

# Plant Bioreactors for Pharmaceuticals

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## Abstract

Plant bioreactors are attractive expression systems for economic production of pharmaceuticals. Various plant expression systems or platforms have been tested with certain degrees of success over the past years. However, further development and improvement are needed for more effective plant bioreactors. In this review we first summarize recent progress in various plant bioreactor expression systems and then focus on discussing protein compartmentation to unique organelles and various strategies for developing better plant bioreactors.

## Introduction

The demand for recombinant therapeutic molecules for clinical applications is increasing significantly over the past decades. The biotechnologists have been trying to develop new promising biofactories for economic production of therapeutic molecules. Besides the conventional microbial and animal cell culture systems, recent progress in using plants as bioreactors for producing recombinant proteins provides a promising and rich source for various bioactive molecules. Plant bioreactors are cost-effective and easy for agricultural scale-up. As a result, certain expensive biopharmaceuticals

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**Abbreviations:** CaMV, cauliflower mosaic virus; CT, cytoplasmic tail; ER, endoplasmic reticulum; PSV, protein storage vacuole; PVC, prevacuolar compartment; TIP, tonoplast intrinsic protein; TMD, transmembrane domain; VSD, vacuolar sorting determinant; VSR, vacuolar sorting receptor; OB, oil body.

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such as human lysosomal enzymes can be produced in plant bioreactors and this is particularly applicable in developing countries. Plant bioreactors have the advantages of having post-translational modifications and lacking of contamination by animal pathogens (Giddings, 2001; Giddings *et al.*, 2000; Kusnadi *et al.*, 1998; Lienard *et al.*, 2007; Ma *et al.*, 2003).

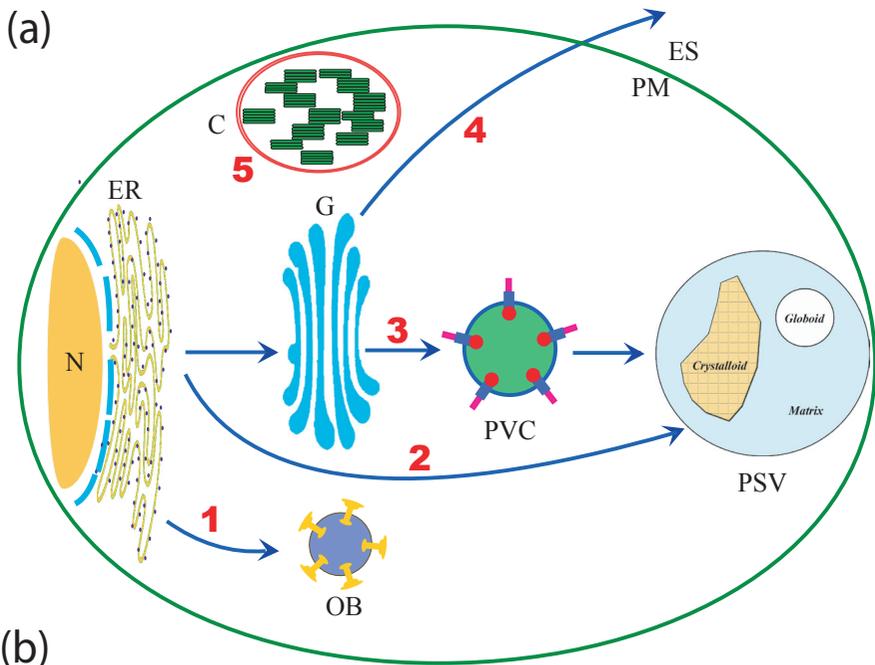
More recently, several strategies and new expression systems have been developed and tested in plants for their feasibility to realize plant-derived pharmaceutical molecules (Ma *et al.*, 2003). The first plant-produced pharmaceutical product, the human growth hormone, was produced in transgenic tobacco in 1986 (Barta *et al.*, 1986). Since then, diverse plant species including cereals, legumes, fruits and vegetables were exploited for the production of recombinant molecules such as human biopharmaceuticals, recombinant antibodies and recombinant subunit vaccines proteins (Ma *et al.*, 2003). In addition, six types of antibodies and two vaccine candidates that were derived from plants have already reached the preclinical testing stage and clinical trials, respectively (Ma *et al.*, 2003).

