Arabidopsis Tic40 Expression in Tobacco Chloroplasts Results in Massive Proliferation of the Inner Envelope Membrane and Upregulation of Associated Proteins

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**Arabidopsis Tic40 Expression in Tobacco Chloroplasts Results in Massive Proliferation of the Inner Envelope Membrane and Upregulation of Associated Proteins**

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**ABSTRACT**

The chloroplast inner envelope membrane (IM) plays essential roles in lipid synthesis, metabolite transport, and cellular signaling in plants. We have targeted a model nucleus-encoded IM protein from *Arabidopsis thaliana*, pre-Tic40-His, by relocating its expression from the nucleus to the chloroplast genome. Pre-Tic40-His was properly targeted, processed, and inserted. It attained correct topology and was folded and assembled into a TIC complex, where it accounted for up to 15% of the total chloroplast protein. These results confirm the existence of a novel pathway for protein targeting to the IM. Tic40-His overexpression resulted in a massive proliferation of the IM (up to 19 layers in electron micrographs) without significant effects on plant growth or reproduction. Consistent with IM proliferation, the expression levels of other endogenous IM proteins (IEP37, PPT, Tic110) were significantly (10-fold) upregulated but those of outer envelope membrane (Toc159), stromal (hsp93, cpn60), or thylakoid (LHCP, OE23) proteins were not increased, suggesting retrograde signal transduction between chloroplast and nuclear genomes to increase lipid and protein components for accommodation of increased accumulation of Tic40. This study opens the door for understanding the regulation of membrane biogenesis within the organelle and the utilization of transgenic chloroplasts as bioreactors for hyperaccumulation of membrane proteins for biotechnological applications.

**INTRODUCTION**

Chloroplasts are highly complex organelles that perform a vast array of essential metabolic processes in plants and algae, including photosynthesis, amino acid and lipid metabolism, and secondary product synthesis. The biogenesis and differentiation of chloroplasts is dependent upon the expression of genes encoded in the chloroplast and the nuclear genomes. The majority of nucleus-encoded chloroplast proteins are synthesized in the cytoplasm and imported into the organelle via the TOC-TIC translocation systems of the chloroplast envelope (Inaba and Schnell, 2008; Jarvis, 2008). In many cases, these proteins are further targeted to subcompartments of the organelle (e.g., the thylakoid membrane and lumen or inner envelope membrane [IM]) by additional targeting systems that function downstream of the import apparatus (Schunemann, 2007).
Much progress has been made in understanding the molecular mechanism of TOC-TIC function and the subsequent targeting of proteins to the thylakoid membrane. The TOC-TIC system consists of multisubunit complexes within the outer membrane and IM (Inaba and Schnell, 2008). These complexes physically associate to mediate preprotein recognition by binding preprotein transit peptides in the cytoplasm and provide direct transport of polypeptides from the cytoplasm to the chloroplast stroma via linked protein-conducting channels. Nucleus- and chloroplast-encoded proteins are targeted from the stroma to thylakoids by at least four protein-trafficking systems (Schunemann, 2007). These systems all correspond to those found in Gram-negative bacteria and presumably were conserved from the original endosymbiont during chloroplast evolution.

In contrast with the protein-import and thylakoid-targeting systems, our knowledge of the pathways and molecular mechanisms of protein targeting and integration at the IM are very limited. The IM contains a complex array of proteins, including enzymes involved in lipid synthesis, the production of secondary products for plant defense and cellular signaling, and transporters that mediate the exchange of metabolites and the import of nucleus-encoded proteins into the organelle (Block et al., 2007). As such, knowledge of the biogenesis of the IM is central to understanding the metabolic and communication networks that link chloroplasts with other cellular activities.

