



Production of recombinant proteins through sequestration in chloroplasts: a strategy based on nuclear transformation and post-translational protein import

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Abstract

Recently, plants have emerged as a lucrative alternative system for the production of recombinant proteins, as recombinant proteins produced in plants are safer and cheaper than those produced in bacteria and animal cell-based production systems. To obtain high yields in plants, recombinant proteins are produced in chloroplasts using different strategies. The first strategy is based on chloroplast transformation, followed by gene expression and translation in chloroplasts. This has proven to be a powerful approach for the production of proteins at high levels. The second approach is based on nuclear transformation, followed by post-translational import of proteins from the cytosol into chloroplasts. In the nuclear transformation approach, foreign genes are stably integrated into the nuclear genome or transiently expressed in the nucleus by non-integrating T-DNA. Although this approach also has great potential for protein production at high levels, it has not been thoroughly investigated. In this review, we focus on nuclear transformation-based protein expression and its subsequent sequestration in chloroplasts, and summarize the different strategies used for high-level production of recombinant proteins. We also discuss future directions for further improvements in protein production in chloroplasts through nuclear transformation-based gene expression.

Keywords Chloroplast · Plant-produced recombinant proteins · Nuclear transformation of foreign genes · *Agrobacterium*-mediated transformation · Protein import

Introduction

Advances in molecular biology and genetic engineering, together with the development of various biotechnological tools, have enabled the production of a large number of protein drugs, vaccines, and industrial enzymes, such as α -amylase and phytase, in living organisms (Bandaranayake and Almo 2014; Chen 2012; Islam et al. 2018; Obembe

et al. 2011; Sohn et al. 2018; Tang and Zhao 2009; Tschofen et al. 2016; Xu et al. 2011). Bacteria and animal cells have been widely used as platforms for the production of various proteins, which are currently available in the market as commercialized products (Bandaranayake and Almo 2014; Chen 2012). However, these conventional production systems have certain shortcomings; for example, the animal cell-based system is expensive and cannot be operated on a large scale (Egelkrout et al. 2012). Recently, plants have received increasing attention as a new platform for the production of recombinant proteins. Compared with animal cells and bacteria, plants have various advantages as protein production systems, such as easy scalability, and potentially zero risk of contamination with human pathogens and bacterial toxins. These advantages have led to the concept of green pharmaceuticals, i.e., pharmaceuticals produced in plants (Egelkrout et al. 2012; Obembe et al. 2011; Xu et al. 2011; Zoschke and Bock 2018).

In plant cells, recombinant proteins are often expressed in subcellular organelles (Jang et al. 1999). In leaf tissues, one of the most attractive organelles for protein production is the

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chloroplast. In mesophyll cells of mature leaves, the number of chloroplasts is as high as 100, and the area occupied by a chloroplast is second to that occupied by a vacuole (Daniell et al. 2016; Zoschke and Bock 2018). Moreover, unlike vacuoles, chloroplasts have neutral pH and minimally active proteolysis (Zoschke and Bock 2018). Thus, chloroplasts are ideal for the storage of recombinant proteins in plant cells. This is supported by the Rubisco protein complex, which is stored in chloroplasts and accounts for half of the total cellular proteins in leaf tissues (Vitlin Gruber and Feiz 2018).

Two different approaches have been developed for recombinant protein production in chloroplasts. One approach is based on the integration of the target gene into the chloroplast genome via homologous recombination, followed by transcription and translation in chloroplasts (Adem et al. 2017). Many excellent review papers have been published on this topic (Adem et al. 2017; Chebolu and Daniell 2009; Daniell et al. 2009, 2016; Jin and Daniell 2015; Olejniczak et al. 2016; Scotti et al. 2012); therefore, this approach is not covered in this review but referred to in these papers.

The second approach is based on nuclear transformation; the transgene is either transiently expressed in the plant cell nucleus or stably integrated into the plant nuclear genome, and the translated protein is imported into the chloroplasts from the cytosol (Cui et al. 2011; Gelvin 2003; Zahin et al. 2016). Like the chloroplast transformation method, this approach also has great potential for the production of foreign proteins in chloroplasts; however, it has not received much attention and has not been fully explored for the production of recombinant proteins. Compared with research conducted on the chloroplast transformation approach, only a few research articles have been published on import of recombinant protein into the chloroplast using nuclear transformation technologies. In this review, we summarize advantages and weaknesses of the nuclear transformation-based approach for the import of recombinant proteins from the cytosol to the chloroplast for the large-scale production.