Recombinant proteins expressed in plant bioreactors are subjected to compartmentation and degradation in the plant cell. Even though various successful expression systems have been reported in plant bioreactors including the use of seed protein storage vacuoles or oil-bodies, cell suspension cultures, root exudates and chloroplasts (Borisjuk *et al.*, 1999; Conrad and Fiedler, 1998; Downing *et al.*, 2006; Giddings *et al.*, 2000; Komarnytsky *et al.*, 2000; van Rooijen and Moloney, 1995), a critical problem in plant bioreactors thus far is the low yield of the recombinant proteins due to the degradation system in plants. As a result, one of the possible solution or improvement is to co-express the target protein together with protease inhibitor to protect the protein from being degradation and thus leading to high yield of the recombinant proteins in transgenic plants (Kim *et al.*, 2007; Komarnytsky *et al.*, 2006; Rivard *et al.*, 2006; Xu *et al.*, 2004).

*Figure 1* shows several representative plant organelles for protein compartmentation of pharmaceuticals and their targeting or delivery strategies in plant bioreactors, where Golgi-bypassing of recombinant proteins to reach seed protein storage vacuole (PSV) would avoid unwanted plant-specific complex glycan modification. Thus, here we will discuss recent progress in various plant bioreactor expression systems, with the emphasis on considering various unique strategies of protein compartmentation in plant bioreactors.

### **Seed-based bioreactor platform**

Seeds provide a suitable environment for storing recombinant proteins in plant bioreactors because plant seeds have high rates of protein synthesis and accumulate a large amount of proteins during seed development (Bewley and Black, 1994). Most soluble proteins in seeds are stored in a specific compartment termed the protein storage vacuole (PSV) or protein body to be used upon seed germination (Herman and Larkins, 1999). Therefore, seed bioreactors offer many advantages for producing recombinant proteins, including their stable accumulation in a small volume and the easy long-term storage of protein avoiding degradation at ambient temperature (Ma *et al.*, 2003; Stoger *et al.*, 2005). For example, antibodies produced in seeds have shown no loss of activity for at least five months at room temperature (Stoger *et al.*,



(b)

Pathway	Storage Organelle	Targeting Mechanisms
<b>1</b>	 Oil body	 Oleosin
<b>2</b>	 Protein storage vacuole	Golgi-bypassing via specific targeting sequences (e.g. alpha-TIP)
<b>3</b>	 Protein storage vacuole	 Vacuolar sorting receptor
<b>4</b>	Extracellular space	N-terminal signal peptide of secretory protein via default pathway
<b>5</b>	 Chloroplast	Transit peptide or organelle transformation

**Figure 1.** The plant bioreactor system.

Plant bioreactor via protein compartmentation within a plant cell. Shown are various intracellular organelles or extracellular spaces (ES) that can be used to store the recombinant proteins expressed in a plant bioreactor. The expressed recombinant proteins can reach their final destination for compartmentation and modification via distinct vesicular transport pathways. Golgi-bypassing of recombinant proteins for PSV targeting would avoid unwanted plant-specific complex glycan modification.

Targeting strategies in plant bioreactors. Shown are five targeting mechanisms that can be used to deliver the expressed recombinant proteins to reach the specific storage compartments in plant bioreactors. ER, endoplasmic reticulum; G, Golgi; PSV, protein storage vacuole; OB, oil body; C, chloroplast; N, nucleus; PM, plasma membrane; ES, extracellular space; PVC, prevacuolar compartment.