The bulk of IM proteins are nucleus-encoded in vascular plants, with only one or two possible exceptions (Ferro et al., 2002, 2003; Froehlich et al., 2003). At least two pathways for IM targeting have been proposed for nucleus-encoded proteins that initially engage the TOC-TIC system for import from the cytoplasm. The first, or so-called stop-transfer pathway, is directly coupled to the process of protein import at the envelope (Li et al., 1992; Brink et al., 1995; Knight and Gray, 1995). The stop-transfer models predict that proteins are directly integrated into the IM during translocation through the TIC translocon. In this scenario, the protein-conducting channel of the TIC complex would sense the presence of transmembrane helices and allow lateral diffusion of the helices into the lipid bilayer. The second pathway is proposed to function independently of the protein-import process. In this so-called postimport pathway, IM proteins are fully imported into the chloroplast stroma via the TOC-TIC system and inserted into the IM from the stroma via an unidentified translocon (Lubeck et al., 1997; Li and Schnell, 2006; Tripp et al., 2007; Chiu and Min Li, 2008). The most detailed evidence in support of the postimport pathway has come from studies using Arabidopsis thaliana pre-Tic40, a single-pass transmembrane protein that functions as a cochaperone in the import apparatus (Chou et al., 2006). Pre-Tic40 normally is nucleus-encoded and imported into the organelle after synthesis in the cytoplasm. Previous studies have shown that pre-Tic40 targeting to the IM involves a soluble intermediate that inserts into the IM from the chloroplast stroma after import from the cytoplasm (Li and Schnell, 2006; Tripp et al., 2007). Targeting to the IM involves two-step proteolytic processing that removes the transit peptide in the stroma and an additional N-terminal sequence at the IM.

The postimport hypothesis predicts that a subset of proteins are targeted to the IM independently of the protein-import apparatus, raising the possibility that these proteins would successfully integrate into the IM if their genes were introduced into the chloroplast genome and the proteins were expressed in the stroma. The possibility of expressing IM proteins from the plastid genome provides a direct experimental approach to test the postimport model. Furthermore, a plastid expression system for IM proteins opens up the possible use of the IM as a target for the expression of recombinant membrane proteins of agronomic and biotechnological interest. The IM is a common feature of plastid types in all plant tissues, and the maternal inheritance of plastids in all crop species eliminates the concerns associated with nuclear transgenes. The transgenic chloroplast system has been used to express several soluble proteins, including vaccine antigens and human blood proteins (Kamarajugadda and Daniell, 2006; Verma and Daniell, 2007). Similarly, transgenic chloroplasts have been used to express several proteins to confer herbicide, insect, or disease resistance and drought or salt tolerance (Daniell et al., 2005).
In this study, we aimed to test the postimport targeting hypothesis and explore the potential of expressing IM proteins in chloroplasts by engineering the gene encoding *Arabidopsis* pre-Tic40 into the plastid genome of tobacco (*Nicotiana tabacum*). The strategy was based on our success in expression of the pre-EPSP synthase (Daniell et al., 1998) or pre-small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Dhingra et al., 2004) in chloroplasts. We demonstrate that plastid-encoded pre-Tic40 is properly processed and targeted and successfully assembled into a functional complex at the IM. Tic40 accumulates to 15% of the total chloroplast protein, resulting in a massive proliferation of the IM. Despite these dramatic changes in chloroplast ultrastructure, transgenic lines exhibited growth and fertility similar to control plants. This study strongly supports the postimport pathway and establishes the potential of chloroplast genetic engineering to stably express membrane proteins within the organelle.

