBI as a fusion partner for one-step ATPS purification in plants. This is also, to our knowledge, the first reported example of hydrophobins being retained in the ER, which appears to induce the formation of PB-like organelles. Initially, it was not clear whether the GFP-HFBI could be easily recovered in a soluble form from the PBs. However, despite being encapsulated into PBs within the plant cell, the GFP-HFBI fusion protein was highly soluble and was easily recovered from the agroinfiltrated leaf extracts using a simple phosphate-buffered saline (PBS)-based extraction buffer without any detergents.

The composition of the extraction buffer can have a major impact on the ATPS purification efficiency (Selber et al., 2001). Acidic sodium acetate buffers (pH 5.0–5.5) have been used successfully with ATPSs (Linder et al., 2004; Lahtinen et al., 2008), but these buffers offered a low recovery when attempting to extract GFP-HFBI from the N. benthamiana leaves (data
not shown). This result can likely be explained by the precipitation of many plant proteins at a pH below 6 (Joensuu et al., 2009) and by the instability of GFP at an acidic pH (Patterson et al., 1997). At a pH of 6.5, the 200 mM sodium acetate and PBS buffers worked equally well for extracting GFP-HFBI and TSP; however, the ATPS was more effective with the PBS-based buffer.

Agrimul NRE 1205 has been previously shown to be an effective general surfactant for ATPSs (Linder et al., 2004), and it has been used to purify HFBI-GFP fusion from insect cells (Lahtinen et al., 2008). In our study, various Agrimul concentrations were evaluated to optimize the ATPS for leaf extracts. To summarize, the purification efficiency of the hydrophobin fusions is greater when using higher surfactant concentrations. On the other hand, lower concentrations of surfactants are most beneficial when higher concentration factors are required, since less water is entrapped in the surfactant phase during formation of the ATPS (Selber et al., 2001). As a result, we were able to capture GFP-HFBI to concentrations of 10 mg mL\(^{-1}\) when the plant proteins were extracted with 2% Agrimul. Although we tested many surfactants for purifying the GFP-HFBI from leaf extracts via ATPS (data not shown), the Agrimul surfactant offered the best compromise between recovery efficiency and concentration factor. The hydrophobin fusion proteins were efficiently back-extracted to the buffer phase from the surfactant phase with the addition of isobutanol, which has been demonstrated to provide robust and good recovery yields (Linder et al., 2001). Although we did not observe denaturation of our target proteins following isobutanol extraction, this may be a concern for other more labile proteins. Thus, the surfactant removal step may require optimization for certain target proteins (Linder et al., 2004). The highest recovery of GFP-HFBI was 91% when using 8% surfactant, comparable to the efficiency of recovery obtained with StrepII affinity columns, which in our experience has been the most efficient affinity chromatography method for capturing recombinant proteins from leaf extracts. However, the SDS-PAGE analysis of the StrepII-purified GFP-HFBI showed a minor coelution of Rubisco large subunit (data not shown), which was not detected after the ATPS. A sequential ATPS-StrepII purification was also performed, but this scheme was unable to increase the purity of GFP-HFBI or to separate the GFP-HFBI dimers or trimers from the monomeric form (data not shown).

To study ATPS for low-accumulating plant recombinant proteins, a leaf extract from wild-type plants was spiked with different concentrations of purified GFP-HFBI. Again, the ATPS was very selective and showed efficient recovery, with 64% of the GFP-HFBI recovered when starting at a low concentration of 0.1% of TSP. This was considerably higher than the recovery of HFBI-GFP obtained from insect cells (Lahtinen et al., 2008). This may be explained by the differences in the orientation of the HFBI fusion (i.e. N terminal versus C terminal) and the absence of a flexible linker region in the insect expression construct.

For selective purification of proteins, affinity chromatography has attained a lot of interest. However, this method is typically only suitable for purifying low quantities of high-value products. Compared with other separation techniques, the major advantage of hydrophobin ATPS technology is the ease by which the process can be scaled up to large industrial levels of protein production and purification. Furthermore, an ATPS is a powerful and robust tool in any downstream processing scheme, since it offers a good volume reduction with high selectivity and yield. Purification with ATPS does not require centrifugation, as efficient phase separation can be obtained by gravity settling. In fact, centrifugation is not the optimal means for separation of surfactant-based systems due to the low interfacial tension and high friction in the separator, which hinders the essential coalescence of the droplets (Selber et al., 2001). Previously, HFBI fusion proteins have been successfully purified from 1,200-L fungal culture supernatants by gravity settling, without a reduction in yield or phase partition coefficient (Selber et al., 2004).