2000). Therefore, protein-rich cereal crops, grain legumes and oilseeds are attractive seed-based platforms for the production of recombinant proteins with a particularly bright future in agricultural application. Due to the high grain yield and capability of the rapid scale-up, maize in cereal crops has recently been used to produce enzymes,

recombinant antibodies and vaccine candidates (Hood, 2002; Hood *et al.*, 2002; Streatfield *et al.*, 2003). Legumes, seeds of pea and soybean, with ~40% protein content, have also been used to produce recombinant proteins including the single-chain Fv fragment antibody and humanized anti-herpes simplex virus 2 monoclonal antibody under the control of seed-specific promoter (Perrin *et al.*, 2000; Zeitlin *et al.*, 1998). On the other hand, due to its easy transformation and short regeneration time, *Arabidopsis thaliana* has also been used as a model to express pharmaceutical proteins before administering to crops for large-scale field application. For example, a human lysosomal enzyme alpha-L-iduronidase that can be used in treating lysosomal storage disorders has been successfully expressed with high enzymatic activity in *Arabidopsis thaliana* seeds (Downing *et al.*, 2006). Furthermore, to control the expression of recombinant proteins limited to seed bioreactors, various seed specific promoters, such as the glutelin promoter Gt-1 in rice, globulin-1 in maize and aleurone-specific promoters from barley, have been used in several application with success (Hood *et al.*, 2003; Hwang *et al.*, 2001; Hwang *et al.*, 2003).

Seeds have shown an attractive and competitive system for the economic production of pharmaceutical molecules and clinical enzymes. However, many factors could affect their final effective applications including the specific expression in seeds, subcellular storage environment and posttranslational modifications (Li and Jiang, 2006). Promising progress in the last decades has been the improvement in the efficiency of molecular farming in seeds, where the seed specific promoters and manipulation of protein sorting pathway have been studied intensively.

#### SEED PROTEIN STORAGE VACUOLE AS BIOREACTOR

Protein storage vacuoles (PSVs) are the major compartments for the storage of recombinant proteins in seed bioreactors (*Figure 1*). PSV in most seeds contains three morphologically and biochemically distinct subcompartments: the matrix, globoid and crystalloid. Individual PSV subcompartments possess unique properties and environments suitable for storing various recombinant proteins: the matrix is the major deposit tank of the soluble storage proteins; the globoid has acidic environment containing phytic acid or oxalate crystals; and the crystalloid lattice structure might be composed of storage proteins (Jiang *et al.*, 2000). Thus pharmaceutical hydrolytic enzyme can be targeted to PSV globoid with a lytic environment for enzyme stable accumulation and can also be separated from the storage proteins in both matrix and crystalloid, where the protein stability and enzymatic activity can be maintained as well.

Soluble proteins reach the seed PSV and its subcompartment via specific transporting pathways because they contain unique targeting signals (Jiang and Rogers, 2003). Thus, these specific targeting signals can be used to target the expressed recombinant proteins to PSV and its subcompartments via distinct transport pathways for specific protein modification (Jiang and Sun, 2002). For example, the transmembrane domain (TMD) and cytoplasmic tail (CT) of BP-80 can direct a reporter from prevacuolar compartment (PVC) to PSV globoid, whereas the combination of BP-80 TMD and the CT of alpha-tonoplast intrinsic protein (TIP) sequences target the same reporter to PSV crystalloid in transgenic tobacco seeds (Jiang *et al.*, 2002). Similarly, AFVY, a vacuolar sorting determinant of phaseolin, can deliver reporter or recombinant protein to PSV

matrix that is separated from the globoid and crystalloid. *Figure 2* shows examples of known sequences that can be used for delivering recombinant proteins to distinct PSV subcompartments in seed bioreactors. In fact, when a soluble recombinant protein with anchored PSV targeting signal was expressed in transgenic tobacco seeds, it was correctly targeted and stably accumulated in seed PSVs abundantly (*Figure 3*).