**RESULTS**

**Chloroplast Vector Design**

The pre-Tic40-His construct used in this study includes a C-terminal 6-His tag and a 76–amino acid N-terminal transit peptide that is normally required for import from the cytoplasm. The transit peptide is removed during import and targeting by a two-step process involving the stromal processing peptidase and an unknown peptidase at the IM that is related to bacterial type I signal peptidases (Li and Schnell, 2006; Tripp et al., 2007). We chose to express pre-Tic40-His because its dual processing provides a convenient marker for synthesis in the stroma (stromal processing peptidase cleavage) and insertion into the IM (final maturation). The pLD-utr-pre-Tic40-His vector used in this study for chloroplast transformation (Figure 1B) is based on the universal chloroplast vector concept that has been used successfully in our laboratory (Verma and Daniell, 2007; Verma et al., 2008) for the expression of many transgenes into the tobacco chloroplast genome. The genes of interest are integrated into the spacer region between the *trnI* and *trnA* genes through homologous recombination of the flanking sequences between the transformation vector and the native chloroplast genome. This site of integration has several unique advantages (Daniell et al., 2004; Verma et al., 2008). The constitutive promoter 16S rRNA ribosomal promoter regulates the expression of the *aadA* (for aminoglycoside 3’ adenyltransferase) gene. The *psbA* promoter and 5’ untranslated region containing several ribosomal binding sites were engineered to enhance transcription and translation of the pre-Tic40-His gene. The transcript was stabilized by incorporating the *psbA* 3’ untranslated region.

**Transgene Integration into the Chloroplast Genome**

Transplastomic plants were obtained as described previously (Daniell et al., 2004; Verma et al., 2008). Several shoots emerged from leaves at 3 to 6 weeks after bombardment with gold particles coated with pLD-utr-pre-Tic40-His plasmid in the first round of selection (Figure 1C). The second round of selection advanced shoots toward homoplasmy (Figure 1D), and the third round of selection in root induction medium (Figure 1E) established independent transgenic lines. PCR analysis using two sets of primers, 3P/3M and 5P/2M, confirmed the transgene integration and site-specific integration of transgenes into chloroplast. As illustrated in Figure 1B, the 3P primer annealed to the native chloroplast genome upstream of the site of integration and the 3M primer annealed to the *aadA* gene, producing a 1.65-kb PCR product, while the 5P and 2M primers annealed to the *aadA* gene and the *trnA* coding sequences, respectively, which produced a 3.2-kb PCR product. All of the transplastomic lines produced the expected PCR products, while no amplification was observed in the wild type (Figures 1F and 1G).

**Evaluation of Homoplasmy**

DNA gel blot analysis was performed to confirm site-specific integration of the pLD-utr-pre-Tic40-His...
cassette into the chloroplast genome and to determine homoplasmy (plants containing only transformed chloroplast genomes). Total plant DNA was isolated from the rooted plants and digested with the enzyme SmaI, which should generate a 4.014-kb fragment in the wild type (Figure 1A) or a 7.078-kb fragment in transplastomic lines (Figure 1B) when hybridized with a 0.81-kb flanking sequence probe. The 32P-labeled trnI-trnA probe hybridized with 4.014- and 7.078-kb fragments in DNA from the wild-type and transplastomic lines, respectively, confirming site-specific integration of the transgenes in the spacer region between the trnI and trnA genes (Figure 2A). Furthermore, the absence of a 4.014-kb fragment in the transgenic lines confirmed that homoplasmy had been achieved (within the levels of detection), even in transplastomic lines in the T0 generation. Transplastomic lines were transferred to jiffy pellets and kept under high humidity initially for 2 weeks and then moved to the greenhouse. DNA gel blot analysis of T1 transplastomic lines confirmed that all of the transgenic lines maintained homoplasmy (Figure 2B).

**Phenotypic Evaluation and Fertility of Transgenic Lines**

The phenotypes of transplastomic lines appeared to be normal (Figure 2C). Transgenic seeds germinated and grew into green plants, while wild-type plants were bleached on medium containing spectinomycin (Figures 2D and 2E). The lack of transgene segregation suggests that transgene is maternally inherited by the progeny. Plant phenotype and fertility were investigated in wild-type and transgenic lines. Previous studies have shown that certain transgenic proteins reduce plant fertility when they are toxic or affect cellular or chloroplast metabolism. For example, male-sterile plants with shorter stamens and no viable pollen were produced when β-keto thiolase was expressed in transgenic chloroplasts (Ruiz and Daniell, 2005). However, pre-Tic40-His transplastomic lines exhibited normal growth and fertility compared with wild-type plants (Figure 3). Both wild-type and transplastomic lines showed similar stamen length and abundance of pollen grains, and both had normal floral development, fertilization, and pod development (Figure 3). Photosynthesis and plant pigments were not significantly different between the wild-type and transplastomic lines. Chlorophyll contents in the wild type and the pre-Tic40-His transplastomic lines are shown in Table 1.