CONCLUSION

In summary, HFBI fusions were able to increase the accumulation of GFP in plants through the formation of novel PBs, which allows for the stable storage of large amounts of heterologous proteins in the confined space of the cell. Furthermore, HFBI fusions also provide a simple, effective, and scalable means for the purification of plant recombinant proteins by surfactant-based ATPS. Hydrophobin fusion technology enhances the value of plant-based expression systems and now opens new possibilities for studying protein overexpression in plants.

MATERIALS AND METHODS

Plant Expression Constructs

The coding sequences of GFP (Cormack et al., 1996) and HFBI (Nakari-Setälä et al., 1996) were constructed using a combined ligase chain reaction/PCR approach (Au et al., 1998). This technique utilizes a set of overlapping oligonucleotides designed by the Web-based program Gene2Oligo (Rouillard et al., 2004) to assemble synthetic genes. The iCUP translational enhancer (Wu et al., 2001) and the Pribi secretory signal peptide from tobacco (Nicotiana benthamiana; Cutt et al., 1988) were fused in-frame to the GFP sequence by homology overlap PCR. For the GFP-HFBI construct, a flexible (GGGS)\(^3\) linker containing a Ksl restriction site was included between the GFP and HFBI sequences. A StrepII purification tag (Schmidli et al., 1996; WSHPQFEK) and a KDEL ER-retrieval signal were included at the C-terminal end of both constructs by extension PCR. To assist in subsequent cloning steps, Xhol and SacI restriction sites were incorporated at the 5’ and 3’ ends of the completed constructs. To demonstrate the removal of the HFBI portion from the fusion protein, a TEV protease cleavage site (ENLYFQ/G) was cloned between the GFP and HFBI portions to create a GFP-TEV-HFBI fusion. The final constructs were moved into the plant binary expression vector pCaMterX (Harris and Geddie, 2001), where the coding sequences were placed under the control of the dual-enhancer cauliflower mosaic virus 35S promoter (Kay et al., 1987) and the Agrobacterium tumefaciens nos (Bevan et al., 1983) or soybean (Glycine max) vspB (Mason et al., 1988) terminator. For the expression
of GOx, the GFP-(GGGS)₃ linker portion of the GFP-HFBI expression cassette (vsvp terminator) was replaced with a tobacco-optimized (Gemeart) version of the gene coding for *Aspergillus niger* GOx (Kriechbaum et al., 1989; Frederick et al., 2009). We are grateful to Joséf Burgyán for providing the p19 vector.

**Transient Expression in N. benthamiana Leaves**

The expression constructs were electroporated into *Agrobacterium* strain EHA105 (Hood et al., 1993). The *Agrobacterium* suspensions were infiltrated into the leaves of 3- to 6-week-old *N. benthamiana* plants as described previously (Kapila et al., 1997; Yang et al., 2000; Marillonnet et al., 2005). Briefly, the saturated overnight bacterial cultures carrying the GFP expression construct were used to inoculate leaves of 5- to 6-week-old tobacco plants as described (Vorobjev et al., 1993). The infiltrated plants were maintained in a controlled growth chamber at 22°C, with a 16-h photoperiod at 120 µmol m⁻² s⁻¹ for 2 to 10 d. To account for the variability between plants, leaves, and the position on the leaf, comparably sized leaves from eight different plants were infiltrated and then sampled by collecting four leaf discs (7.1 mm in diameter) for each construct at each time point. The same leaves were also used to collect separate pooled samples (one disc per leaf) for the SDS-PAGE analysis followed by GelCode staining (Pierce). For each leaf sample, the TSP was extracted and the TSP and GFP concentrations were quantified as described previously (Conley et al., 2009a).

**Protoplast Preparation**

Protoplasts were prepared from the agroinfiltrated leaves by digesting thin leaf sections in Man-PP buffer (0.5 M mannitol, 2% Suc [w/v], 0.5% MES [w/v], and 0.5% Cellulase Onozuka R10 and 0.2% Mazerzyme R10 [w/v]; Yakult). After 18 h, the digestion mixture was filtered through 100-µm nylon mesh and centrifuged at 500 g for 10 min at 22°C. The pellet was resuspended in Man-PP with 50% Percoll (Sigma) and covered with layers of Man-PP with 20% Percoll and Man-PP alone. After density centrifugation (1,000 g, 10 min, 22°C), the intact protoplasts were recovered from the Man-PP (20% Percoll)-Man-PP interface.