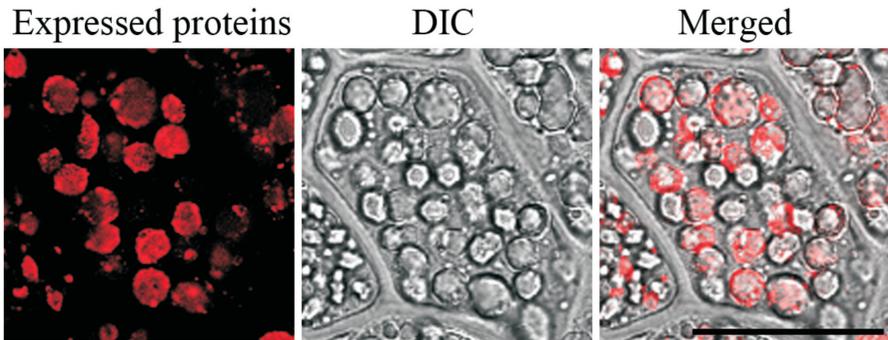
	Fusion structure	Expected destination
(a)		PSV crystalloid
		PSV globoid
		PSV matrix
(b)		Recombinant protein
		Transmembrane domain of BP-80
		C-terminal of -TIP
		C-terminal of BP-80
		AFVY signal from phaseolin

**Figure 2.** Membrane anchors for PSV targeting in seed bioreactors. Shown are various unique plant protein sequences that can be used as anchors to target for delivery of the recombinant proteins to specific PSV subcompartments for accumulation in seed bioreactors.

#### SEED OIL BODY AS BIOREACTOR

In addition to seed PSV, seed oil body (OB) is also an attractive bioreactor because it stores large amount of macromolecules (Boothe *et al.*, 1997; Huang, 1996; Huang, 1992; Murphy, 1990; van Rooijen *et al.*, 1995; and Siloto *et al.*, 2006). In plant seeds, OBs are surrounded by a unique protein termed oleosin with high density that function in maintaining the structural integrity of OBs and providing a recognition signal for lipase binding during oil mobilization in seedlings (Huang, 1996; Murphy, 1993). Oleosins are 15-26 kDa hydrophobic proteins highly enriched in OBs (Huang, 1996; Murphy, 1993). The small size of oleosin makes it an ideal carrier for heterologous protein production in plant bioreactor. For example, when a fusion protein containing oleosin and the  $\beta$ -glucuronidase was expressed in transgenic plants, it was successfully targeted and stably accumulated on seed OBs (Hsieh and Huang, 2004; van Rooijen *et al.*, 1995).

The use of OBs as the storage organelles in plant bioreactor has many advantages. Firstly, OBs exist in diverse tissues including seeds, pollen and fruits but especially



**Figure 3.** Stable accumulation of recombinant proteins in protein storage vacuoles (PSVs) of transgenic tobacco seeds. Mature seeds from transgenic tobacco plants expressing a recombinant protein were fixed and labeled with specific antibodies, followed by detection using rhodamine-conjugated secondary antibodies with a confocal microscope. Red signals indicated the expressed recombinant proteins, while the morphology of the seed PSVs (as indicated by arrows) were shown in the DIC (differential interference contrast) image. Scale bar = 10  $\mu\text{m}$ .

enrich in oilseeds (Huang, 1996; Huang, 1992; Ting *et al.*, 1998; Vance and Huang, 1987; Wu *et al.*, 1997). Secondly, via the fusion with oleosins, the recombinant proteins (fused with oleosins) can be easily purified from the OBs system, where OBs can be separated from other cellular components by using a flotation centrifugation method in downstream processing due to the high ratio of triacylglycerides in OBs (van Rooijen *et al.*, 1995). Thus, after centrifugation of grounded oilseeds, intact OBs associated with oleosin fusion proteins can be highly concentrated in the upper part, making it easy for protein recovery and further purification. Using this system, hirudin, a pharmaceutical protein commonly used as anticoagulants to prevent thrombosis, has been successfully expressed and purified in seed of *Brassica napus* (Boothe *et al.*, 1997; Murphy, 1990; Tzen *et al.*, 1997) and *Brassica carinata* (Chaudhary *et al.*, 1998). Such OBs-derived hirudin also show high stability, in which no degradation of the fusion protein has been observed even after two years of seed storage (Boothe *et al.*, 1997; Murphy, 1990; Tzen *et al.*, 1997). In *Brassica napus* seed, triacylglycerides account for 40-45% of its dry weight while oleosin contributes 8-10% of total seed proteins (1.6-2.0% of seed dry weight) (Boothe *et al.*, 1997), thus making it an ideal platform for an OB bioreactor.