**Expression and Localization of pre-Tic40-His in Transgenic Tobacco**

SDS-PAGE and immunoblot analysis of total leaf extracts confirmed expression of the pre-Tic40-His protein in two of the transgenic lines (TC42 and TC59) (Figure 4). A major 45-kD polypeptide is present in TC42 and TC59 extracts (Figure 4A, lanes 4 and 7, arrow). This polypeptide reacts with anti-His6 antibodies (Figure 4B), confirming that it corresponds to Tic40-His. An immunoreactive band is not observed in extracts from wild-type plants (Figure 4B, lanes 1 to 3). The Tic40-His polypeptide cofractionates with the native IM protein IEP37, consistent with the membrane localization of Tic40 (Figure 4, lanes 5 and 8). By contrast, the soluble stromal protein, large subunit of Rubisco (LSu), is enriched in soluble fractions (Figure 4B, middle panel).

In order to confirm that pre-Tic40-His was expressed within chloroplasts, chloroplasts were isolated from transgenic TC59 tobacco plants. The 45-kD imido black–stained band that reacted with the anti-His6 antibody was enriched in chloroplast fractions, confirming localization within this organelle (Figure 5, compare lanes 2 with 5). This pattern is similar to the chloroplast markers IEP37 and LSu but distinct from actin, which is found exclusively in whole cell lysates (Figure 5, bottom panels). After extraction of the chloroplast samples with alkaline carbonate, the majority of Tic40-His and IEP37 remained with the membrane fraction, whereas LSu was enriched in the soluble fraction (Figure 5, compare lanes 6 and 7). These results confirm the membrane integration of Tic40-His.

Pre-Tic40 from *Arabidopsis* is normally processed in two steps to generate mature Tic40 (Li and Schnell, 2006; Tripp et al., 2007). The first processing step is performed by the stromal processing peptidase during import from the cytoplasm to remove residues 1 to 42 of the N-terminal transit peptide (Li and
This generates a stromal intermediate (int-Tic40) that is further processed to mature Tic40 at the IM during the integration process (Li and Schnell, 2006). Comparison of chloroplast-expressed pre-Tic40-His in transgenic line TC59 with in vitro translated markers corresponding to pre-Tic40-His, int-Tic40-His, and mature Tic40-His demonstrated that the vast majority of the transgenic protein had been processed to mature Tic40-His (Figure 5, compare lanes 1 and 2). Although no pre-Tic40-His was detected, a minor species corresponding to int-Tic40-His was observed (Figure 5, lower panel, lanes 5 and 6), suggesting that the transgenic protein was processed in two steps, similar to pre-Tic40 imported from the cytoplasm.

The proper targeting of Tic40-His is supported by the observations that it is membrane-associated and processed to its mature form. To confirm these data, we examined the suborganellar localization and assembly of Tic40-His directly. We separated isolated chloroplast membranes by sucrose gradient centrifugation and probed the distribution of Tic40-His in fractions enriched in IM and thylakoid proteins. Figure 6A shows that Tic40-His is highly enriched in membrane fractions containing the IM markers Tic110 and IEP37. This localization is distinct from LHCP, a thylakoid membrane marker. Taken together, these data support our conclusion that pre-Tic40-His is synthesized, processed, and properly targeted to the IM when expressed from the plastid genome.

