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Minireview

Metabolic pathway engineering by plastid transformation is a powerful tool for production of compounds in higher plants

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Abstract Plastid transformation is a powerful tool for the production of useful compounds in higher plants through metabolic engineering, because it has many advantages over conventional nuclear transformation: high-level foreign protein accumulation, no need for a transit peptide, absence of gene silencing, and convenient transgene stacking in an operon. Plastid transformation has recently yielded remarkable results in the production of highly valued biopharmaceutical proteins and in conferring herbicide and insect resistance. Metabolic pathway engineering by plastid transformation has also produced higher levels of useful compounds than nuclear transformation. Furthermore, recent reports have shown the functional regulation of transgene expression from the plastid genome. In this review, we have focused on the progress of plastid transformation in material production from the aspect of biosynthetic pathway engineering, discussing the issues for future expansion of plastid transformation.

Key words: Astaxanthin, carotenoid, chloroplast, photosynthesis, plastid transformation, metabolic engineering.

Higher plants produce biomass such as cellulose and starch, and industrially applicable substances such as amino acids, fatty acids, isoprenoids, and flavonoids, from the carbon dioxide (CO_2) that is fixed in chloroplasts. Chloroplasts are the site of photosynthesis, where carbon atoms are primarily assimilated using chemical energy (ATP and NADPH) converted from harvested light energy. In chloroplasts, sucrose, most amino acids, fatty acid precursors (acyl-acyl carrier protein (ACP)), isoprenoids such as monoterpenes, and carotenoids are synthesized. Thus, for the improvement of production of these substances in higher plants through metabolic pathway engineering by genetic transformation, the enhancement of metabolic flux in chloroplasts is one of the effective strategies. The chloroplast is one of the organelles categorized as plastids, encompassing proplastids, the progenitor of all plastid types, chloroplasts (green plastids), chromoplasts (yellow or red plastids, found in some fruits and flowers), and different types of white plastids such as amyloplasts (containing starch) and elaioplasts (containing oil) (Gillham, 1994). In several crop plants, such non-green plastids are the storage site for metabolites in sink tissues (seeds, roots, tubers, and fruits). Accordingly, metabolic pathway engineering in non-green plastids is also expected to be a tool for the overproduction of useful compounds.

In engineering plastid metabolic pathways using a conventional *Agrobacterium*-mediated nuclear genome transformation method, it has been necessary to import foreign proteins translated in the cytosol into the plastids by the addition of a transit peptide-coding sequence such as the sequence of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit to the 5′-terminus of the transgene to allow the translocation of foreign proteins across the plastid membrane (Misawa et al. 1993). The transit peptide needs to be proteolytically removed by endogenous proteases to yield a mature functional protein in plastids. However, nuclear transformants can potentially accumulate unprocessed proteins that are not functional and that can interfere with the expected results (Jayaraj et al. 2007), because transit-peptide scission efficiency is dependent on the amino acid sequence around the connecting region with the target protein (Robinson and Ellis 1985; Wasmann et al. 1988).

Abbreviations: ACP, acyl carrier protein; AS, anthranilate synthase; CPL, chorismate pyruvate-lyase; DXR, deoxyxylulose phosphate reductoisomerase; DXP, deoxyxylulose phosphate; GUS, β-glucuronidase; MEP, methylerythritol phosphate; PHB, polyhydroxybutyrate; pHBA, p-hydroxybenzoic acid; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; T7RNAP, T7RNA polymerase; UTR, untranslated region.

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Table 1. Useful compounds produced through transplastomic engineering

Compound	Foreign protein	Production level in transplastomic plants	Literature reference
Astaxanthin	β-Carotene ketolase/ β-Carotene hydoxylase	5.44 mg g^{-1} dry weight in leaves of *N. tabacum* (total carotenoid level 2.1-fold higher than wild type)	Hasunuma et al. (2008a)
β-Carotene	Lycopene β-cyclase	0.2861 mg g^{-1} dry weight in fruits of *S. lycopersicum* (4-fold higher than wild type)	Wurbs et al. (2007)
p-Hydroxybenzoic acid	Chorysmate pyruvate lyase	26.5% dry weight in old leaves of *N. tabacum*	Viitanen et al. (2004)
Isoprenoids	Deoxyxylulose phosphate reductoisosmerase	The level of total chlorophyll, total carotenoid, solanesol and sitosterol is 23.5%, 16.1%, 32.2%, 25.0%, 243.0% higher than wild type in leaves of *N. tabacum*	Hasunuma et al. (2008b)
Polyhydroxybutyrate	β-Ketothiolase/	1.7% dry weight in leaves of *N. tabacum*	Lössl et al. (2003)
	Acetoacetyl-CoA reductase/	0.16% dry weight in leaves of *N. tabacum*	Arai et al. (2004)
	PHB polymerase	1.383 % dry weight in leaves of *N. tabacum*	Lössl et al. (2005)
Tryptophan	Anthranilate synthase α-subunit	350 nmol g^{-1} fresh weight in leaves of *N. tabacum* (10-fold higher than wild type)	Zhang et al. (2001)