Expression of target genes in the nucleus for protein production in chloroplasts

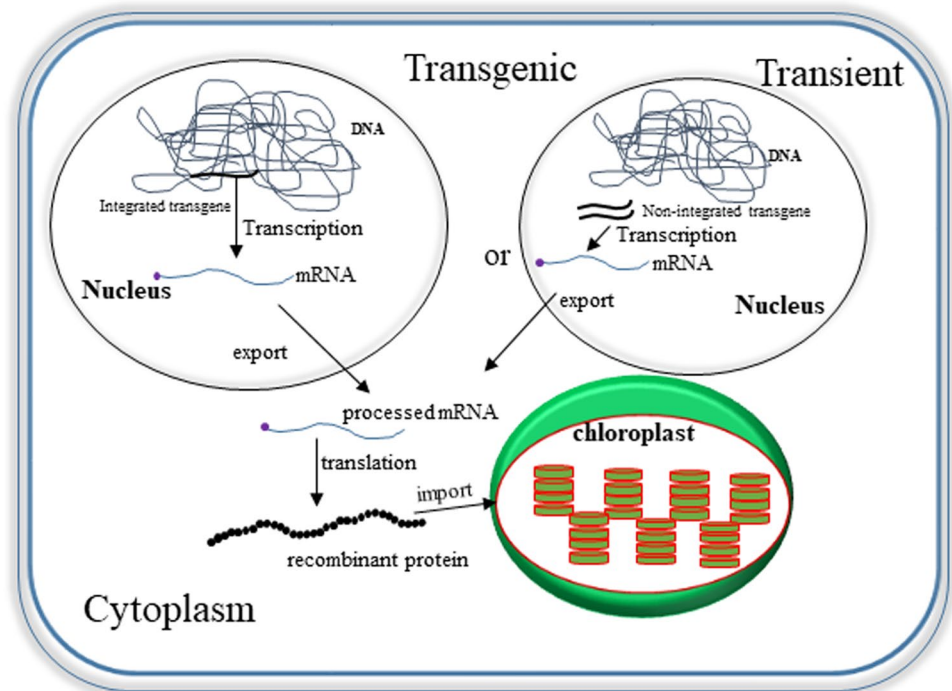
Agrobacterium tumefaciens, a phytopathogenic Gram-negative soil bacterium, has been used for delivering transgenes into the nucleus of plant cells. A substantial region of the tumor-inducing (Ti) plasmid of *Agrobacterium*, referred to as the transfer DNA (T-DNA), is integrated into the host plant genome (Gelvin 2003; Hwang et al. 2017a; Smith and Townsend 1907). Plant transformation is performed using the binary vector system, which was developed based on the T-DNA of *Agrobacterium*. Bacterial genes within the T-DNA are replaced with the gene of interest and delivered into the host nucleus during transformation. Recently, it has

been shown that *Agrobacterium* can be used to transform not only tobacco but also a large number of other plant species (Guo et al. 2018). Thus, there is almost no restriction on plant species for foreign gene expression via nuclear transformation. To produce recombinant proteins in chloroplasts via nuclear transformation, two different approaches have been developed, based on the status of the transgene in the nucleus. In the first approach, the transgene is stably integrated into the nuclear genome, whereas, in the second approach, the transgene is transiently expressed in the nucleus by the unintegrated T-DNA (Fig. 1). Although both approaches rely on nuclear gene expression, there are several differences that distinguish these two approaches when used for recombinant protein production in plants.

Stable expression system for foreign proteins

Stable integration of the transgene in the nuclear genome requires the generation of transgenic plants. First, the transgene is introduced into a binary vector. Subsequently, the binary vector carrying the transgene is introduced into *A. tumefaciens*, which transfers the T-DNA from the binary vector into the host cells, where the T-DNA is integrated into the nuclear genome. In this approach, a marker gene is integrated into the nuclear genome along with the gene of interest, thereby allowing the screening of transformed cells from which transgenic plants are regenerated. This is followed by the selection of homozygous plants for the production of proteins encoded by transgenes. The advantage of this approach is that the production of plant biomass expressing the recombinant proteins is as simple as growing plants in a greenhouse, and does not require any additional steps such as agroinfiltration. Elite lines selected on the basis of performance are propagated via seeds, thus requiring minimal maintenance. Thus, this approach is the most suitable for large-scale protein production. However, the disadvantage of this approach is that the generation of homozygous lines is a time-consuming process. In addition, this approach can be used for the production of transgenic in only a limited number of plant species. Furthermore, individual transgenic plants produced using this approach show high variability in transgene expression. To obtain transgenic plants expressing the transgene at high levels, it is necessary to screen a large number of independent transgenic plants; therefore, this method is labor intensive. Moreover, the expression level of a single copy of the transgene stably integrated into the host genome is generally lower than that resulting from the transient transgene expression post-agroinfiltration, and the expression of transgenes is often silenced after several generations. To address these problems, many new approaches have been developed recently. These include the use of matrix attachment sequences (to ensure transgene expression independent of the integration site; (Halweg et al.

Fig. 1 Stable or transient expression of foreign genes from the nuclear genome and post-translational import of foreign proteins into chloroplasts. Two methods are used for transgene expression following nuclear transformation. In the first method, T-DNA harboring the transgene is stably integrated into the nuclear genome, thereby giving rise to transgenic plants, and in the second method, the non-integrated transgene is expressed from the nucleus and subsequently proteins are synthesized on cytosolic ribosomes and post-translationally imported into chloroplasts



2005), strong artificial transcription factors (Li et al. 2017; Lowder et al. 2018), inducible gene silencing suppressors, transcription terminators [with high transcription efficiency; (Csorba et al. 2015)], and 5' untranslated regions [with high translational efficiency; (Kim et al. 2013; Li et al. 2017)]. These advances in the expression of transgenes in the host cell nucleus provide a new strategy for high-level protein production in chloroplasts. In addition, according to the current regulation of genetically modified (GM) plants, it is important to identify homozygous transgenic plants carrying a single-copy insertion of the target gene in the intergenic region. Thus, this approach would best fit the current regulation of GM plants for commercialization.