The Arabidopsis Tic40 contains a single transmembrane helix at its N-terminal region, and the bulk of the protein extends into the stroma (Chou et al., 2003). In isolated inside-out inner membrane vesicles, the hydrophilic domain is exposed and therefore sensitive to protease digestion (Li and Schnell, 2006). To confirm that overexpressed Tic40-His attains the correct topology, isolated inner membrane vesicles were digested with thermolysin and the sensitivity of inner membrane proteins was examined by immunoblotting (Figure 6B). As controls, we examined the sensitivity of two additional endogenous inner membrane proteins. Tic110 contains a short N-terminal membrane anchor with the bulk of the protein exposed to the stroma and is therefore susceptible to protease digestion in IM vesicles (Inaba et al., 2003). IEP37 is anchored at its C terminus and extends into the intermembrane space between the outer and inner envelopes (Motohashi et al., 2003). In isolated IM vesicles, IEP37 is largely insensitive to protease digestion because the bulk of the polypeptide is located within the vesicle lumen. As expected, Tic40-His and the endogenous control, Tic110, are sensitive to protease treatment of inside-out vesicles (Figure 6B, compare lanes 1, 2, and 3). By contrast, IEP37 is largely resistant to protease treatment (Figure 6B, compare lanes 1, 2, and 3) (Motohashi et al., 2003). In addition to full-length IEP37, a slightly smaller polypeptide is also present after digestion due to the partial cleavage of the short C-terminal tail of the protein. The resistance of IEP37 to proteolysis is not due to intrinsic stability, because it was digested when the membrane barrier was disrupted by nonionic detergent (Figure 6B, compare lanes 3 and 4). Therefore, we conclude that Tic40-His attains the correct topology when expressed in the stroma and inserted into the inner envelope.

As a final step to confirm that Tic40-His obtained its native structure, we examined its ability to interact with partner proteins at the inner envelope. Tic40 functions as a membrane-anchored cochaperone within the TIC translocon of the envelope protein-import apparatus. It aids in coordinating the transfer of preproteins from the translocon to the stromal chaperone machinery during the protein-import process (Chou et al., 2006). Previous studies have demonstrated that a minor fraction of Tic40 interacts with a TIC complex containing Tic110 and the stromal Hsp93 chaperone under steady state conditions (Inaba et al., 2005; Chou et al., 2006). To test if Arabidopsis Tic40-His could interact with this complex in transgenic tobacco, transgenic chloroplasts were treated with a covalent cross-linker to stabilize protein–protein interactions, dissolved with nondenaturing detergents, and subjected to immunoprecipitation with anti-Tic110 antibodies. Figure 6C demonstrates that Tic40-His assembles into a TIC complex containing endogenous Hsp93 and Tic110 that is similar to those previously identified in pea (Pisum sativum) and Arabidopsis (lanes 4 and 7) (Nielsen et al., 1997; Chou et al., 2003). These interactions are...
specific because another abundant protein, IEP37, was not detected in the immunoprecipitates. These results demonstrate that a transgenic membrane protein can be expressed, targeted, integrated, and properly folded into its native conformation when expressed from the chloroplast genome.

Examination of the protein profiles of total leaf extracts or chloroplast fractions (Figures 4 and 5) indicated that Tic40-His was accumulating at levels that made it easily detectable by imido black staining. Quantification of the protein content in the TC59 samples indicated that Tic40-His accumulated to ∼15% of total chloroplast protein (Figure 5), demonstrating the remarkable efficiency of the postimport pathway. IM proteins normally constitute <1% of total chloroplast protein, raising the question of the effects of Tic40-His overexpression on the IM. Consistent with IM proliferation, the expression levels of other endogenous inner membrane proteins in line TC59 were significantly upregulated. IEP37, Tic110, and the triose phosphate-phosphate translocator were upregulated ∼10 fold compared with untransformed plants, as estimated by semiquantitative immunoblotting (Figure 7). The levels of the outer envelope membrane marker Toc159 and two stromal markers, hsp93 and cpn60, were not significantly different in transgenic and control plants (Figure 7). Likewise, the thylakoid proteins LHCP and OE23 appeared to be unchanged (Figure 7). Examination of isolated chloroplasts from wild-type and TC59 plants by differential interference contrast light microscopy demonstrated an alteration in chloroplast structure in the transgenic plants (Figure 8). In contrast with the uniform spherical morphology of wild-type chloroplasts, the transgenic chloroplasts contained a distended bulge surrounding internal whorl-like structures (Figure 8, top panels, indicated by arrows). Immunofluorescence with anti-His antibodies to detect Tic40-His showed a peripheral localization pattern with intense staining within the whorls of the bulge areas (Figure 8, middle panels). This pattern was distinct from the internal chlorophyll autofluorescence signal exhibited by thylakoid membranes (Figure 8, bottom panels) and is consistent with localization to the envelope. Anti-His signal was not observed in wild-type chloroplasts (Figure 8, left panels).