Plastids are cellular organelles with their own genome and transcription-translation machinery, which can be genetically engineered by insertion of foreign genes. After plastid transformation of higher plants was first achieved in tobacco (*Nicotiana tabacum*) in 1990 (Svab et al. 1990), elements of transformation techniques such as the gene transfer method, vector construction, positive selection and marker gene elimination have been improved using tobacco as a model plant (reviewed in Maliga 2004; Verma and Daniell 2007).

Plastid transformation offers several advantages over nuclear transformation. First, the expression of a transgene from the plastid genome eliminates the need for a transit peptide. Secondly, because the plastid genome is highly polyploid (500–10,000 copies of the genome per cell) (Bendich 1987), the expression level of transgenes is likely to be high, and thus has the potential to confer high levels of protein accumulation compared to transgene expression from the nuclear genome. For instance, the expression of *Bacillus thuringiensis cry2*Aa2 operon in tobacco chloroplasts under the control of the 16S ribosomal RNA (*rrn*) promoter caused accumulation of the protein at levels representing 45.3% of total soluble protein in mature tobacco leaves (De Cosa et al. 2001). This ability of plastid transformation enables boosting metabolic flux more drastically than nuclear transformation when the flux is enhanced by increasing rate-limiting enzyme activity (Hasunuma et al. 2008a; Viitanen et al. 2004; Zhang et al. 2001). Third, since plastids possess a prokaryotic gene expression system, polycistronic multigene expression would be achieved under the control of a single promoter, which enables enhancing sequential metabolic reactions in a single transformation procedure (Hasunuma et al. 2008a; Lössl et al. 2005). This has another advantage: there is no need to use several selection markers. Fourth, the site-specific integration of a transgene into the plastid genome by homologous recombination eliminates positional effects that are frequently observed with nuclear transformation (Lee et al. 2003). Fifth, no gene silencing has been observed in genetically engineered plastids. In general, positional effects due to random transgene integration and gene silencing confer unwanted variability in expression levels between transgenic lines. This not only limits the level of transgene expression but also makes it difficult to analyze the direct effects of foreign gene expression. The homologous recombination of the plastid genome prevents the disruption of endogenous genes, and assessment of gene expression effects on metabolic changes in transplastomic plants is more straightforward than in nuclear transformants (Hasunuma et al. 2008b; Zhang et al. 2001). Finally, since it is widely believed that the plastid genome is maternally inherited, transgene flow via pollen is greatly repressed, which provides an additional safeguard for transgene containment (Daniell 2002; Zhang et al. 2001).

Thus, the plastid transformation technique has remarkable characteristics as a tool for the production of useful compounds through metabolic engineering. In Table 1, the list of compounds produced by plastid transformation is shown. In this review, we summarize the successful examples and current picture of plastid transformation, and discuss the issues that should be overcome to enhance the material production capacity of higher plants.

Successful production of useful compounds through transplastomic engineering

Plastid transformation can lead to higher accumulation levels of enzymes than nuclear transformation, which enables overproduction of target metabolites by enhancement of enzyme activity. Viitanen et al. (2004) generated transplastomic tobacco expressing a chorismate pyruvate-

lyase (CPL) gene, *ubiC*, from *Escherichia coli* under the control of a tobacco-derived *psbA* promoter. The transformed tobacco showed 250-fold higher CPL activity compared with a nuclear transformant expressing the same CPL gene (Siebert et al. 1996), and also contained 50-fold higher levels (26.5% dry weight) of p-hydroxybenzoic acid (pHBA) glucose conjugates, the product of the CPL reaction, in the leaves than in those of the nuclear transformant. pHBA is much in demand as a major monomer in liquid crystal polymers. CPL, which is absent in higher plants, synthesizes both pyruvate and pHBA from chorismate, an intermediate of the shikimate pathway that produces aromatic amino acids such as phenylalanine, tyrosine and tryptophan in plastids. In the nuclear transformants that express the *E. coli* CPL gene, CPL specific activity showed positive correlation to the level of pHBA glucose conjugates in the leaves (Siebert et al. 1996), suggesting that the CPL reaction controls the level of the pHBA conjugates.

