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Review

The plant vesicular transport engineering for production of useful recombinant proteins

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Abstract

The molecular breeding of plants that have been genetically engineered for improved disease resistance and stress tolerance has been undertaken with the goal of improving food production. More recently, it has been realized that transgenic plants can serve as bioreactors for the production of proteins or compounds with industrial or clinical uses. Several different recombinant enzymes and antibodies have been produced in this manner. To maximize the potential of industrial plants as a production system for proteins, efficient expression systems utilizing promoters that optimize transgene expression, 5′-untranslated region elements for efficient translation, and appropriate post-translational modifications and localization must be developed. This review summarizes successful examples of the production of recombinant enzymes, antibodies, and vaccines using signal peptides that direct vesicular localization in transgenic plants. We further discuss the modulation of recombinant protein localization to the endoplasmic reticulum, vacuolar system, or extracellular compartments by varying the signal peptide.
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Keywords: Vesicular transport system; Transgenic plants; Secretory production of enzymes and antibodies; Signal peptide; Peroxidase

1. Introduction

Foreign genes can be introduced into any plant species, and it is possible to selectively breed plants for the expression of specific characteristics. Herbicide-resistant crops, insect resistant crops, long-life tomatoes, and soybeans with increased oleic acid content have already been developed, and some of these plants are available in the market. The industrial production of amino acids and polyhydroxy alkanoate in plants may be put to practical use in the near future. From an economical point of view, the production cost of biomaterials using plants is less than that for compounds produced through fermentation. For plants, solar energy, CO_2 and inorganic chemicals are the only materials needed once suitable seeds have been produced, but equipment, substrate, and electric energy are constantly needed for materials produced by fermentation. Transgenic plants are produced by introducing a limited number of genes that lead to desired phenotypes. The generation of transgenic plants with more valuable and useful traits requires the consideration of not only the structure of genes encoding proteins of interest, but also technologies that control transgene expression. Of these factors, the chosen promoter is an essential factor that drives the transcription of the transgene. The promoter of cauliflower mosaic virus (CaMV) 35S RNA gene [1] is widely used for constitutive and high-level expression of foreign genes in many kinds of plants. It is possible that organ or tissue specific promoters can be used for these purposes, as well [2]. Use of a 5′-untranslated region (UTR) as a *cis*-element is also important for the efficient translation of polypeptides from mRNA. Chimeric constructs containing an appropriate promoter, 5′-UTR, and the gene of interest can be inserted into the T-region of a binary vector and introduced into the chromosomal DNA of plant cells by *Agrobacterium*-mediated transformation [3].

2. Production of recombinant proteins in the plant vesicular transport system

The development of a highly efficient system for the production of exogenous proteins in plants requires

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Table 1
Useful enzymes and proteins production in transgenic plants

Proteins (origin)	Host plants	Signal peptides (origin)	Reference
Invertase (yeast)	Tobacco and *Arabidopsis*	Protease inhibitor II (potato)	[13]
Seed albumin (pea)	Tobacco	Phytohemagglutinin (soybean)	[14]
ChiA (*Serratia marcescens*)	Tobacco	PR1b (tobacco) and own S.P.	[15]
α-Amylase (*Bacillus licheniformis*)	Tobacco	PR-S (tobacco)	[16]
T4 lysozyme (bacteriophage)	Potato	α-Amylase (barley)	[17]
Phytase (*Aspergillus niger*)	Tobacco	PR-S (tobacco)	[18]
α-Trichosanthin (medical plant)	Tobacco	Own S.P.	[19]
Erythropoietin (human)	Tobacco	Own S.P.	[20]
Xylanase (*Clostridium thermocellum*)	Tobacco	Protease inhibitor	[21]
α1-Antitrypsin (human)	Suspension culture of rice cells	α-Amylase (rice)	[22]
Collagen (human)	Tobacco	PR1b (tobacco)	[23]
(1,3-1,4)-β-Glucanase	Barley	D hordein (barley)	[24]
Spider silk protein	Tobacco and potato	Legumin + KDEL	[25]
Follicle stimulating hormone (human)	Tobacco	Own S.P.	[26]
GM-CSF (human)	Suspension culture of tobacco cells	Own S.P.	[27]
Sucrose isomerase (*Erwinia rhapontici*)	Tobacco	Protease inhibitor II (tobacco)	[28]
Interleukin subunits (human)	Suspension culture of tobacco cells	Own S.P.	[29]
Alkaline phosphatase	Hairy root of tobacco	Own S.P.	[30]
β-Casein (human)	Potato	Own S.P.	[31]
α-Lactalbumin (human)	Tobacco	Own S.P.	[32]
Lactoferrin (human)	Maize	Sporamin (sweet potato)	[33]
Methylmercury lyase (bacteria)	*Arabidopsis*	Extensin (tobacco) + KDEL	[34]
Peroxidase C (horseradish)	Suspension culture of tobacco cells	Own S.P.	[35]