To investigate chloroplast ultrastructure in more detail, we examined chloroplasts in wild-type and transgenic lines by transmission electron microscopy (Figure 9). Wild-type chloroplasts exhibit typical envelope morphology containing tightly apposed outer membranes and IMs with roughly equivalent surface areas (Figure 9, wild type). By contrast, chloroplasts from both transgenic lines exhibit a massive expansion of the inner envelope (Figure 9, TC59 and TC42). The membrane appears to invaginate and form stacks or whorls underneath the outer membrane, up to 19 layers. These structures are remarkably similar to the Tic40-His–labeled bulges observed in the light microscopic images in Figure 8. The shape of transgenic chloroplasts is altered due to such invaginations compared with the lens-shaped organelles observed in untransformed chloroplasts. We conclude that the bulge structures containing Tic40-His observed by immunofluorescence microscopy correspond to the invaginated membrane whorls present in the electron microscopic images. Taken together, these data confirm that the IM specifically proliferates in response to overexpression of Tic40-His. The response includes the upregulation of other nucleus-encoded IM proteins, suggesting that IM proliferation involves a coordinated increase in both the lipid and protein components to provide increased membrane surface area to compensate for increased levels of Tic40.

**DISCUSSION**

Our results provide strong evidence for the existence of a postimport pathway for the targeting of nucleus-encoded proteins to the IM. Transfer of the gene encoding pre-Tic40-His to the plastid genome resulted in high-level expression of the protein in the chloroplast stroma. Remarkably, pre-Tic40 was efficiently and properly inserted into the IM from its site of synthesis in the stroma, demonstrating that insertion of this class of proteins can occur independent of the protein-import process. The molecular machinery mediating the postimport pathway remains to be identified, but our results demonstrate the existence of a
unique targeting pathway from the chloroplast stroma that is distinct from the conservative thylakoid protein-targeting systems.

The chloroplast envelope is estimated to account for ~1% of total chloroplast protein and <10% of chloroplast membrane surface area under normal conditions (Block et al., 2007). The fact that overexpressed Tic40-His alone can accumulate to ~15% of total chloroplast protein and induce dramatic membrane proliferation provides key insights into the regulation of membrane biogenesis in this organelle. Such proliferation of membranes as the result of overexpression of one of the proteins has been observed in the yeast endoplasmic reticulum/nuclear envelope (Wright et al., 1988) and in the plant endoplasmic reticulum (Gong et al., 1996), and in both cases the membranes had similar appearances to the chloroplast inner envelope shown in this study. Chloroplasts are the major source of fatty acids in plant cells, and together with the endoplasmic reticulum they generate the majority of cellular and chloroplast membrane lipids (Benning, 2008). The IM is the primary site of lipid synthesis, and it plays a direct role in the generation of constituents for all three chloroplast membranes. Despite this interconnection, our data indicate that the biogenesis of the three chloroplast membrane systems is independently regulated. In the case of the inner envelope, membrane expansion is coupled to the levels of membrane protein synthesis.