Another example of the effectiveness of plastid transformation for the overproduction of useful compounds is the generation of astaxanthin-producing transplastomic plants through carotenoid pathway engineering. A transplastomic tobacco that expresses both β-carotene ketolase and β-carotene hydroxylase genes from the marine bacterium *Brevundimonas* sp. strain SD212 accumulated large quantities of astaxanthin at concentrations of up to 5.44 mg g^{-1} dry weight, corresponding to 74% of the total carotenoid (Hasunuma et al. 2008a). This greatly exceeds the levels reached in previous nuclear transformants (Gerjets and Sandmann 2006; Gerjets et al. 2007; Mann et al. 2000; Ralley et al. 2005). Astaxanthin, one of the carotenoids synthesized by some bacteria and fungi, is a highly valued red pigment. This pigment has been industrially exploited as a feed dye, particularly as a feed supplement in poultry farming and aquaculture. Also, since the diverse biological functions of astaxanthin include involvement in the anti-oxidation of low-density lipoprotein (Iwamoto et al. 2000), anticancer activities (Tanaka et al. 1994), singlet oxygen-quenching activity (Tatsuzawa et al. 2000), and enhancement of immune responses (Jyonouchi et al. 1995), its use in the pharmaceutical, food and feed industries is expected to increase dramatically in the near future. Astaxanthin can be synthesized by the introduction of keto and hydroxyl moieties at the 4,4′ and 3,3′ positions of the β-ionone rings of β-carotene. This can be achieved by two enzymes, a β-carotene ketolase and a β-carotene hydroxylase. Since plants are devoid of a β-carotene ketolase, apart from very few exceptions, such as *Adonis* flowers (Cunningham and Gantt 2005), the functional expression of a heterologous β-carotene ketolase gene in plants is required for the production of astaxanthin. In order to produce astaxanthin, transgenic plants have so far been generated through conventional engineering by *Agrobacterium*-mediated gene transfer, but the yield of astaxanthin is low and far from a practicable level because of the low conversion efficiency of β-carotene to astaxanthin. Plastid transformation circumvented the difficulties by increasing the conversion efficiency; it produced large quantities of astaxanthin, which changed the color of the aerial part from green to reddish brown. Interestingly, there was no significant difference in the size of the aerial part between the transformants and wild-type plants at the final stage of their growth. The photosynthetic rate of the transformants was also similar to that of wild type under high light conditions. The growth phenotype has a positive significance in increasing the yield of astaxanthin per plant, because the yield depends on the plant size. Furthermore, this study is one of the examples showing success in multigene expression by a single promoter. Higher plants exhibit β-carotene hydroxylase activity, which is considered to be the rate-limiting step of the carotenoid (xanthophyll) biosynthetic pathway because the activity of β-carotene hydroxylase has a positive correlation with the pool size of xanthophylls (Johnson et al. 2007). Therefore, the overproduction of astaxanthin was attributed to the co-expression of *Brevundimonas* β-carotene hydroxylase and β-carotene ketolase genes. The quantity of astaxanthin is higher in the plants co-expressing these two genes compared with a transformant expressing only the β-carotene ketolase gene.

Zhang et al. (2001) succeeded in the overproduction of tryptophan by targeting a nuclear anthranilate synthase (AS) α-subunit gene to the tobacco plastid genome. AS is the controlling enzyme of the tryptophan biosynthetic pathway (Radwanski and Last 1995), converting chorismate to anthranilate. AS holoenzymes are tetramers consisting of two α- and β-subunits encoded by separate nuclear genes and synthesized in the cytosol as precursor proteins with a plastid-targeting transit peptide. After entering the plastid, the transit peptide is cleaved and the mature subunits are assembled into a holoenzyme (Bohlmann et al. 1995). Biosynthesis of tryptophan is tightly controlled, not only through end product tryptophan feedback of the AS enzyme, but also by the regulation of the abundance of AS mRNAs (Radwanski and Last 1995). To avoid posttranscriptional regulation, Zhang et al. (2001) integrated a tobacco cDNA (*ASA2*) (Song et al. 1998) encoding a feedback-insensitive AS α-subunit into the tobacco plastid genome through site-specific insertion. A high level of *ASA2* mRNA was observed in the transplastomic plants but not in the wild-type plants. The transplastomic plants exhibited a higher level of the AS α-subunit and AS enzyme activity that was less sensitive to tryptophan feedback inhibition, leading to a 10-fold increase in tryptophan level of the leaves compared with wild-type plants. On the other hand, the tryptophan level in transgenic tobacco plants expressing the same *ASA2* cDNA from the nuclear