The OB bioreactor is not a perfect system. For example, the fusion protein might not be fully released from digested OBs that cause a decrease in productivity (Parmenter *et al.*, 1996). Furthermore, truncated forms of recombinant proteins - including hirudin - have also observed, increasing the complexity of the recombinant proteins for downstream processing (Boothe *et al.*, 1997). In addition, the cost of cleavage of oleosin from the recombinant protein is another concern for commercial application of the oleosin partitioning technology, despite the recent success in using immobilized proteases (Boothe *et al.*, 1997; Kuhnel *et al.*, 2003).

### Secretion strategy in plant bioreactors

Transgenic plants and seeds as bioreactors may also possess some disadvantages, including the risk of contamination from field-grown environments, high cost of

downstream processing and purification of recombinant proteins. Thus, the secretion platforms have also recently been developed using suspension culture cells and root systems to target the pharmaceutical molecules into the cultured media, thus providing an effective and economic system for protein purification in downstream processing.

#### RECOMBINANT PROTEIN EXPRESSION USING PLANT SUSPENSION CULTURES

Plant suspension cultured cells provide a fast system for producing secondary metabolites, biologically active recombinant proteins and antibodies (Francisco *et al.*, 1997; Terashima *et al.*, 1999; Torres *et al.*, 1999). The recombinant proteins expressed can either be transported to subcellular organelles or be secreted into extracellular space via the default pathway in plant suspension cultured cells (Denecke *et al.*, 1990; Liu *et al.*, 1997). The latter secretion method provides a cost-effective strategy for stable accumulation and purification of the secreted recombinant proteins. In addition, suspension cultures also provide a fast screening platform for testing various recombinant proteins before they are further expressed in transgenic plants in seed bioreactors.

The plant cell wall was previously described as a significant barrier for secretion of proteins with size larger than 30 kDa (Carpita *et al.*, 1979; Fischer *et al.*, 1999). When expressed in transgenic tobacco BY-2 cells, a human protein erythropoietin was found to fail to be secreted into the cultured medium (Matsumoto *et al.*, 1995). However, our recent study has demonstrated that a 80-kDa human lysosomal protein, under the control of the 35S CaMV constitutive promoter and a signal peptide from a plant protein, can be successfully expressed and secreted into the cultured media of transgenic tobacco BY-2 cells. These secreted proteins have been shown to acquire N-linked glycosylation and had very high enzymatic activity, a result demonstrating the correct folding of this BY-2 cells-derived human lysosomal enzyme (Fu *et al.*, 2008, our unpublished data).

During transit through the Golgi apparatus, recombinant protein expressed in plant suspension cultures may acquire complex-glycan modifications that are highly immunogenic to human. Several strategies have been proposed to solve this potential problem in a plant bioreactor, including inhibition of the plant Golgi enzyme glycosyltransferases, expression of the mammalian glycosyltransferases and manipulation of the vesicular transport pathway via Golgi-bypassing (Gomord and Faye, 2004; Wang *et al.*, 2006).