Transient expression system for foreign proteins

In this approach, the transgene carried by the non-integrating T-DNA system is only transiently present and expressed in the nucleus of leaf cells post-agroinfiltration through mutant *Agrobacterium* strains (Fig. 1; Gelvin 2003; Kapila et al. 1997; Sheludko 2008). Thus, this approach is not subject to the limitations of transgene integration in the nuclear genome, such as the lengthy process of transgenic plant selection and the effect of the insertion site on transgene expression (Marillonnet et al. 2005; Sheludko et al. 2007; Sheludko 2008; Vaquero et al. 1999). Using this approach, recombinant proteins can be produced within a few days, typically ranging from 3 to 10 day post-agroinfiltration. Thus, the transient expression system is a rapid method for the production of recombinant proteins compared with

the stable transformation system. This approach would be particularly suitable for the production of recombinant vaccines in the event of viral disease outbreaks (Chen et al. 2013). The most important advantage of this approach is the high-level protein production compared with the transgenic approach. The transgene copy number in the nucleus is higher in transiently transformed plants than that in single-copy transgenic plants, leading to higher protein production (Huang and Mason 2004; Komarova et al. 2010; Sheludko 2008). If the binary vector is based on DNA or RNA viruses, the copy number of the target gene or mRNA, respectively, is amplified greatly, thus leading to high-level protein production (Cañizares et al. 2006; Huang et al. 2009; Lico et al. 2008; Marillonnet et al. 2005; Sheludko 2008). For example, the transient expression of a foreign gene using the MagnICON vector, an RNA virus-based expression vector, followed by import of translated foreign proteins into chloroplasts led to the expression level as high as that obtained with the transplastomic expression approach (Marillonnet et al. 2004, 2005). Moreover, gene silencing can be avoided by the coexpression of gene silencing suppressors (Csorba et al. 2015). Furthermore, it is possible to express multiple genes simultaneously, enabling the production of protein complexes composed of hetero-multimeric subunits (Pineo et al. 2013).

However, this approach also has certain disadvantages compared with the transgenic approach. In the transient expression approach, a new *Agrobacterium* culture is needed each time recombinant protein production is required, and plants must be infiltrated individually. Moreover,

agroinfiltration is a laborious process compared with the sowing of seeds in the transgenic approach. Thus, the overall cost of biomass production using the transient expression approach is much higher than that using the transgenic approach. Another point of concern is that the efficiency of protein production varies among individual plants transformed in one experiment and among plants transformed in different experiments; this could be a major problem when the consistency of protein production is important, as in the case of pharmaceuticals (Fujiuchi et al. 2016). Another disadvantage of this method is the use of *Agrobacterium*, a Gram-negative species of bacteria. Recently, plant systems have been advocated as a safe system with no or very low level of endotoxins (Islam et al. 2018). However, with the use of *Agrobacterium*, the plant system is no longer considered an endotoxin-free system, and additional processing is needed to remove endotoxins of *Agrobacterium* origin from the purified proteins. It should be noted that the above-mentioned issues are not specific to protein production in chloroplasts; instead, these issues represent the general problems of this approach when used for recombinant protein production in plants.

Protein import into chloroplasts from the cytosol after translation

Successful application of nuclear transformation for foreign protein production in chloroplasts is critically dependent on the efficiency of protein import into chloroplasts after translation in the cytosol. Thus, it is important to understand how a large amount of protein is imported into chloroplasts from the cytosol for high-level protein accumulation. The capacity to deliver proteins from the cytosol to the chloroplast may not be a limiting factor, because more than 3000 different proteins are imported into chloroplasts from the cytosol (Jarvis 2008; Lee et al. 2013; Li and Chiu 2010). Moreover, the three most highly abundant proteins in plant leaf cells, Rubisco large subunit (RbcL), Rubisco small subunit (RbcS), and chlorophyll a/b-binding protein (Cab), occur in chloroplasts. Two of these three proteins, RbcS and Cab, are imported into chloroplasts from the cytosol. Thus, the first step for high-level protein production in chloroplasts via nuclear transformation is to identify a signal sequence that is highly efficient in delivering foreign proteins into chloroplasts. Since transit peptides are cargo-independent (Zhang and Glaser 2002; Zybailov et al. 2008), the selection of a highly efficient transit peptide is critical for the accumulation of proteins at high levels in chloroplasts. Transit peptides of RbcS and Cab proteins have been widely used for protein production in chloroplasts (Shen et al. 2017) examples below). However, transit peptides are highly variable in sequence and protein import efficiency. Small sequence motifs present in the transit peptide play crucial roles in

the efficiency of protein import into chloroplasts. Thus, it is possible that the modulation of these sequence motifs affects the protein import efficiency (Lee et al. 2006, 2018). Consistent with this possibility, a recent report showed that modified chloroplast transit peptides improve the import of diverse *E. coli* enzymes such as EcTSR and EcGCL into rice chloroplasts (Shen et al. 2017). Thus, the identification of transit peptides with high protein import efficiency could potentially enhance recombinant protein production in chloroplasts via nuclear transformation.

Another important issue in transit peptide-mediated delivery of proteins into chloroplasts is the removal of transit peptide from the target recombinant protein after import into chloroplasts. During or after import of precursors into chloroplasts, the N-terminal transit peptide is cleaved off by signal propeptide peptidases (SPPs) within chloroplasts (Chen and Li 2007; Richter and Lamppa 1998). However, the entire signal sequence is not removed from the target proteins after import, because the cleavage site of SPPs is located within the transit peptide (Emanuelsson et al. 1999; Lee et al. 2008). Thus, target proteins contain many extra residues derived from the transit peptide. It is possible that the N-terminal region relative to the SPP cleavage site of the transit peptide can be used as a signal sequence for delivering proteins into chloroplasts. However, this may decrease the import efficiency, as the sequence downstream of the cleavage site also contributes to the import efficiency (Lee et al. 2008). Another approach for completely removing the transit peptide from the target protein after import into chloroplasts is to include a new proteolytic cleavage site between the transit peptide and target protein. For example, enterokinase removes the remaining region of the transit peptide when the enterokinase recognition site is inserted between the transit peptide and N-terminus of the target protein. Enterokinase cleaves the C-terminal end of the cleavage site, leaving no extra residues (Hosfield and Lu 1999; Skala et al. 2013).