genome was only 3 times greater than that of wild-type plants (Tsai et al. 2005). The higher tryptophan productivity in the transplastomic tobacco was achieved by relocating the *ASA2* gene from the nucleus to the plastid genome. This study also suggested that the abundance of α-subunits encoded by the plastidic *ASA2* might stabilize the available β-subunits, resulting in more functional AS holoenzymes.

Thus, when the metabolic flux of target products is controlled by the abundance of metabolic enzymes in plastids, massive accumulation of the enzyme by plastid transformation has the potential to enhance the level of target metabolites compared with nuclear transformation. However, when a foreign protein does not catalyze a rate-limiting step in its metabolic pathway, transformants accumulate excess protein that is unnecessary for metabolic engineering (Hasunuma et al. 2008b). Deoxyxylulose phosphate reductoisomerase (DXR), which converts deoxyxylulose phosphate (DXP) to methylerythritol phosphate (MEP), is the first committed step of the plastidial isoprenoid biosynthetic pathway, the MEP pathway, because DXP is a precursor not only of isoprenoids but also of cofactor thiamine pyrophosphate (Julliard and Douce 1991). Plant isoprenoids are of industrial, nutritional and medicinal importance as additives, organic materials, vitamins and ingredients in medicine (Cane 1999). To improve isoprenoid production in higher plants, transplastomic tobacco was generated that expressed the DXR gene from the cyanobacterium *Synechosystis* sp. strain PCC6803 from the plastid genome. However, the level of isoprenoids such as chlorophyll *a*, lutein, β-carotene and solanesol in the transplastomic plants was only 23.5%, 16.1%, 32.2%, 25.0% higher than that in wild-type plants, although the transformants accumulated large quantities of foreign DXR (5.4–7.4% of total soluble protein) and showed 350-fold higher DXR activity than wild type (Hasunuma et al. 2008b). This result indicates that the isoprenoid content in plastids is controlled more by alternative factors such as DXP supply and other rate-limiting enzymes than by DXR. The increased rate of total chlorophyll and carotenoid amount in the transplastomic tobacco plants was similar to that in a DXR-overexpressing *Arabidopsis* nuclear transformant in which DXR activity was comparatively very low (Carretero-Paulet et al. 2006), indicating that for the overproduction of isoprenoids in transgenic tobacco plants, a large increase in DXR activity would not be necessary. Recently, a fatty acid Δ^9-desaturase gene from either a wild potato species (*Solanum commersonii*) or the cyanobacterium *Anacystis nidulans* was integrated into the tobacco plastid genome (Craig et al. 2008), but the generated transformants showed no significant difference in fatty acid composition compared to nuclear transformants (De Palma et al. 2008). Those studies demonstrate that, in order to produce a large quantity of a useful compound in plants through metabolic engineering, it is very important to manipulate rate-limiting enzymes in the metabolic pathway accurately, an objective that is not confined to just plastid transformation.

