Chapter 9
Transient Expression Using Agroinfiltration and Its Applications in Molecular Farming

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Abstract Transient expression via agroinfiltration and/or viral vectors has quickly emerged as the preferred expression system for plant-made recombinant proteins. Transient expression can serve as a valuable research tool for finding optimal expression parameters before tedious and time-consuming production of stable transgenic plants or it can be scaled up to commercial production scale with vacuum infiltration. This technology is poised to compete with conventional production systems, large-scale production facilities are currently available and others are in the process of development. This chapter will introduce background and rationale in development of transient expression systems in plants and summarize the latest developments and examples in this area.

9.1 Introduction

To satisfy the increasing rigor of industrial production, a recombinant protein production system must fulfill several criteria: scalability, simple and inexpensive purification methods, short generation and production timelines, high production rates, capability to carry out co- and post-translational modifications, biosafety and product reproducibility.

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Currently, no classical recombinant expression system satisfies all of these requirements. For example, complex recombinant proteins produced in microbial cell culture are not always properly processed or folded resulting in a protein incapable of biological activity. Accordingly, prokaryotic expression systems have been utilized for the production of simple recombinant proteins that do not require extensive post-translational modifications such as insulin, interferon and human growth hormone (Walsh and Jefferis 2006). Due to the limitations of microbial production systems, focus has been placed towards optimization of competing eukaryotic expression systems, yeast, fungi and mammalian cultures. These production systems possess disadvantages such as hyperglycosylation for yeast and fungi, and the potential of harboring human pathogens coupled with scalability and ethical concerns for mammalian cell culture and transgenic animals. These limitations combined with growing demand for therapeutic and industrial proteins have contributed to the emergence of plants as a much safer, low-cost alternative for the production of biologically active recombinant proteins. Plant expression systems can perform post-translational modifications and produce a variety of functional mammalian proteins and industrial enzymes. The production of correctly folded and assembled multi-subunit proteins such as antibodies in plant cells is a good illustration that plants possess the ability to produce and assemble complex mammalian proteins (Nuttall et al. 2005).

9.2 Plant Expression Systems

Stable transformation of plants has been until recently the preferred method of overexpression of recombinant proteins. In stably transformed plant tissue, *Agrobacterium* strains introduce recombinant genes into the plant nuclear genome through virulence factors coded from the Ti-plasmid. The portion of the Ti plasmid which contains the gene of interest is delineated by left and right T-DNA border sequences and is randomly integrated into the plant nuclear genome (Zambryski 1988). This method is largely utilized by most plant scientists; however it has proven more successful for dicotyledonous plants. A major advantage of stable transgenic plants is that the heterologous protein production trait is heritable, resulting in a permanent resource, allowing for simple and rapid scale-up and almost unlimited and sustainable production capacity only requiring planting of seeds in a large area and harvesting it (Gray et al. 2009). Disadvantages of using transgenic plants as production platform include the long developmental phase required to regenerate and analyse the transformants, unpredictable expression due to chromosomal position effects associated with random gene insertion, recombinant protein stability issues leading to low accumulation levels, and physiological effects on the host plant such as toxicity of the recombinant protein (Hobbs et al. 1990; Krysan et al. 2002).

Since the first idea of expressing vaccines in edible plant organs such as bananas and potatoes, much has been accomplished in the area of producing recombinant

proteins in plants. It has become clear that food crops cannot be used for the production of pharmaceutical proteins due to the risk of contaminating the food supply. As well, the idea of oral administration of plant material has evolved to oral administration of purified and quality-controlled products because of the need for accurate dosing which would not be possible with raw plant materials (Rybicki 2010). This is due to the variability in expression levels between plants, within the same plant, and depending on the physiological condition of each plant. Therefore, even though vaccines such as the hepatitis B surface antigen (HBsAg) had been successfully produced in potato and had shown to be effective in human clinical trials (Thanavala et al. 2005), attention was redirected to non-food plants and expression in transient systems that allow faster timelines and higher expression levels than stable transgenic plants. Several breakthroughs in transient expression have been reported in recent years that allow for unprecedented expression levels within 1–2 weeks. Such systems rely on two classes of plant pathogens, plant viruses and *Agrobacterium*, a bacterial soil pathogen.