Demonstration of foreign gene expression and sequestration of translated proteins into chloroplasts via nuclear transformation technologies

Stable expression of foreign proteins

Several studies report successful production of recombinant proteins in chloroplasts using the nuclear transformation strategy (Table 1). These include production of β -glucuronidase (GUS), green fluorescent protein (GFP), and *E. coli* phosphoenolpyruvate synthetase and xylanase in various plant species such as petunia, potato, rice, and tobacco (Hyunjong et al. 2005; Kavanagh et al. 1988; Köhler

Table 1 List of proteins expressed via nuclear transformation, followed by transit peptide-mediated protein import into chloroplasts

Transit peptide	Protein	Expression level	Host plant	References
Cab	β -glucuronidase		Tobacco	(Kavanagh et al. 1988)
RbcS	Glutathione reductase, phosphinothricin acetyl transferase		Tobacco	(Creissen et al. 1995)
recA	GFP		Tobacco	(Köhler et al. 1997)
RbcS	Phosphoenolpyruvate synthetase	0.1% TSP	Potato	(Panstruga et al. 1997)
RbcS	GFP	10% TSP	Rice	(Jang et al. 1999)
gbss	GFP strictosidine synthase		Tobacco cell lines	(Hoppmann et al. 2002)
RbcS	Polyhydroxybutyrate		Maize	(Zhong et al. 2003)
RbcS	Xylanase	4.8% TSP	Arabidopsis	(Hyunjong et al. 2005)
RbcS	Cry1Ac	2% TSP	Rice	(Kim et al. 2009)
Cab	Endoglucanase Cel5A	5.2% TSP	Tobacco	(Kim et al. 2010)
rCTP	EcTSR EcGCL		Rice	(Shen et al. 2017)
RbcS	HPV16-L1	11% TSP	Tobacco	(Maclean et al. 2007)
RbcS	p24	230 μ g/kg	Tobacco	(Meyers et al. 2008)

RbcS Rubisco small subunit, *Cab* chlorophyll *alb*-binding protein, *gbss* granule-bound starch synthase, *recA* chloroplast transit peptide, *rCTP* rice chloroplast transit peptide, *HPV16* human papilloma virus type 16, *GFP* green fluorescent protein, *TSP* total soluble protein

et al. 1997; Panstruga et al. 1997). The purified GUS protein from petunia showed 95% bioactivity (Kavanagh et al. 1988). Transit peptides of *Arabidopsis* or rice RbcS or Cab proteins were used for the delivery of these proteins to chloroplasts. The level of protein accumulation in chloroplasts showed high variability, depending on the protein and plant species. For example, in potato, *E. coli* phosphoenolpyruvate synthetase accounted for approximately 0.1% of the total soluble protein (TSP) (Panstruga et al. 1997). However, xylanase and GFP were expressed at much higher levels, reaching approximately 4.5% and 10% of the TSP in transgenic tobacco and rice plants, respectively. In addition, HPV16-L1 or p24 antigen produced in transgenic tobacco plants accounted for 11% of the TSP or 230 μ g/kg (fresh weight; fw) of plant biomass (Maclean et al. 2007; Meyers et al. 2008). To date, the level of HPV16-L1 protein produced in transgenic tobacco plants is the highest level of protein production achieved in chloroplasts through nuclear transformation (Jang et al. 1999). Hoppmann et al. (2002) used tobacco cell lines to produce GFP and *Catharanthus roseus strictosidine* synthase (Str1); these proteins accumulated to very high levels when delivered to chloroplasts using the transit peptide of potato granule-bound starch synthase (*gbss*). In addition, several enzymes have been produced using this approach. Polyhydroxybutyrate (PHB) enzymes of *Alcaligenes eutrophus* were produced in chloroplasts of transgenic maize (Zhong et al. 2003). Hyperthermostable endoglucanase Cel5A and a protein encoded by the truncated *cry1Ac* gene were produced to approximately 5.2% and 2% of the TSP in transgenic tobacco and rice plants, respectively (Kim et al. 2009, 2010). Moreover, Cel5A produced in chloroplasts was highly efficient in the degradation

of its substrate, carboxy-methyl cellulose (CMC) (Kim et al. 2010). In these three studies, the transit peptide of RbcS was used to deliver proteins to chloroplasts. Together, these studies support the potential of recombinant protein production in chloroplasts using stable transformation.

Transient expression of foreign proteins

Recently, transient expression of foreign genes, followed by protein import into chloroplasts, was used for high-level protein production in plants (Table 2). For example, bioactive human growth hormone (hGH) and Norwalk virus capsid proteins were expressed to 0.1% of the TSP and approximately 0.1 mg/kg (fw) of plant biomass, respectively (Gils et al. 2005). The truncated Gag (p17/p24) and p24 capsid

Table 2 List of proteins expressed in tobacco via agroinfiltration, followed by protein import into chloroplasts using the transit peptide of the small subunit of Rubisco (RbcS)

Protein	Expression level	References
GFP		(Marillonnet et al. 2004)
Human growth hormone	0.1% TSP	(Gils et al. 2005)
HPV16-L1	4.5% TSP	(Maclean et al. 2007)
NVCP	0.11 mg/g	(Santi et al. 2008)
p24	1 mg/kg	(Meyers et al. 2008)
HPV16-L1	1710 mg/kg	(Pineo et al. 2013)
HPV16-L1	250 mg/kg	(Zahin et al. 2016)
LALF32-51-E7	0.44% TSP	(Yanez et al. 2017)
LALF32-51-E7		(Yanez et al. 2018)