#### RECOMBINANT PROTEIN EXPRESSION USING THE HAIRY ROOT SYSTEM

The hairy root system, generated by the infection of the soil bacterium-*Agrobacterium rhizogenes* (Chilton *et al.*, 1982; Shanks and Morgan, 1999), is one of the most mature systems using plant tissue for the production of biopharmaceuticals (Sivakumar, 2006). The fast growth property, long-term genetic and biosynthetic stability have made "hairy root" an excellent bioreactor system. Hairy root can synthesize the same components as those of the intact roots of transgenic plants (Choi *et al.*, 2006; Hashimoto and Yamada, 1987; Shanks *et al.*, 1999). Several biopharmaceuticals have been successfully produced by the hairy root system (Banerjee *et al.*, 1998; Borisjuk *et al.*, 1999; Jouhikainen *et al.*,

1999; Wongsamuth and Doran, 2000), where 100-fold enhancement of scopolamine was achieved in *Hyoscyamus muticus* L. hairy root culture (Jouhikainen *et al.*, 1999). In addition, the secretion system of hairy root, called “rhizosecretion”, also offers a simplified method for the isolation of recombinant proteins from simple hydroponic medium (Borisjuk *et al.*, 1999; Sivakumar, 2006), where recombinant proteins can be collected continuously over the life span of a transgenic plant (Denecke *et al.*, 1990; Sivakumar, 2006). Silencing of transgenes is a common problem in the hairy root system and thus a major commercial obstacle to its application (Sivakumar, 2006).

### **Chloroplast as a bioreactor**

Chloroplasts are the most general organelles in plants cells and eukaryotic algae. Since the establishment of the chloroplast transformation system in 1990 (Svab *et al.*, 1990), chloroplasts have been considered as an ideal organelle to produce recombinant proteins.

Two different methods have been tested to produce and target heterologous proteins in chloroplast. One strategy is to stably insert the foreign gene into nuclear chromosomes and then target the expressed proteins into chloroplast via transit peptide, a chloroplast targeting signal (Comai *et al.*, 1988; Della-Cioppa *et al.*, 1986; Rangasamy and Ratledge, 2000). The other approach is to directly target and express the genes into the chloroplast genome (Daniell *et al.*, 2005a; Tregoning *et al.*, 2003). This transplastomic expression is the most common and successful method for the production of recombinant proteins using the chloroplast bioreactor, where many chloroplast-derived biopharmaceutical proteins including insulin, interferons, and somatotropin have been evaluated (Arlen *et al.*, 2007; Ruhlman *et al.*, 2007; Staub *et al.*, 2000).

#### ADVANTAGES AND PROGRESS OF THE CHLOROPLAST BIOREACTOR

The molecular farming of recombinant proteins using a chloroplast bioreactor has several advantages, including high level of protein expression, high protein stability, easy manipulation and highly abundance of chloroplasts in green leaves. Upon chloroplast transformation with high copies of target gene stably integrated into the chloroplast genome, transgenic plants can accumulate recombinant proteins as high as 46% of the total leaf proteins (Arlen *et al.*, 2007; De Cosa *et al.*, 2001). Benefiting from the prokaryotic organization of the chloroplast genome (Arlen *et al.*, 2007; Murphy, 2007; Svab *et al.*, 1990), nearly no gene silencing has been observed in chloroplast bioreactor even when the accumulation of transcripts is 169 times higher in chloroplast than that in nuclear (Lee *et al.*, 2003). In addition, the chloroplast genome can be precisely manipulated because of its small size, in which exogenous genes can be site-specifically integrated into the chloroplast genome via homologous recombination using a DNA sequence derived from the chloroplast genome (Daniell *et al.*, 1998; Sidorov *et al.*, 1999). Furthermore, the simultaneous expression of multiple genes in a single transformation is also feasible via genetic engineering. After transcription, foreign proteins can also be properly folded (Arlen *et al.*, 2007; Glenz *et al.*, 2006; Ruhlman *et al.*, 2007; Staub *et al.*, 2000).