9.2.1 Agrobacterium Infiltration

In agroinfiltration, recombinant *Agrobacterium tumefaciens* bacteria harbouring a binary expression vector are introduced directly into plant leaves using vacuum infiltration or direct syringe injection. Following infection, single-stranded T-DNA is transferred from the *Agrobacterium* to the plant cells. Once moved into the plant cell by bacterial and plant encoded proteins, this T-DNA is trafficked to the nucleus with the aid of chaperones. Only a small percentage is integrated into the host chromosomes leading to stable transformed cells that can subsequently be regenerated into transgenic plants (Zambryski 1988). The long-term fate of the T-DNAs that do not integrate into chromosomes is unclear; however, it appears that the free T-DNA molecules are transcriptionally competent, thus providing an opportunity for a short-lived burst of recombinant protein production and harvest (Voinnet et al. 2003).

The significantly reduced production timeline and convenience of agroinfiltration technique yields results within 2–5 days, making transient plant-based expression an attractive option for the production of proteins. Thus, agroinfiltration can be used to rapidly evaluate the activity of expression constructs and to produce small amounts of recombinant protein for functional analysis (Wroblewski et al. 2005). These timelines compare very favourably to the time- and resource-intensive process of generating stable transgenic plants, which usually takes 3–6 months. The transient expression system is also flexible, as it allows for the expression of multiple genes simultaneously, and provides a reliable and reproducible indicator of expression construct performance, since it avoids the positional effects normally associated with stable transgenic plants (Kapila et al. 1997).

9.2.2 Virus-Based Expression

Virus-based expression of recombinant proteins is the subject of another chapter in this book (Wang, Chap. 10), thus we will very briefly describe virus-based systems and focus on recent developments where deconstructed viruses are Agro-infiltrated and result in very high expression levels.

The first virus-based expression vectors were simple gene replacement vectors, in which a foreign gene of interest replaced the capsid protein gene of a virus. These vectors were limited in the expression of these genes and although they could move from cell to cell, they could not move systemically in plants. Eventually, plant RNA viruses were constructed to express a foreign gene in addition to all required viral genes, so after inoculation, the plant virus would systemically infect all cells of the plants and generate multiple transcripts of the transgene (Pogue et al. 2002).

More recently, a new system relying on agroinfiltration of deconstructed viral vectors has shown the highest levels of expression achieved in any system. This system, called magnifection, was developed by the German biotechnology company Icon Genetics (now a subsidiary of Bayer Innovation). Magnifection combines the high transfection efficiency of *Agrobacterium* with high expression yield of deconstructed viral vectors, leading to accumulation levels up to 5 g recombinant protein per kg of fresh leaf weight (FLW), equivalent to about 50% TSP (Gleba et al. 2005, 2007). The process consists of an infiltration of whole mature plants with a diluted suspension of *Agrobacterium* carrying T-DNAs encoding viral replicons. The bacteria carry on the function of primary infection while the virus provides cell-to-cell spread, amplification and high-level expression. This system allows the expression of heteromeric recombinant proteins such as monoclonal antibodies (mAbs). For this, the magnICON® system uses two non-competitive viral vectors based on turnip vein clearing tobamovirus (TVCV) and potato virus X (PVX). One vector carries the heavy chain while the other vector carries the light chain. Both vectors are co-infiltrated into *N. benthamiana* where both chains are expressed and assembled into a fully functional mAb (Giritch et al. 2006; Hiatt and Pauly 2006).

The scale up for this technique is essentially the same as for agroinfiltration requiring an infiltration apparatus. Because of very high expression levels in this system, proteins can be produced in a contained facility minimizing biosafety-related risk. The company Kentucky BioProcessing (KBP), in collaboration with Bayer Innovation and Icon Genetics, has adapted the MagnICON system to infiltrate kilograms of plants per hour, allowing the production of 25–75 g of antibody to be produced per greenhouse lot in about 2 weeks (Pogue et al. 2010).