HPV16 human papilloma virus type 16, *NVCP* Norwalk virus capsid protein, *GFP* green fluorescent protein, *TSP* total soluble protein

subunit proteins of human immunodeficiency virus type 1 (HIV-1) were produced at very high levels, exceeding 1 mg/kg (fw) of plant biomass (Meyers et al. 2008). Moreover, p17/p24 proteins boosted T-cell and humoral responses in mice that had been primed with a *gag* DNA vaccine (Meyers et al. 2008). Another example of a viral protein produced in chloroplasts is HPV16 L1; Maclean et al. (2007) produced 137 mg HPV16 L1 per kg (fw) of plant biomass. Moreover, L1 proteins were properly assembled into virus-like particles (VLPs) in *N. benthamiana*, which are highly immunogenic in target animals and can be developed into effective vaccines; thus, this result demonstrates the potential of this approach for vaccine development. Another study showed further improvement in the productivity of HPV16 L1 VLPs using the MagnICON vector (Zahin et al. 2016); using this vector, 250 mg HPV16 L1 protein was produced per kg (fw) of plant biomass. VLPs of the human papilloma virus (HPV) are composed of both L1 and L2 proteins, and are thought to exhibit higher immunogenicity than L1 alone (Zahin et al. 2016). Indeed, coexpression of *HPV16 L1* and *L2* genes encoding the major and minor coat proteins, respectively, resulted in the accumulation of 1710 mg HPV16 VLPs per kg (fw) of plant biomass (Pineo et al. 2013). In other studies, Yanez et al. (2017, 2018) demonstrated the production of HPV16 E7 to high levels in plants. Together, these studies demonstrate that transient transgene expression, followed by protein import into chloroplasts, is a powerful approach for high-level recombinant protein production in plants.

Potential production of *N*-glycosylated proteins in chloroplasts

Chloroplasts originally evolved from cyanobacteria and, therefore, do not have the ability to synthesize glycosylated proteins. However, many pharmaceutical proteins are *N*-glycosylated (Gomord et al. 2010; Solá and Griebenow 2010). These *N*-glycans are important for the stability of proteins. Moreover, in certain cases, *N*-glycans are crucial for the biological activity of proteins. Proteins are *N*-glycosylated in the endoplasmic reticulum (ER) via a system involving a large number of proteins (Ceriotti et al. 1998). However, it has been shown that certain chloroplast proteins such as α -type carbonic anhydrases and amylases are *N*-glycosylated (Burén et al. 2011; Chen et al. 2004; Faye and Daniell 2006; Lehtimäki et al. 2015). Moreover, α -carbonic anhydrases in *Arabidopsis* are one of the most abundant proteins in chloroplasts, suggesting that a large amount of foreign proteins can be transported to chloroplasts via this route. Recent studies show that *N*-glycosylated chloroplast proteins use a special route to reach chloroplasts (Lehtimäki et al. 2015). These proteins are first targeted to the ER and then to the Golgi apparatus. In the ER, proteins are *N*-glycosylated, and

N-glycans are modified to the complex type in the Golgi apparatus (Chen et al. 2004; Villarejo et al. 2005). Subsequently, *N*-glycosylated proteins are diverted from the Golgi apparatus to chloroplasts. The exact mechanism of trafficking from the Golgi to the chloroplast has not been elucidated. These data suggest that this pathway can be used for the import of *N*-glycosylated foreign proteins in chloroplasts in a large-scale production. To use this pathway for recombinant protein production, proteins must be produced via nuclear transformation. However, this pathway has not yet been used for the import of foreign proteins in chloroplasts.

Conclusion and perspectives

Proteins are one of the most useful biomaterials. Advances in molecular biology and genetic engineering have enabled the use of proteins in a wide range of applications such as biopharmaceuticals, vaccines, antibodies, and industrial enzymes. With the sequencing of the entire genomes of numerous organisms, ranging from bacteria to human, the source of protein-coding genes has increased substantially. In addition, recombinant DNA technology has enabled the generation of new genes by de novo synthesis or by combining domains from naturally existing genes. To ensure that recombinant proteins are used in a wide range of applications, the method of protein production must be economical and highly scalable. For these reasons, plants have recently gained popularity as a platform for recombinant protein production. Chloroplasts are considered excellent subcellular organelles in plants for the production of recombinant proteins at high levels. Two main approaches have been developed for protein production in chloroplasts: chloroplast transformation, which has emerged as a highly cost-effective method, and nuclear transformation, followed by protein import into chloroplasts, which has not been explored extensively for the production of foreign proteins. Transient transgene expression via agroinfiltration has been shown to produce target proteins at extremely high levels in chloroplasts, suggesting that nuclear transformation-based expression is also an excellent option for protein production by import of translated recombinant proteins into chloroplasts. In contrast to transient expression, stable integration of a foreign gene in the nuclear genome has a clear disadvantage for protein production, because proteins are expressed from a single copy of the gene. However, it is still possible to achieve a high level of protein production from limited copy numbers of genes. For example, in *Arabidopsis*, only two copies of the *RbcS* gene have been used for the accumulation of *RbcS* protein to extremely high levels in chloroplasts. New innovative approaches are needed for improving the level of protein production in chloroplasts. One possible