Several approaches have been tested to address the issue of protein stability inside the chloroplast. For example, overexpression of the chaperone crystal protein of *Bacillus thuringiensis* that would form the cuboidal crystal structure to protect the expressed foreign protein from degradation resulted in 128-fold accumulation of recombinant proteins in the chloroplast bioreactor (De Cosa *et al.*, 2001). Similarly, the expression of human serum albumin protein in chloroplast can be increased to 500-fold due to the formation of inclusion bodies (Fernandez-San Millan *et al.*, 2003). Furthermore, fusion protein techniques can enhance the stability of foreign protein, where human insulin accumulated up to 16% of the total soluble proteins when it was expressed as a fusion with Cholera toxin B-subunit protein in transgenic plants (Ruhlman *et al.*, 2007). To make sure that high mRNA levels correlate well with high level of protein accumulation in chloroplast, specific expression cassettes in chloroplast transformation has been used to obtain an optimal protein levels in leaves of transgenic plants (Arlen *et al.*, 2007). In addition, to minimize the deleterious effects or burden on the transgenic plants caused by high level expression of the heterologous proteins (Arlen *et al.*, 2007; Magee *et al.*, 2004), inducible promoters have been used to control the transgenes for restricted expression of the recombinant protein to certain timeframe (Streatfield, 2007).

Chloroplast bioreactors for the production of recombinant proteins used to be limited to certain tissue types and species (Ruf *et al.*, 2001; Sidorov *et al.*, 1999; Svab *et al.*, 1990). However, great progress has been made to overcome the limitations of chloroplast transformation, thus expanding chloroplast bioreactor to several important economy crops including soybean, carrot, lettuce, rice, and oilseed (Daniell *et al.*, 2005b; Dufourmantel *et al.*, 2004; Hou *et al.*, 2003; Kumar *et al.*, 2004; Lee *et al.*, 2006; Lelivelt *et al.*, 2005; Ruhlman *et al.*, 2007). In terms of safety, the chloroplast bioreactor would have a low risk of environmental and food chain contamination because chloroplast genes are maternally inherited in most angiosperm plants and transgenic plants derived from chloroplast transformation are not disseminated by pollen (Arlen *et al.*, 2007; Daniell *et al.*, 2002).

### **Co-expression of proteinase inhibitors in plant bioreactors**

One of the major obstacles in the plant bioreactor is the low yield of recombinant proteins (de Wilde *et al.*, 2007; Ma *et al.*, 2003; Twyman *et al.*, 2003), which are susceptible to proteolytic degradation in transgenic plant cells (De Neve *et al.*, 1993; Doran, 2006; Stevens *et al.*, 2000). Therefore, reducing protease activity in transgenic plants is one of the potential approaches to improve the yield of recombinant proteins.

Proteinase inhibitors (PIs) can be added directly to transgenic plants, cultures and/or to protein extraction buffers to prevent unwanted protein degradation. For example, Bacitracin was applied to the suspension culture of transgenic tobacco (*Nicotiana tabacum*) cells, resulting in a 2.18-fold improvement in yield of recombinant human granulocytemacrophage colony-stimulating factor (hGM-CSF) (Lee *et al.*, 2003). The addition of PIs in transgenic tobacco BY-2 suspension culture also reduced the proteolysis of the recombinant *Desmodus rotundus* salivary plasminogen activator alpha1, improving the accumulation of intact product (Schiermeyer *et al.*, 2005). Although natural or synthetic exogenous PIs could provide protection against the

extracellular proteases, the use of these additives results in an increased cost due to the need of additional purification steps to remove them. In addition, exogenous PIs are less effective in protection of recombinant proteins against the intracellular proteases that are responsible for degradation of recombinant proteins within the plant cells.