9.2.3 Silencing and Its Suppression

In transgenic plants, post-transcriptional gene silencing (PTGS) is observed as the reduction in steady-state levels of transcript being coded from a foreign DNA sequence (Voinnet et al. 1999). This reduction is caused by an increased turnover of target RNA, with the transcription level of corresponding genes remaining unaffected.

RNA silencing triggered by the presence of transgenes requires an RNA-dependant RNA polymerase (RdRp)-like protein to catalyze synthesis of RNA complementary to the target species (Dalmay et al. 2000). Double-stranded RNA is then recognized by a specific nuclease and cleaved to produce 21–23 nucleotide RNA species (Zamore et al. 2000). These small RNAs are proposed to associate with nuclease-like proteins and serve as guides for sequence specific cleavage of target RNA transcripts (Voinnet et al. 1999).

PTGS can be avoided by expressing simultaneously the gene of interest and a suppressor of silencing. Individual plant viruses seem to produce their own suppressor of silencing and the characterization of a large number of suppressors is currently in progress (Lienard et al. 2007). The best characterized suppressor of silencing is the p19 protein, encoded by tomato bushy stunt virus (TBSV). Through agroinfiltration methods, recombinant protein expression has been greatly improved when co-infiltrated in the presence of p19 (Voinnet et al. 2003), up to 50-fold. Use of this method provides a simple route for increasing recombinant protein production in any plant species which is amenable to agroinfiltration.

9.2.4 Syringe vs Vacuum Infiltration

Initially, transient expression by agroinfiltration was developed for assessing the ability of various constructs to induce the expression of recombinant proteins. The best performing constructs would then be used to produce stable transgenic lines. For this, an *Agrobacterium* suspension is infiltrated into the abaxial side of the leaves, the plant most frequently used for these experiments is *N. benthamiana*, but *Nicotiana tabacum* can also be used, as well as other plants such as *Arabidopsis thaliana*, tomato or lettuce (Wroblewski et al. 2005). In addition to the choice of plant species, the developmental stage and physiological status of infiltrated plants can have a major effect on expression levels of target proteins. When co-infiltrated with a suppressor of PTGS such as HcPro from potato virus Y, expression levels can be as high as 1.5 g recombinant protein/kg of LFW (Vézina et al. 2009). Because of the high expression levels and the speed of protein production using *Agrobacterium* syringe infiltration, this method has been scaled up for vacuum infiltration of kilogram amounts of tissue. About half the amount of recombinant protein was obtained using vacuum infiltration in a side by side comparison with syringe infiltration (Vézina et al. 2009). Nevertheless, vacuum infiltration has now been adopted by several groups including Medicago Inc. for the production of influenza vaccines (D'Aoust et al. 2010) and automated by Kentucky BioProcessing, LLC for the magnifection system to infiltrate kilograms of plants/hour (Pogue et al. 2010).

9.3 Applications in Molecular Farming

This section will briefly describe advances in plant-made pharmaceuticals developed for treatment of human diseases, with a focus on transient expression, which seems to be method of choice for fast high level production of complex proteins.

Detailed reviews are available for each of these categories, and not all produced proteins will be discussed, rather a sample of the most successful developed examples will be described.

9.3.1 Vaccines

9.3.1.1 Hepatitis B Virus

Hepatitis B surface antigen (HBsAg) has been produced in transgenic potatoes and shown to be immunogenic in a human clinical trial as a booster in previously immunized human volunteers (Thanavala et al. 2005). However, low expression levels and concerns about using food crops for the production of pharmaceutical proteins triggered the investigation of alternative plant hosts and ways to improve expression levels. *Agrobacterium* infiltration for transient expression of HBsAg was first reported in 2004 by Huang and Mason (Huang and Mason 2004) who used this method for the evaluation of antigen conformation with and without a fusion partner. To optimize the transient expression system, the same authors used the deconstructed MagnICON viral vectors, and showed that HbsAg properly assembled into dimers and virus-like particles and accumulated to 300 mg/kg LFW. Further, immunization of mice with partially purified HBsAg elicited HBsAg-specific antibodies (Huang et al. 2008). In another attempt of developing a hepatitis B vaccine, the MagnIcon vectors were used for expressing the hepatitis B core antigen (HBc) in *N. benthamiana.* HBc accumulated to 2.38 g/kg LFW, assembled into virus-like particles which were immunogenic in mice (Huang et al. 2006). In an attempt at developing other expression vectors, the same group developed a deconstructed viral vector based on the geminivirus bean yellow dwarf virus (BeYDV) to transiently produce HBc to levels up to 1 g/kg LFW (Chen et al. 2011).