approach is to use RbcS as a fusion partner to derive the production of foreign proteins at high levels. Accumulation of foreign proteins has been improved by several fold using the full-length RbcS protein as a fusion partner rather than using only the transit peptide of RbcS (Hwang et al. 2017b). Based on the design principle of transit peptides, synthetic transit peptides with high efficiency in protein import into chloroplasts could be generated to deliver a large amount of protein into chloroplasts (Lee and Hwang 2018). In addition, the nuclear transformation approach could be explored for producing *N*-glycosylated proteins in chloroplasts. Although considerable research has been conducted for demonstrating the production of recombinant proteins at high levels in chloroplasts by importing them into chloroplasts, no recombinant proteins produced in chloroplasts have yet been commercialized. We expect that this promising approach will soon be used for the production of commercial protein products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Adem M, Beyene D, Feyissa T (2017) Recent achievements obtained by chloroplast transformation. *Plant Methods* 13:30
- Bandaranayake AD, Almo SC (2014) Recent advances in mammalian protein production. *FEBS Lett* 588:253–260
- Burén S, Ortega-Villasante C, Blanco-Rivero A, Martínez-Bernardini A, Shutova T, Shevela D, Messinger J, Bako L, Villarejo A, Samuelsson G (2011) Importance of post-translational modifications for functionality of a chloroplast-localized carbonic anhydrase (CAH1) in *Arabidopsis thaliana*. *PLoS One* 6:e21021
- Cañizares MC, Liu L, Perrin Y, Tsakiris E, Lomonosoff GP (2006) A bipartite system for the constitutive and inducible expression of high levels of foreign proteins in plants. *Plant Biotechnol J* 4:183–193
- Ceriotti A, Duranti M, Bollini R (1998) Effects of *N*-glycosylation on the folding and structure of plant proteins. *J Exp Bot* 49:1091–1103
- Chebolu S, Daniell H (2009) Chloroplast-derived vaccine antigens and biopharmaceuticals: expression, folding, assembly and functionality. *Curr Top Microbiol Immunol* 332:33–54
- Chen R (2012) Bacterial expression systems for recombinant protein production: *E. coli* and beyond. *Biotechnol Adv* 30:1102–1107
- Chen KY, Li H (2007) Precursor binding to an 880-kDa Toc complex as an early step during active import of protein into chloroplasts. *Plant J* 49:149–158
- Chen M-H, Huang L-F, H-m Li, Chen Y-R, Yu S-M (2004) Signal peptide-dependent targeting of a rice α -amylase and cargo proteins to plastids and extracellular compartments of plant cells. *Plant Physiol* 135:1367–1377
- Chen Q, Lai H, Hurtado J, Stahnke J, Leuzinger K, Dent M (2013) Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. *Adv Tech Biol Med* 1:103
- Creissen G, Reynolds H, Xue Y, Mullineaux P (1995) Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco. *Plant J* 8:167–175
- Csorba T, Kontra L, Burgyán J (2015) Viral silencing suppressors: tools forged to fine-tune host-pathogen coexistence. *Virology* 479:85–103
- Cui C, Song F, Tan Y, Zhou X, Zhao W, Ma F, Liu Y, Hussain J, Wang Y, Yang G, He G (2011) Stable chloroplast transformation of immature scutella and inflorescences in wheat (*Triticum aestivum* L.). *Acta Biochim Biophys Sin* 43:284–291
- Daniell H, Singh ND, Mason H, Streatfield SJ (2009) Plant-made vaccine antigens and biopharmaceuticals. *Trends Plant Sci* 14:669–679
- Daniell H, Lin CS, Yu M, Chang WJ (2016) Chloroplast genomes: diversity, evolution, and applications in genetic engineering. *Genome Biol* 17:134
- Egelkrot E, Rajan V, Howard JA (2012) Overproduction of recombinant proteins in plants. *Plant Sci* 184:83–101
- Emanuelsson O, Nielsen H, Von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8:978–984
- Faye L, Daniell H (2006) Novel pathways for glycoprotein import into chloroplasts. *Plant Biotechnol J* 4:275–279
- Fujiuchi N, Matoba N, Matsuda R (2016) Environment control to improve recombinant protein yields in plants based on *Agrobacterium*-mediated transient gene expression. *Front Bioeng Biotechnol* 4:23
- Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Mol Biol Rev* 67:16–37
- Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y (2005) High-yield production of authentic human growth hormone using a plant virus-based expression system. *Plant Biotechnol J* 3:613–620
- Gomord V, Fitchette AC, Menu-Bouaouiche L, Saint-Jore-Dupas C, Plasson C, Michaud D, Faye L (2010) Plant-specific glycosylation patterns in the context of therapeutic protein production. *Plant Biotech J* 8:564–587
- Guo M, Ye J, Gao D, Xu N, Yang J (2018) *Agrobacterium*-mediated horizontal gene transfer: mechanism, biotechnological application, potential risk and forestalling strategy. *Biotechnol Adv* 37(1):259–270
- Halweg C, Thompson WF, Spiker S (2005) The Rb7 matrix attachment region increases the likelihood and magnitude of transgene expression in tobacco cells: a flow cytometric study. *Plant Cell* 17:418–429
- Hoppmann V, Di Fiore S, Zimmermann S, Emans N (2002) The potato granule bound starch synthase chloroplast transit peptide directs recombinant proteins to plastids. *J Plant Physiol* 159:1061
- Hosfield T, Lu Q (1999) Influence of the amino acid residue downstream of (Asp) 4Lys on enterokinase cleavage of a fusion protein. *Anal Biochem* 269:10–16