**Protein Purification**

For the two-phase separation, TSP was extracted from GFP (5 dpi) and GFP-HFBI (8 dpi) plants by grinding the agroinfiltrated leaves with a mortar and pestle in liquid nitrogen. Six volumes (w/v) of PBS was added, and the ice-cold homogenate was clarified twice by centrifugation (20,000 g, 5 min, 4°C) to obtain the TSP. The TSP was then prewarmed to 22°C in a water bath for 10 min. Agramul NRE 1205 surfactant (chemical formula C₁₂-₁₈EO₅ [Cognis]; 2%, 4%, or 8% [w/v]) was mixed with the TSP for 10 min at room temperature. The phases were then allowed to separate for 1 h at 22°C in a water bath. After a centrifugation step (3,000 g, 5 min, 22°C), the upper phase was recovered. To wash the detergent from the fraction containing the protein of interest, the upper phase was then extracted with isobutanol (one-half of the original extract volume) for 10 min, and the phases were left to separate for 30 min at 22°C and centrifuged as stated above. An 8% surfactant concentration was used in the GFP-HFBI spiking experiment. The StreptII purification was performed with 1-mL Streptactin Macroprep affinity columns (IBA) according to the manufacturer's instructions. To demonstrate the removal of the HFBI portion from the GFP-TEV-HFBI fusion, ATPS-purified GFP-TEV-HFBI protein was cleaved with TurboTEV (Eton Bioscience) according to the manufacturer’s instructions for 60 h at 4°C. A second round of ATPS was performed to separate the released GFP from the uncleaved GFP-TEV-HFBI fusion and cleaved HFBI tag.

**Confocal and Electron Microscopy**

A TCS SP2 confocal laser scanning microscope (Leica Microsystems) equipped with a 63× water-immersion objective was used to examine the subcellular localization of GFP in leaf epidermal cells and protoplasts. For the imaging of GFP expression and chlorophyll autofluorescence, excitation with a 488-nm argon laser was used, and fluorescence was detected at 500 to 525 nm and 630 to 690 nm, respectively. The agroinfiltrated leaves were immunogold labeled and examined by electron microscopy as described previously (Conley et al., 2009b).

**Kinetic GOx Assay**

GOx activity was determined by a kinetic assay described elsewhere (Carwein et al., 1970; Kahle et al., 1970; Werner et al., 1970). Briefly, hydroperoxide generated during the GOx-catalyzed oxidation of D-Glc to D-glucono-δ-lactone was utilized by horseradish peroxidase (Sigma) as a cosubstrate in the oxidation of ABTS [diazonium-2,2′-azoino-bis(3-ethyl-benzthiazoline-4-sulfonate); Roche]. ABTS oxidation rates were measured in 300-µL reaction volumes on 96-well plates as an increase in ΔA₄₅₀ using a microtiter plate reader (Varioskan; Thermo Fisher Scientific). The reaction solutions were prepared by dissolving ABTS, D-Glc, and horseradish peroxidase in a 10 mM sodium phosphate buffer (pH 7.0) to concentrations of 0.91 mM, 1 mM, and 0.76 µM, respectively. ABTS oxidation was monitored over a 5-min period using 15-s sampling intervals. A commercial *Aspergillus niger* GOx (Sigma) was used as a reference for the measurements.

**Statistical Analysis**

The statistical analysis was performed with SPSS 16.0 for Windows. The normal distribution of the data was confirmed with the Lilliefors's test. Two-tailed Student’s t test adjusted for unequal variances was used to analyze the data. Statistical significance level for all the tests was defined as 0.05.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number Z68124 for the gene encoding HFBI.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** The concentrations of TSP and GFP in the *N. benthamiana* leaf extracts expressing GFP and GFP-HFBI when coinfiltrated with the posttranscriptional gene-silencing suppressor p19.

**Supplemental Figure S2.** Release and purification of GFP from the GFP-TEV-HFBI fusion using TEV protease digestion followed by ATPS.

**Supplemental Video S1.** A Z-stack scan of a protoplast expressing the GFP-HFBI fusion protein.

**Supplemental Video S2.** A Z-stack scan of a leaf epidermal cell expressing the GFP-HFBI fusion protein.

**Supplemental Video S3.** Time-lapse imaging of GFP-HFBI purification by an ATPS.

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