improvements in the relevant existing technology. In addition to controlling transcription and translation, regulating the intracellular localization of proteins is also crucial. Expression of recombinant protein in chloroplasts has been successful in the past, but the enzymatic machinery available to modify these proteins is limited [4]. For recombinant proteins that require post-translational modifications (e.g. glycosylation) for their activity, expressing proteins through the vesicular transport system could be useful. In plants, at least two types of vacuoles are known to exist, the protein storage vacuole (PSV) and the lytic vacuole (LV). The PSV is a useful candidate as a target of foreign protein accumulation. Two types of vacuolar sorting determinants (VSD), the sequence-specific VSD (ssVSD) and the C-terminal VSD (ctVSD), are responsible for sorting proteins to the LV and PSV, respectively [5]. ssVSDs have been identified in sweet potato sporamin and barley proaleurain [6,7], while ctVSDs have been identified in the proregions of storage proteins, including barley lectin and brazil nut 2S albumin [8,9], and of pathogenesis-related proteins including chitinase, β-1,3-glucanase, and osmotin [10,11]. ssVSDs have a consensus amino acid motif, N-P-I-R, but there is no such conserved motif for the ctVSDs. There are few reports of recombinant proteins targeted to the PSV, and further investigation into the use of these newly-identified sorting signals must be undertaken to develop a high-level protein accumulation system in plants.

The default pathway for vesicular protein transport is secretion in eukaryotic cells. It has been reported that carboxyl-terminal propeptide (CTPP) deletion mutants of vacuolar chitinase, β-1,3-glucanase and AP24, which possess only an NTPP, were secreted from transgenic tobacco cells (*Nicotiana tabacum*) to outside of the cell [11,12]. Generally, the secretion of foreign proteins becomes possible by the attachment of the signal peptide (propeptide) at the N-terminus of protein that allows entry into the vesicular transport system. In this case, secretory production includes the accumulation of recombinant proteins in the cell wall region, apoplastic space, or endoplasmic reticulum (ER). Numerous successful reports of recombinant protein production using signal peptides in transgenic plants are listed in the tables (Table 1 for enzymes including some useful peptides, Table 2 for vaccines, and Table 3 for antibodies). The expression of recombinant antibody molecules in plants has been previously reviewed [68]. Varying the signal sequence of recombinant proteins can affect the degree of protein production. The efficiency of secretion of heterologous proteins in transgenic tobacco could be improved by replacing the heterologous endogenous signal peptide with the signal peptide of tobacco [15,64]. It has been reported that attachment an ER retrieval signal, KDEL [69], increased the production level of recombinant protein targeted to the secretory pathway in transgenic plants [39,45,49,50,54]. In the production of seed protein vicilin and scFv, attachment of the KDEL sequence resulted in an approximately 100-fold increase in the amount of protein produced [63,70]. This high level of accumulation is thought to result from KDEL-mediated ER retention.