9.3.1.2 Human Papilloma Virus

Human papilloma viruses are responsible for causing cervical cancers in women, and are implicated in anogenital and head and neck tumors in both men and women (Bosch et al. 2002). Vaccination is the most efficient way for fighting HPV infections, and two prophylactic vaccines are now available, Gardasil produced in yeast by Merck, and Cervarix produced in insect cells by GlaxoSmithKline. Both vaccines protect against the two high risk HPV types 16 and 18 which cause 70% of all cervical cancers (Bosch et al. 2008). Several groups have focused their efforts at making HPV vaccines in plants as a way to significantly reduce costs of production. The most successful attempt achieved expression levels of 0.5 g/kg LFW (17% TSP) in a transient agroinfiltration system, co-expressing the tomato spotted wilt virus non-structural small silencing suppressor protein, and targeting the HPV-16 L1 protein to the chloroplast (Maclean et al. 2007). Virus-like particles were formed and parenteral administration of concentrated plants extracts elicited high titer antisera

in the same range as those reported for human trial subjects injected with commercial vaccines (Giorgi et al. 2010). A further 50% increase in expression levels was achieved by the same group upon the use of replicating geminivirus sequences in the agro-infiltrated binary vector (Regnard et al. 2010).

9.3.1.3 HIV

The type I human immunodeficiency virus (HIV) is responsible for the acquired immunodeficiency syndrome (AIDS) and currently infects more than 40 million people worldwide, and is continuing to spread, mainly in sub-Saharan Africa. The development of an effective vaccine is essential for controlling the epidemic, and treatments such as neutralizing antibodies for treating infected patients are needed. In either case, large amounts of recombinant proteins are required, and plants can be an inexpensive system for their production, provided accumulation levels are high enough (Rybicki 2010). Several efforts have focused on the production of HIV antigens as chimeric proteins in plants, including structural proteins Gag (and its component proteins p24, p17, and p17/p24) and Env and regulatory proteins Tat and Nef (De Virgilio et al. 2008; Karasev et al. 2005; Meyers et al. 2008; Yusibov et al. 1997; Zhou et al. 2008). Only two of these antigens accumulated to high enough levels, Nef when fused to zeolin accumulated to 1.5% TSP and p24-Nef when expressed from the chloroplast genome accumulated to 40% TSP in petite Havana, a small laboratory tobacco cultivar, and to 6% TSP in a high biomass tobacco cultivar (Marusic et al. 2009; Zhou et al. 2008). Similarly, the Gag-derived p17/p24 fusion protein could be expressed to 5 mg/kg FW by agroinfiltration when targeted to the chloroplast (Meyers et al. 2008).

Another approach for controlling the transmission of HIV consists in producing griffithsin, a potent viral entry inhibitor from the red alga Griffithsia (Mori et al. 2005). This protein is a lectin that targets the high-mannose glycans displayed on the surface of HIV envelope glycoproteins, and inactivates the virus on contact (Emau et al. 2007; Ziółkowska et al. 2006). Griffithsin was produced in *N. benthamiana* through infection with a tobacco mosaic virus-based vector at extremely high levels of more than 1 g/kg of LFW in just 12 days. Plant-made griffithsin was shown to be active, directly virucidal, and capable of blocking cell to cell HIV transmission (O'Keefe et al. 2009).