- Huang Z, Mason HS (2004) Conformational analysis of hepatitis B surface antigen fusions in an *Agrobacterium*-mediated transient expression system. *Plant Biotech J* 2:241–249
- Huang Z, Chen Q, Hjelm B, Arntzen C, Mason H (2009) A DNA replicon system for rapid high-level production of virus-like particles in plants. *Biotechnol Bioeng* 103:706–714
- Hwang H-H, Yu M, Lai E-M (2017a) *Agrobacterium*-mediated plant transformation: biology and applications. *Arabidopsis Book* 15:e0186
- Hwang I, Kang HJ, Sohn EJ, Lee YJ, Berggren P-O, Yoo YJ, Kim JY (2017b) Method of highly expressing target protein from plants using RbcS fusion protein and method of preparing composition for oral administration of medical protein using target protein expression plant body. In: US patent application publication No. US2017/0335361 A1
- Hyunjong B, Lee D-S, Hwang I (2005) Dual targeting of xylanase to chloroplasts and peroxisomes as a means to increase protein accumulation in plant cells. *J Exp Bot* 57:161–169
- Islam MR, Kwak JW, Js Lee, Hong SW, Khan MRI, Lee Y, Lee Y, Lee SW, Hwang I (2018) Cost-effective production of tag-less recombinant protein in *Nicotiana benthamiana*. *Plant Biotechnol J* 17(6):1094–1105
- Jang I-C, Nahm BH, Kim J-K (1999) Subcellular targeting of green fluorescent protein to plastids in transgenic rice plants provides a high-level expression system. *Mol Breed* 5:453–461
- Jarvis P (2008) Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytol* 179:257–285
- Jin S, Daniell H (2015) The engineered chloroplast genome just got smarter. *Trends Plant Sci* 20:622–640
- Kapila J, De Rycke R, Van Montagu M, Angenon G (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci* 122:101–108
- Kavanagh TA, Jefferson RA, Bevan MW (1988) Targeting a foreign protein to chloroplasts using fusions to the transit peptide of a chlorophyll *a/b* protein. *Mol Gen Genet* 215:38–45
- Kim EH, Suh SC, Park BS, Shin KS, Kweon SJ, Han EJ, Park SH, Kim YS, Kim JK (2009) Chloroplast-targeted expression of synthetic cry1Ac in transgenic rice as an alternative strategy for increased pest protection. *Planta* 230:397–405
- Kim S, Lee D-S, Choi IS, Ahn S-J, Kim Y-H, Bae H-J (2010) *Arabidopsis thaliana* Rubisco small subunit transit peptide increases the accumulation of thermotoga maritima endoglucanase Cel5A in chloroplasts of transgenic tobacco plants. *Transgenic Res* 19:489–497
- Kim Y, Lee G, Jeon E, Ej Sohn, Lee Y, Kang H, Dw Lee, Kim DH, Hwang I (2013) The immediate upstream region of the 5'-UTR from the AUG start codon has a pronounced effect on the translational efficiency in *Arabidopsis thaliana*. *Nucleic Acids Res* 42:485–498
- Köhler RH, Cao J, Zipfel WR, Webb WW, Hanson MR (1997) Exchange of protein molecules through connections between higher plant plastids. *Science* 276:2039–2042
- Komarova TV, Baschieri S, Donini M, Marusic C, Benvenuto E, Dorokhov YL (2010) Transient expression systems for plant-derived biopharmaceuticals. *Expert Rev Vaccines* 9:859–876
- Lee DW, Hwang I (2018) Evolution and design principles of the diverse chloroplast transit peptides. *Mol Cells* 41:161
- Lee DW, Lee S, G-j Lee, Lee KH, Kim S, Cheong G-W, Hwang I (2006) Functional characterization of sequence motifs in the transit peptide of *Arabidopsis* small subunit of rubisco. *Plant Physiol* 140:466–483
- Lee DW, Kim JK, Lee S, Choi S, Kim S, Hwang I (2008) *Arabidopsis* nuclear-encoded plastid transit peptides contain multiple sequence subgroups with distinctive chloroplast-targeting sequence motifs. *Plant Cell* 20:1603–1622
- Lee DW, Jung C, Hwang I (2013) Cytosolic events involved in chloroplast protein targeting. *Biochim Biophys Acta* 1833:245–252
- Lee DW, Yoo Y-J, Razzak MA, Hwang I (2018) Prolines in transit peptides are crucial for efficient preprotein translocation into chloroplasts. *Plant Physiol* 176:663–677
- Lehtimäki N, Koskela MM, Mulo P (2015) Posttranslational modifications of chloroplast proteins: an emerging field. *Plant Physiol* 168:768–775
- Li H, Chiu CC (2010) Protein transport into chloroplasts. *Annu Rev Plant Biol* 61:157–180
- Li Z, Zhang D, Xiong X, Yan B, Xie W, Sheen J, Li J-F (2017) A potent Cas9-derived gene activator for plant and mammalian cells. *Nat Plants* 3:930
- Lico C, Chen Q, Santi L (2008) Viral vectors for production of recombinant proteins in plants. *J Cell Physiol* 216:366–377
- Lowder LG, Zhou J, Zhang Y, Malzahn A, Zhong Z, Hsieh T-F, Voytas DF, Zhang Y, Qi Y (2018) Robust transcriptional activation in plants using multiplexed CRISPR-Act2. 0 and mTALE-act systems. *Mol Plant* 11:245–256
- Maclean J, Koekemoer M, Olivier A, Stewart D, Hitzeroth I, Rademacher T, Fischer R, Williamson A-L, Rybicki E (2007) Optimization of *human papillomavirus* type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. *J Gen Virol* 88:1460–1469
- Marillonnet S, Giritch A, Gils M, Kandzia R, Klimyuk V, Gleba Y (2004) In planta engineering of viral RNA replicons: efficient assembly by recombination of DNA modules delivered by *Agrobacterium*. *Proc Natl Acad Sci USA* 101:6852–6857
- Marillonnet S, Thoeringer C, Kandzia R, Klimyuk V, Gleba Y (2005) Systemic *Agrobacterium tumefaciens*-mediated transfection of viral replicons for efficient transient expression in plants. *Nat Biotechnol* 23:718
- Meyers A, Chakauya E, Shephard E, Tanzer FL, Maclean J, Lynch A, Williamson A-L, Rybicki EP (2008) Expression of HIV-1 antigens in plants as potential subunit vaccines. *BMC Biotechnol* 8:53
- Obembe OO, Popoola JO, Leelavathi S, Reddy SV (2011) Advances in plant molecular farming. *Biotechnol Adv* 29:210–222
- Olejniczak SA, Łojewska E, Kowalczyk T, Sakowicz T (2016) Chloroplasts: state of research and practical applications of plastome sequencing. *Planta* 244:517–527
- Panstruga R, Hippe-Sanwald S, Lee Y-K, Lataster M, Lipka V, Fischer R, Liao YC, Häusler RE, Kreuzaler F, Hirsch H-J (1997) Expression and chloroplast-targeting of active phosphoenolpyruvate synthetase from *Escherichia coli* in *Solanum tuberosum*. *Plant Sci* 127:191–205
- Pineo CB, Hitzeroth II, Rybicki EP (2013) Immunogenic assessment of plant-produced human papillomavirus type 16 L1/L2 chimaeras. *Plant Biotech J* 11:964–975
- Richter S, Lampka GK (1998) A chloroplast processing enzyme functions as the general stromal processing peptidase. *Proc Natl Acad Sci USA* 95:7463–7468
- Santi L, Batchelor L, Huang Z, Hjelm B, Kilbourne J, Arntzen CJ, Chen Q, Mason HS (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. *Vaccine* 26:1846–1854
- Scotti N, Rigano MM, Cardi T (2012) Production of foreign proteins using plastid transformation. *Biotechnol Adv* 30:387–397
- Sheludko YV (2008) *Agrobacterium*-mediated transient expression as an approach to production of recombinant proteins in plants. *Recent Pat Biotechnol* 2:198–208
- Sheludko Y, Sindarovska Y, Gerasymenko I, Bannikova M, Kuchuk N (2007) Comparison of several *Nicotiana* species as hosts for high-scale *Agrobacterium*-mediated transient expression. *Biotechnol Bioeng* 96:608–614