Recent progress in plastid transformation technology for metabolic engineering

The engineering of a nutritionally important metabolic pathway via plastid transformation seems a very worthwhile challenge. Until recently, the application of plastid transformation to metabolic pathway engineering was restricted to the model species tobacco, which can be transformed relatively easily. The procedures are technically demanding, and the construction of a plantlet regeneration system from either leaf explants or callus that are damaged by gene transfer manipulation such as particle bombardment and chemical treatment is required. Both selection agents and growth conditions have great influence on the regeneration of transformants. The implementation of plastid genome engineering in agriculture will depend both on methodological improvements that make the technology routine in important food crops, and on success with the expression of transgenes in edible plant organs such as tubers and fruits, which often contain non-green plastid types (amyloplasts and chromoplasts). In recent years, protocols for some food crops have been established (Dufourmantel et al. 2004; Kanamoto et al. 2006; Kumar et al. 2004; Ruf et al. 2001; Sidorov et al. 1999). Wurbs et al. (2007) succeeded in altering carotenoid biosynthesis in tomato (*Solanum lycopersicum*) fruits, which produced an elevated content of provitamin A (β-carotene), an important antioxidant and essential vitamin for human nutrition. This is the first report of material production in a vegetable crop through metabolic pathway engineering using plastid transformation, where the expression of the lycopene β-cyclase gene from the bacterium *Erwinia herbicola* under the control of the plastid *atpI* promoter led to an approximately 4-fold increase in β-carotene content in the transplastomic fruits compared with that in the wild type. Lycopene β-cyclase converts lycopene to β-carotene. In the transplastomic tomato, the level of lycopene decreased, which changed the color of fruits from red to orange. Also, no metabolic change was observed in the leaves, indicating that the metabolic engineering was fruit-specific. However, in a previous report (Rosati et al. 2000), transgenic tomato that expressed the *Arabidopsis* lycopene β-cyclase from the nuclear genome under the control of a fruit-specific phytoene desaturase promoter showed a 7-fold-higher β-carotene content in the fruit than wild type, indicating that tomato fruits have the potential to accumulate β-carotene to higher levels than

observed in the transplastomic plant. In order to achieve a further increase in the level of β-carotene by plastid transformation, a systematic analysis of gene expression in such non-green types of plastids is needed to identify expression elements (e.g., promoters, 5′ and 3′ untranslated regions (UTR)) that are particularly suitable to trigger high-level plastid transgene expression in non-leafy tissues (Bock 2007).

Although plastid transformation has the potential to produce high levels of proteins, constitutive expression of transgene products can occasionally be deleterious to plant health because of toxicity or interference with their metabolism (Magee et al. 2004; Tregoning et al. 2003). Interaction between heterologous products and metabolism in different growth stages reduces potential plant productivity or even inhibits selection of primary transformants. For instance, constraints such as growth inhibition and male sterility have been observed in plastid transformants containing the *phb* operon, encoding genes required for the production of polyhydroxybutyrate (PHB), a class of biodegradable polyesters, under the control of constitutive expression promoters such as the plastid *psbA* and *rrn* promoters (Arai et al. 2004; Lössl et al. 2003). In the bacterium *Ralstonia eutropha*, PHB was synthesized from acetyl-CoA by a sequence of three enzymatic reactions (Slater et al. 1988). First, condensation of two molecules of acetyl-CoA is catalyzed by β-ketothiolase to form acetoacetyl-CoA. Acetoacetyl-CoA reductase then reduces acetoacetyl-CoA to β-hydroxybutyryl-CoA, which is then polymerized by PHB polymerase to PHB. In R. *eutropha*, the genes for these enzymes are organized in a single operon. Expressing the transgene at a particular developmental stage has been required, but the missing functional regulation of plastid gene transcription illustrates the problem that plastid transformation technology alone does not allow any tuning of transgenes inserted into the plastid genomes. Consequently, Lössl et al. (2005) constructed a trans-activation system to regulate transcription of the *phb* operon. In this system, *phb* operon expression from the plastid genome was controlled by a nuclear-located ethanol-inducible T7RNA polymerase (T7RNAP) that was targeted to plastids using the Rubisco small subunit transit peptide. The ethanol-inducible gene-control system was based on the *alcA* promoter and transcription factor ALCR for the alcohol dehydrogenase regulon of *Aspergillus nidulans* (Caddick et al. 1998). The plastid *phb* operon was driven by a T7 promoter so that spraying of the transgenic plants with ethanol induced expression of the T7RNAP in the nucleus, which in turn switched on transcription of the *phb* operon in plastids. In this report, PHB in the leaves of the transformants treated with 5% ethanol reached a level (1383 ppm in dry weight) similar to that in previous transplastomic tobacco that expressed the *phb* operon constitutively. Without ethanol induction, the development of flowers and fertile seeds was possible. The transcription of the T7RNAP gene followed ethanol induction within 24 h and the accumulation of PHB reached a maximum level between 14 and 21 days after induction. This was the first report on inducible trans-activation of transgenes introduced into the plastid genome, although there was a slight degree of leakiness. On the other hand, PHB was accumulated at levels of up to 14.3% dry weight in young leaves of transgenic *Arabidopsis* plants that express PHB biosynthetic genes from the nuclear genome by chemical (ecdysone analog) induction (Kourtz et al. 2007). Therefore, further improvement of the gene expression system will be required to achieve higher accumulation of inducible PHB in transplastomic plants.