9.3.1.4 Influenza

Influenza viruses evolve rapidly and require the development of a new vaccine every year for the seasonal influenza season. The probability of occurrence of a pandemic influenza and the identity of the virus causing it are unknown, and therefore the ability of organizations to prepare and stockpile such vaccines is very limited. Once the virus is identified, it usually takes about 6 months to produce the vaccine product which consists of inactivated viruses grown in eggs (D'Aoust et al. 2010). These concerns have triggered interest in developing influenza vaccines in plants using the quick

agroinfiltration technology. Antigenic domains of H5 from strain A/Vietnam/04 (H5N1) and of H3 from A/Wyoming/3/03 (H3N2), both fused to a carrier protein were produced by agroinfiltration in *N. benthamiana.* The immunogenicity of H3 was demonstrated in a ferret study and showed that a single dose in combination with the neuraminidase antigen induced a strong immune response (Mett et al. 2008; Musiychuk et al. 2007). However, the dose of 200 μg required to induce the immune response was much higher than industry standards. Agroinfiltration of the hemagglutinin (HA) domain spanning the outside of the viral envelope from the human seasonal influenza virus A/Wyoming/03/03 (H3N2) and several highly pathogenic H5N1 avian strains was also reported (Shoji et al. 2008, 2009a, b). These studies showed that specific expressed domains induced a significant immune response in mice with the addition of an adjuvant. However, (Shoji et al. 2009a) showed that three high doses were necessary for protecting ferrets against a lethal challenge with the homologous strain. A more successful strategy was developed by the Canadian biotechnology company Medicago Inc., which involves the expression of the entire HA protein from H1N1 strain A/New Caledonia/20/99 and H5N1 strain A/Indonesia/5/05. They found that virus-like particles are produced, bud off the plasma membrane and accumulate between the plasma membrane and the cell wall (D'Aoust et al. 2008). They also showed that the VLPs are more immunogenic than the HA protein that had not assembled into VLPs (D'Aoust et al. 2009). Further, all mice injected with two doses of 0.5 μg of H5-VLPs were protected against a lethal challenge of a different H5N1 isolate (D'Aoust et al. 2008). Medicago Inc. further adapted the agroinfiltration method for scaling up their production capacity and are able to agro-infiltrate batches of 1200–1500 plants weekly from which 25 kg of leaf biomass can be harvested and the VLPs are subsequently purified (D'Aoust et al. 2010). This system was tested for speed of production during the outbreak of the H1N1 pandemic in the spring of 2010, and this group showed that they were capable of obtaining VLPs only 3 weeks after the sequence of the novel A/H1N1 strain A/California/04/09 became available. Further, they showed that the produced VLPs were highly efficacious as a vaccine in a mouse study that lasted another 6 weeks. Therefore, a pandemic VLP vaccine can be produced in plants much faster than by conventional vaccine manufacturing (D'Aoust et al. 2010). These positive results, and the unpreparedness for quickly facing an influenza pandemic have influenced the U.S. Defence Department to invest $21 million US in Medicago Inc, which is now building a large manufacturing facility in North Carolina to grow tobacco plants and produce about 40 million doses of seasonal flu vaccine per year, or 120 million doses of pandemic flu vaccine for the U.S. market (http://www.cbc.ca/news/health/story/2010/11/24/flu-vaccine-tobacco-plants-medicago.html).

9.3.2 Antibodies

Among the biopharmaceutical drugs in development, monoclonal antibodies constitute the fastest growing group because of their outstanding specificities (Aggarwal 2009).

Demand for therapeutic antibodies far exceeds their production capacity in current mammalian expression systems, and plants provide an attractive production platform because they can correctly produce, fold and assemble complex multimeric proteins such as antibodies (Ma et al. 1995). Transient expression in plants using agroinfiltration either with traditional binary vectors (Vézina et al. 2009), with MagnICON vectors (Giritch et al. 2006) or with the use of cowpea mosaic vector hypertranslatable deleted RNA2 (Sainsbury and Lomonossoff 2008) has so far provided the highest expression levels of antibody production of any other plant system; however, glycosylation patterns of secreted monoclonal antibodies are different in plants and can lead to immunogenicity in humans. As well, the approach of retaining the antibodies in the ER which prevents plant specific and immunogenic glycan addition causes their rapid clearance from the blood stream, and causes lower complement-dependent cytotoxicity (Jefferis 2009). Therefore, close attention has been paid to engineering glycosylation pathways in plants to produce "humanized" glycosylation patterns. This has been done by knocking-out endogenous glycosyltransferases and/or knocking-in human glycosyltransferases.