Table 2
Vaccines production in transgenic plants

Vaccines	Host plants	Signal peptides (origin)	References
Rabies virus coat protein	Tomato	Own S.P.	[36]
Hepatitis B surface antigen	Potato	Own S.P. or VSP (soybean) + KDEL	[37]
Hepatitis B surface antigen	Soybean and tobacco	Own S.P.	[38]
Hepatitis B surface antigen	Suspension culture of tobacco cells	VSP (soybean) + KDEL	[39]
Cholera toxin B subunit	Potato	Own S.P. + KDEL	[40]
Cholera toxin B subunit	Tobacco	PR1b (tobacco)	[41]
Human cytomegarovirus glycoprotein B	Tobacco	Gluten (rice)	[42]
Transmissible gastroenteritis virus spike protein	Tobacco	PR (tobacco)	[43]
B subunit of *E. coli* enterotoxin	Tobacco and potato	KDEL	[44,45]
B subunit of *E. coli* enterotoxin	Maize	α-Amylase (barley)	[46]

3. Regulation of vesicular transport route of peroxidase by altered N- and C-terminal propeptides

Peroxidases (EC 1.11.1.7) catalyze the oxidation of certain substrates while carrying out reduction of hydrogen peroxide to water. Isozyme C of horseradish (*Armoracia rusticana*) peroxidase (HRP C) is used as a reporter enzyme in immunoassays, diagnostic assays, and histochemistry assays. The amino acid sequence deduced from *prxC1a* cDNA contained the same sequence as that determined from purified HRP C isozyme [71,72] and revealed that HRP C is initially synthesized as a preprotein containing propeptides at its N- and C-termini. Both the N-terminal propeptide (NTPP) and the C-terminal propeptide (CTPP) are excised during protein maturation. The NTPP contains a cluster of hydrophobic amino acids and possibly functions as a signal peptide for the translocation of HRP C protein into the ER. The CTPP, present only in vacuolar PRX precursors, is thought to be responsible for sorting to vacuoles. The role of the propeptides in the intracellular localization of HRP C1a has been investigated by observing the localization of EGFP-propeptide fusion proteins in cultured tobacco cells [35]. Although intact EGFP was localized to the cytosol and/or nuclear matrix, EGFP fused with both HRP C1a NTPP and CTPP was observed mainly in vacuoles. The NTPP–EGFP fusion protein localized to the ER. These results indicate that NTPP and CTPP of HRP C1a can function as a signal peptide for ER translocation and effect the efficient sorting of proteins into plant cell vacuoles, respectively, in heterologous tobacco cells. Furthermore, a CTPP deletion mutant precursor of HRP C1a (N-PRX) was constructed, and wild-type (N-PRX-C) and mutant precursors were independently produced in cultured tobacco cells (Fig. 1A). Soluble proteins from transgenic cultured tobacco cells and proteins in the culture medium were analyzed by immunoblot analysis with anti-HRP C antibody, and PRX activity was assessed with 4-chloro-1-naphthol after IEF. In N-PRX-C producing cells, active HRP C was detected in cellular extracts, but not in the culture media. However, active HRP C was detected in both the media and cellular extracts from N-PRX producing

Table 3
Antibodies production in transgenic plants

Antibodies	Host plants	Signal peptides (origin)	References
Catalytic IgG1 antibody	Tobacco	With/without own S.P.	[47]
Immunoglobulin VH domain	Tobacco	Pectate lyase B (bacteria)	[48]
Fab fragment to human creatine kinase-MM	*Arabidopsis*	2S Seed storage protein (*Arabidopsis*) + KDEL	[49]
Monoclonal antibody to fungal cutinase	Tobacco	κ Light chain of antibody	[50]
Monoclonal antibody	Tobacco	α-Amylase (barley)	[51]
Hybrid IgA–G to streptococcal cell-surface proteins	Tobacco	Own S.P.	[52,53]
Monoclonal antibody to streptococcal cell-surface proteins	Tobacco		[54,55]
IgG to herpes simplex virus mAb	Tobacco	Extensin (tobacco)	[56]
Monoclonal antibody to human rabies virus	Tobacco	Own S.P. + KDEL	[57]
Anti-phytochrome scFv	Tobacco	PR1a (tobacco)	[58]
Anti-hapten oxazolone scFv	Tobacco	Legumin B4 (*Vicia fava*)	[59]
Anti-abscisic acid scFv	Tobacco	Legumin B4 (*V. fava*) + KDEL	[60,61]
scFv to human creatine kinase-MM	Tobacco	2S Seed storage protein (*Arabidopsis*)	[62]
scFv to *Botrytis cinerea* cutinase	Tobacco	κ Light chain (murine) + KDEL	[63]
scFv to beet necrotic yellow vein virus coat protein	Tobacco	Phytohemagglutinin (soybean) and pectate lyase B (bacteria)	[64]
scFv and mouse/human chimeric antibody to human carcinoembryonic antigen	Tobacco	H/L chain of mAb 24 (murine) + KDEL	[65]
scFv of Ig from mouse B cell lymphoma	Tobacco	α-Amylase	[66]
Anti-CEA (tumor-associated marker) scFv	Wheat and rice	TMV virion-specific mAb 24 heavy chain + KDEL	[67]