Three genetic engineering techniques might be used to reduce the proteolytic degradation of recombinant proteins in plant cells, including 1) removal or replacement of the proteolytic sites from the recombinant proteins, 2) silencing of the genes encoding the major proteases in plant cells, and 3) inhibition of the proteolytic activities by co-expression of PIs. The first technique requires the investigation of the specific amino acid sequences that are responsible for protease cleavage, which is a poorly understood aspect for most plant proteases (Garcia-Lorenzo *et al.*, 2006), whilst change of the recombinant protein's sequence might cause undesired side-effects on recombinant proteins, including shorter half-lives, loss of the biological activities and antigen responses when used as biopharmaceuticals. For the second technique, specific proteases that are responsible for the degradation of recombinant proteins in plant cells need to be identified. This strategy, however, is promising because of the successful applications of protease-deficient *E. coli* (Rozkov and Enfors, 2004) and yeast (Cereghino and Cregg, 2000) strains. Considering the facts that proteases are released in all stages of plant development (Schaller, 2004) and the interaction mechanisms between proteases and PIs have been well established (Christeller, 2005), the third approach, namely co-expression of PIs in transgenic plants, would be a more feasible way to solve the problem of recombinant protein degradation in the plant bioreactor.

Xu *et al.* (2004) have reported that the heterogeneous expression of a PI from nightshade (SaPIN2a) in transgenic lettuce resulted in the inhibition of plant endogenous trypsin-like and chymotrypsin-like activities, suggesting that the heterogeneous expressed PIs could be exploited in the protection of recombinant protein production in transgenic plants. Komarnytsky *et al.* (2006) further confirmed that co-secretion of soybean Bowman-Birk Ser protease inhibitor reduced degradation of the immunoglobulin complexes in the secretion pathway of transgenic tobacco roots and increased antibody production. Rivard *et al.* (2006) also found that transgenic potato expressing either tomato cathepsin D inhibitor (CDI) or bovine aprotinin showed decreased levels of cathepsin D-like and ribulose 1,5-bisphosphate carboxylase/oxygenase hydrolysing activities *in vitro*, and the decreased turnover rates of the selection marker protein neomycin phosphotransferase II (NPT II). More recently, Kim *et al.* (2007) used overlap PCR to synthesis a serine protease inhibitor sPI-II harboring the chymotrypsin and trypsin inhibitor domains in an effort to reduce proteinase activity in rice cell suspension culture and then introduced the sPI-II gene into rice cells, resulting in approximately a 77% reduction of proteinase activity in transformed cell suspension culture when compared to non-transformed culture.

Because some endogenous proteinases play important roles in regulating plant development and growth, it is important to choose specific PI that could effectively inhibit the proteases responsible for the degradation of recombinant proteins without affecting the normal growth of plants or plant cells. Zavala *et al.* (2004) have shown that constitutive overexpression of trypsin PI (TPI) in transgenic *Nicotiana attenuata* incurred large fitness costs, resulting in a large reduction in growth rate, plant height and seed production. Overexpression of a sieve element-localized PI of *Solanum*

*americanum*, SaPIN2a, also caused growth retardation in transgenic plants, which might be due to the impaired differentiation of sieve elements in transgenic plant phloem (Xie *et al.*, 2007).

### Concluding remarks

In conclusion, *Table 1* summarizes various factors that may affect the application of various plant bioreactor systems in producing pharmaceuticals economically. Based on our own research experience and the research results that have been obtained thus far, it is also most likely that a single expression system in plant bioreactor will not fit for producing all pharmaceuticals, and each system may only be suitable for several or even a single recombinant protein. In addition, the physiological difference of various plants or tissues also makes it complicate to apply a single expression system universally. Therefore, case-by-case and proof of concept are still a must in using plant bioreactors to produce recombinant proteins or pharmaceuticals successfully in plant biotechnology.

**Table 1.** Comparison of different plant bioreactor systems.

Bioreactors	Seed	Plant suspension cultured cell	Hairy root system	Oil body	Chloroplast
Overall cost	low	High	high	low	low
Production timescale	long	short	short	long	moderate
Purification difficulty	moderate	Low	low	moderate	moderate
Product stability	high	Low	low	high	moderate
Storage difficulty	dried/ambient	chilled	chilled	dried/ambient	desiccated/frozen
Storage cost	low	High	high	low	high
Shipment cost	low	moderate	moderate	low	moderate
Scale-up capacity	very high	medium	high	very high	very high

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