N. benthamiana plants lacking immunogenic β1,2-xylose and core α1,3-fucose were generated by RNAi and were used to transiently produce the 2G12 HIV antibody (Strasser et al. 2008). The antibody was found to effectively contain homogeneous N-glycans without detectable β1,2-xylose and α1,3-fucose residues. The functional properties of the plant produced antibody were similar to those of a Chinese hamster ovary (CHO)-produced 2G12 antibody. For further humanizing the glycosylation patterns of antibodies produced in plants, a human galactosyltransferase was introduced into the previously produced RNAi line lacking α1,3-fucosyl- and β1,2-xylosyl- transferase (Fuc-T and Xyl-T) activities. Two HIV antibodies, 2G12 and 4E10 were subsequently expressed in the resulting plants and were shown to be fully galactosylated and to possess much more homogeneous glycans than CHO cells were able to produce (Strasser et al. 2009). The resulting antibodies displayed improved virus neutralization potency when compared with other glycoforms produced in plants and Chinese hamster ovary cells.

A similar result was achieved in a very elegant experiment where instead of knocking-down Fuc-T and Xyl-T, Vezina et al. (2009) expressed a chimeric form of the human β1,4-galactosyltransferase (Gal-T) together with a diagnostic antibody, C5-1. The chimeric protein consisted in the fusion of the N-terminus of GNTI to the catalytic domain of human Gal-T. GNTI is typically expressed in the ER and cis-Golgi, upstream from the Fuc-T and Xyl-T in the secretory pathway. This allowed the production of C5-1 completely lacking plant-specific β1,2-xylose and α1,3-fucose glycans presumably because the hybrid Gal-T acted at the earliest stage of complex N-glycan synthesis and inhibited further transfer of plant specific xylose and fucose to the core oligosaccharide (Vézina et al. 2009).

Therefore it is clear that complex antibodies can be transiently produced in plants with appropriate post-translational modifications at high levels and with high activity.

9.3.3 Fusions for Improved Accumulation and Purification

Recently, the use of fusion tags has gained popularity in plant-production platforms. Most common fusion tags in use were originally conceived as aids for isolation and downstream purification such as Arg-tag, His-tag, FLAG-tag, c-myc-tag, GST-tag etc.… (Lichty et al. 2005; Terpe 2003). As well, the use of protein-stabilizing proteins as fusions to "difficult to express" proteins has been successful in improving expression levels such as ubiquitin (Mishra et al. 2006), β-glucuronidase (Dus Santos et al. 2002), cholera toxin B subunit (Molina et al. 2004), and human immunoglobulin α-chains (Obregon et al. 2006). However, three fusion partners have emerged that have shown very significant improvements in accumulation levels and that result in the accumulation of recombinant proteins inside round structures that are reminiscent of seed protein bodies (PBs); these are γ-zein from maize, elastin-like polypeptides of mammalian origin, and hydrophobins from fungi. All three partners can also be used in protein purification either by density centrifugation (γ-zein), inverse transition cycling (ELP) or aqueous two phase separation system (hydrophobins). As well, all three fusion partners were shown to give the best results in plants when expressed transiently by agroinfiltration into *N. benthamiana*.