- Shen BR, Zhu CH, Yao Z, Cui LL, Zhang JJ, Yang CW, He ZH, Peng XX (2017) An optimized transit peptide for effective targeting of diverse foreign proteins into chloroplasts in rice. *Sci Rep* 7:46231
- Skala W, Goettig P, Brandstetter H (2013) Do-it-yourself histidine-tagged bovine enterokinase: a handy member of the protein engineer's toolbox. *J Biotech* 168:421–425
- Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. *Science* 25:671–673
- Sohn EJ, Lee Y, Park N, Park M, Kim NH, Park S, Min K, Gu S, Park Y, Song J (2018) Development of plant-produced E2 protein for use as a green vaccine against *classical swine fever virus*. *J Plant Biol* 61:241–252
- Solá RJ, Griebenow K (2010) Glycosylation of therapeutic proteins. *BioDrugs* 24:9–21
- Tang WL, Zhao H (2009) Industrial biotechnology: tools and applications. *Biotechnol J* 4:1725–1739
- Tschofen M, Knopp D, Hood E, Stöger E (2016) Plant molecular farming: much more than medicines. *Annu Rev Anal Chem* 9:271–294
- Vaquero C, Sack M, Chandler J, Drossard J, Schuster F, Monecke M, Schillberg S, Fischer R (1999) Transient expression of a tumor-specific single-chain fragment and a chimeric antibody in tobacco leaves. *Proc Natl Acad Sci USA* 96:11128–11133
- Villarejo A, Burén S, Larsson S, Déjardin A, Monné M, Rudhe C, Karlsson J, Jansson S, Lerouge P, Rolland N (2005) Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. *Nat Cell Biol* 7:1224
- Vitlin Gruber A, Feiz L (2018) Rubisco assembly in the chloroplast. *Front Mol Biosci* 5:24
- Xu J, Ge X, Dolan MC (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol Adv* 29:278–299
- Yanez RJ, Lamprecht R, Granadillo M, Weber B, Torrens I, Rybicki EP, Hitzeroth II (2017) Expression optimization of a cell membrane-penetrating *human papillomavirus* type 16 therapeutic vaccine candidate in *Nicotiana benthamiana*. *PLoS One* 12:e0183177
- Yanez RJ, Lamprecht R, Granadillo M, Torrens I, Arcalís E, Stöger E, Rybicki EP, Hitzeroth II (2018) LALF32-51-E7, a HPV-16 therapeutic vaccine candidate, forms protein body-like structures when expressed in *Nicotiana benthamiana* leaves. *Plant Biotechnol J* 16:628–637
- Zahin M, Joh J, Khanal S, Husk A, Mason H, Warzecha H, S-j Ghim, Miller DM, Matoba N, Jenson AB (2016) Scalable production of HPV16 L1 protein and VLPs from tobacco leaves. *PLoS One* 11:e0160995
- Zhang XP, Glaser E (2002) Interaction of plant mitochondrial and chloroplast signal peptides with the Hsp70 molecular chaperone. *Trends Pharmacol Sci* 7:14–21
- Zhong H, Teymouri F, Chapman B, Maqbool SB, Sabzikar R, El-Maghraby Y, Dale B, Sticklen MB (2003) The pea (*Pisum sativum* L.) rbcS transit peptide directs the *Alcaligenes eutrophus* polyhydroxybutyrate enzymes into the maize (*Zea mays* L.) chloroplasts. *Plant Sci* 165:455–462
- Zoschke R, Bock R (2018) Chloroplast translation: structural and functional organization, operational control, and regulation. *Plant Cell* 30:745–770
- Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS One* 3:e1994

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