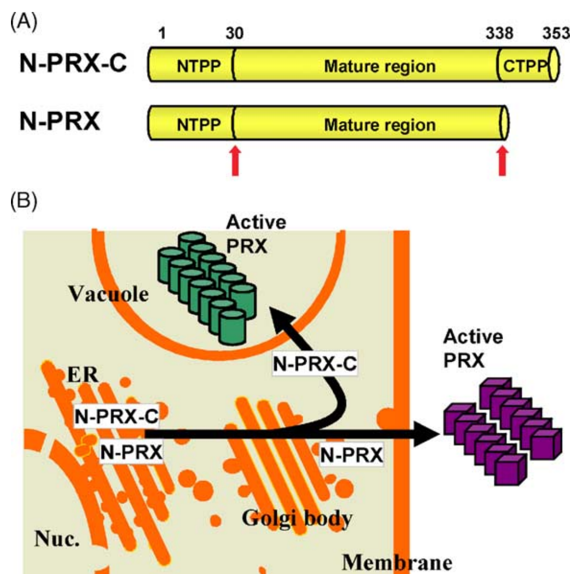

Fig. 1. (A) Construction of proteins introduced into BY2 cells. N-PRX-C is the HRP C1a precursor protein with NTPP and CTPP. N-PRX is a modified precursor protein with a deletion of the CTPP. The amino acid sequence of NTPP is MHFSSSSTLFTCITLIPLVCLILHASLSDA and that of CTPP is LLHDMVEVVDFVSSM. Numbers indicate the amino acid position of the HRP C1a protein, and vertical arrows indicate the cleavage sites of the propeptides. (B) Schematic diagram indicating the localization of N-PRX-C and N-PRX in the vesicular transport system of cultured tobacco cells.

cells (Fig. 1B). These results suggest that the localization of recombinant proteins in the vesicular transport system can be regulated by using NTPP and CTPP of HRP C1a.

The amino acid alignment of HRP C1a NTPP is characteristic of the signal peptide for ER translocation. The signal peptide is divided into three (n, h and c) regions. The h-region is highly hydrophobic and inserted into ER membrane. The polar c-region is on the C-terminal side of h-region and contains helix-breaking proline and glycine residues as well as small uncharged residues in the site of signal peptide cleavage. Positively charged n-region on the N-terminal side of h-region is important for binding with the signal recognition particle (SRP). On the other hand, the amino acid requirement for vacuolar sorting by CTPP is unknown. Even though many peptides are reported to function as vacuolar sorting determinants in plant cells, conserved amino acid sequence has not found in them. A random mutation and selection including novel methods of combinatorial analyses will be useful to clarify amino acid sequence in CTPP which is responsible for vacuolar sorting.

4. Conclusions

The plant vesicular transport system is potentially useful for the production of recombinant proteins that require post-translational modification for their activity, such as antibodies and glycoproteins. The novel high-performance proteins including functional peptides and catalytic antibodies that are created by using method of combinatorial bioengineering, will be useful target of the plant vesicular transport system in near future. Currently, we can retain foreign proteins in the ER or allow for their secretion using different signal peptides. To maximize the level of protein production, in addition to optimization of the promoter and 5′-UTR, the choice of a suitable signal peptide is important. The addition of an ER retrieval signal, KDEL, leads to a high level of accumulation of recombinant proteins in the vesicular transport system. It has also reported that overexpression of the chaperone BiP or protein disulfide isomerase increased secretion levels of recombinant scFvs in budding yeast [73]. The further refinement of systems, the plant vesicular transport engineering, that allow for high levels of recombinant protein production holds great promise for the large-scale production of biologically and clinically useful proteins.

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