9.3.3.1 Zera Fusions

γ-zein, a major constituent of maize storage proteins is able to induce the formation of PBs in seed and vegetative organs of transgenic dicots in the absence of the other zein subunits α-zein and β-zein (Geli et al. 1994). The N-terminal domain of γ-zein, termed Zera® (Era Biotech, Barcelona, Spain), was shown to be sufficient for the formation of PBs and for the increase in accumulation levels of several proteins; for example, epidermal growth factor accumulated to 100-fold higher levels up to 0.5 g/kg LFW, and human growth hormone accumulation increased by 13-fold to 3.2 g/kg LFW (Torrent et al. 2009). 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 toward PB formation in *N. benthamiana* (Llop-Tous et al. 2010). Zera has been very recently used in transient agroinfiltration of *N. benthamiana* leaves for the expression of a zera-xylanase fusion. Expression levels were up to 9% TSP, corresponding to 1.6 g of the fusion/kg LFW. The fused protein was shown to accumulate as biologically active insoluble aggregates inside PBs (Llop-Tous et al. 2011).

9.3.3.2 ELP Fusions

Elastin-like polypeptides (ELP) are synthetic proteins composed of the pentapeptide repeat VPGVG that occur in mammalian elastins (Raju and Anwar 1987; Urry 1988). Various sizes of ELP tags are used by various groups for expression of recombinant protein fusions in plants; for example, (Scheller et al. 2006) showed that a 100-mer ELP tag increased accumulation of a recombinant antibody fragment up to 40 times in tobacco seeds, while (Patel et al. 2007) showed that a 28-mer ELP tag increased

accumulation of interleukin-4 (IL-4) and IL-10 by 85 and 90-fold, respectively, in tobacco leaves. Therefore, (Conley et al. 2009a) produced an ELP size library starting at 5 repeats up to 240 repeats and tested this library with four different proteins by agroinfiltration. In this study the size of the ELP tag was shown to have a significant impact on the accumulation and the purification of recombinant proteins by inverse transition cycling. Smaller tags were more beneficial for protein accumulation, leading to the accumulation of IL-10 to 4.5% TSP, a 1000-fold improvement from the best expressing IL-10 transgenic line previously reported (Menassa et al. 2007), while larger tags allowed a higher recovery rate of the protein fusion by ITC. An ELP size of 30–40 pentapeptide repeats was found to be a good compromise for both accumulation and purification (Conley et al. 2009a). Furthermore, the fusion protein was found to accumulate in novel spherical, membrane bound structures reminiscent of Zera PBs. It is thought that these PBs allow higher accumulation levels by protecting the recombinant protein from the degradative machinery of the cell (Conley et al. 2009b).

9.3.3.3 Hydrophobin Fusions

Hydrophobins are a class of small surface active proteins produced by filamentous fungi, and are thought to be involved in the adaptation of fungi to their environment (Talbot 1999). Hydrophobins coat fungal surfaces and have been proposed to protect against desiccation and wetting and to contribute to spore and conidial dissemination (Linder et al. 2005). These small proteins (ca. 10 kDa) contain a large proportion of hydrophobic amino acids and eight cysteines all of which involved in four intramolecular disulfide bonds (Hakanpaa et al. 2004). The unique surface-active properties of hydrophobins can be transferred to their fusion partners, a property that has been exploited for a rapid and inexpensive surfactant-based aqueous two phase purification system, ATPS (Linder et al. 2004). Recently, we have shown by agroinfiltration of *N. benthamiana* that hydrophobin fusions allow for extremely high accumulation levels of GFP, up to 3.7 g/kg LFW. Similar high expression levels were obtained with glucose oxidase, an enzyme that did not express well in other conventional systems (Bankar et al. 2009; Joensuu et al. 2010). In the same study we also found that GFP-hydrophobin fusion accumulates in PBs, and that infiltrated leaves survived longer when the fusion rather than GFP alone was used in the infiltration. This indicates that PBs not only protect the recombinant protein from degradation as observed with ELP, they also protect cells from the toxicity of highly expressed proteins (Conley et al. 2011; Joensuu et al. 2010).

9.4 Conclusions

Agroinfiltration enhances the value of plant-based expression systems, serves as a very useful tool to boost research in plant biology and opens new possibilities for studying protein overexpression in plants. With the fast production timeline, good

yield and well defined co- and post-translational modifications, agroinfiltration shows promise for plant-derived bioproducts and can now compete with classical eukaryotic expression systems.

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