Future directions

So far, the plastid transformation technique has proven to be an efficient tool for the production of biopharmaceutical proteins, including insulin, interferons and somatotropin, and highly valued antibodies and vaccines, and for the addition of herbicide and insect resistance, because of the high production capacity of plastids, as shown in a number of experiments (reviewed in Verma and Daniell 2007). In this review, we have focused on the progress of the technique in metabolite production by biosynthetic pathway engineering. Since the 1990s, conventional nuclear-genome transformation has generated numerous metabolically engineered plants, but the accumulation levels of target metabolites have generally been far below those required to make plants a practical, economically competitive platform for bioproduction. Plastid transformation has shown the potential to resolve the problem by massive accumulation of the enzyme of interest, although the technique has not been utilized much for metabolic pathway engineering. In the future, with improved elucidation of the control mechanisms of metabolic processes in higher plants, the chance to use plastid transformation will increase because the enhancement of rate-limiting steps in a metabolic flux enables overproduction of target metabolites in plants.

In contrast to molecular farming and resistance engineering, massive accumulation of enzyme is not constantly necessary for metabolic pathway engineering. In some cases, an excess of foreign protein accumulation in chloroplasts constitutes a burden for plants, leading to slowing of growth and pigment deficiency (Magee et al. 2004; Tregoning et al. 2003). In plastid transformation, the desired expression levels can be adjusted by choosing appropriate combinations of plastid expression sequences such as promoters and untranslated region elements, but this approach is in the trial-and-error stage and optimization of the sequences is required. This is because the sequence of the coding region has a strong in-

fluence on expression levels. The fruit-specific expression of a transgene was achieved by the *atpI* promoter (Wurbs et al. 2007), which remains the only example of tissue-specific metabolic engineering. Thus, for future expansion of the use of plastid transformation, catalogues of plastid expression sequences should be required, based on the strength and site of gene expression.

The levels of expressed proteins in plastids are dependent not only on the abundance of transcripts but also on the efficiency of gene translation. Several studies suggest that translation efficiency is of more importance for protein stability than transcript abundance in transgenic chloroplasts (Dhingra et al. 2004). The translation efficiency is influenced by the sequence of both UTR elements and coding regions (Eibl et al. 1999; Tregoning et al. 2003; Zou et al. 2003). Transgenic tobacco lines containing different combinations of UTRs showed 5-fold variation in the *uidA*-mRNA level and approximately 100-fold difference in β-glucuronidase (GUS) activity (Eibl et al. 1999). Zou et al. (2003) has shown that the secondary structure of the *psbA* 5′-UTR stem-loop is required for mRNA stabilization and efficient translation using site-directed mutagenesis. In plastids, the difference in usage of synonymous codons that are not used with equal frequency in protein-coding sequences also affects the translation efficiency (Nakamura and Sugiura 2007). Early observations in *E. coli* and yeast showed that codon usage is positively correlated with tRNA content, especially for highly expressed genes (Ikemura 1985). Based on this correlation, codon optimization generally improved the expression of mammalian proteins in *E. coli* (Gustafsson et al. 2004). Nakamura and Sugiura (2007) showed that translation efficiencies of synonymous codons for phenylalanine and tyrosine were contrary to their codon usage, whereas those for alanine, aspartic acid and asparagine were parallel to their codon usage in tobacco chloroplasts by an *in vitro* translation system. Further analysis of the translation efficiency between synonymous codons will be required for the efficient production of recombinant proteins in plastids. The modulation of the primary sequence as well as the secondary structure of transgene will contribute to the control of the accumulation level of foreign proteins.

Tobacco has been a widely exploited host for plastid transformation because of its ease of use in genetic manipulation (Verma and Daniell 2007). As 1 hectare of tobacco can produce more than 100 metric tons of leaves per year (Verma and Daniell 2007), this plant holds great promise as a host for the production of useful compounds. According to estimates, transplastomic tobacco that expresses both *Brevundimonas* sp. *crtZ* and *crtW* (Hasunuma et al. 2008a) can produce 54.4 kg of astaxanthin per hectare per year. Also, the platform of plastid transformation has recently been expanded to several other plant species. Therefore, for future improvement of useful compound levels per planted area, production in crop plants that possess sink tissue will be desirable.

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