One particularly interesting observation from our study was the apparent coordinated regulation of membrane expansion and membrane protein expression. The induction of membrane proliferation by overexpression of Tic40-His in the stroma resulted in the concomitant upregulation of the expression of other IM proteins, including IEP37 (S-adenosyl methionine–dependent methyltransferase) and the triose phosphate–phosphate translocator, an abundant polytopic metabolite transporter. These data suggest that the regulatory networks for lipid synthesis and protein expression that control IM biogenesis can respond to internal cues from the chloroplast. The upregulation of nuclear genes that code for other IM proteins and membrane proliferation should provide a novel system to understand signal transduction between chloroplast and nuclear genomes. Retrograde signaling factors studied so far include magnesium protoporphyrin, redox signaling, inhibition of plastid gene expression, and accumulation of reactive oxygen species (Woodson and Chory, 2008), and no system is yet available to study the effect of nuclear genes in response to the expression of a specific protein within chloroplasts.

Our results also have significant implications for understanding membrane biogenesis and provide a first step in using chloroplast transformation as a means of expressing and accumulating high levels of native or foreign membrane proteins for structural studies or biomedical applications. Progress in understanding chloroplast biogenesis and gene expression has led to advances in the use of chloroplasts as highly efficient bioreactors for the expression of foreign proteins of biomedical and therapeutic interest. Foreign proteins have been shown to accumulate at levels up to 46% of the total leaf protein (DeCosa et al., 2001), and it is possible to produce up to 360 million doses of fully functional anthrax vaccine in 1 acre of tobacco (Koya et al., 2005). Several other chloroplast-derived vaccine antigens have been shown to be highly immunogenic and to confer protection against pathogen or toxin challenge (Tregoning et al., 2005; Arlen et al., 2008).

Our goal was to extend the success in expressing soluble foreign proteins in chloroplasts to include membrane proteins. Heterologous expression systems in prokaryotes for the expression of membrane proteins have been hampered by different synthesis, targeting, insertion, and folding characteristics in their hosts. Adequate expression of membrane proteins continues to be a major challenge due to the toxic effects that severely reduce cell growth product yields (Wagner et al., 2006, 2007). Therefore, finding suitable expression systems for membrane proteins is highly desirable.

The ability of transgenic chloroplasts to express membrane and soluble proteins makes them ideal targets for metabolic engineering and biotechnology applications. For example, localization of α-tocopherol
(vitamin E) synthesis in the IM (Arango and Heise, 1998) facilitates engineering of an essential dietary nutrient for human and animal health. The high levels of Tic40-His expression with limited effects on organelle function or plant growth and development open the door for the use of transgenic chloroplasts to overexpress membrane proteins for various biomedical applications. Space for accommodation of the overexpressed membrane proteins has been a major bottleneck, and mechanisms that regulate membrane proliferation are poorly understood (Wagner et al., 2006).

In contrast with the thylakoid membrane that is restricted to green tissues, the IM provides a target for constitutively expressed membrane proteins in all plant tissue types. Maternal inheritance of genetically modified chloroplast genomes and the absence of any reproductive structures when foreign proteins are expressed in leaves combine to offer efficient transgene containment and facilitate the safe production of transgenic products in the field (Daniell, 2007). Therefore, transplastomic plants producing human therapeutic proteins have been tested in the field after obtaining USDA–Animal and Plant Health Inspection Service approval (Arlen et al., 2007). These unique advantages make the chloroplast an ideal bioreactor for the expression of membrane proteins for biomedical applications.

**METHODS**

**Vector Construction**

The pET-21d plasmid containing Arabidopsis thaliana pre-Tic40-His (GenBank accession number BT006595) (Li and Schnell, 2006) was digested with NcoI/Sacl to subclone the pre-Tic40-His fragment. This pre-Tic40-His fragment was subcloned into NcoI/Sacl–digested pUC-utr-aphA6 plasmid, replacing the aphA6 gene to generate pUC-utr-pre-Tic40-His. This plasmid was digested with EcoRI to remove the utr-pre-Tic40-His fragment and finally cloned into EcoRI-digested and dephosphorylated chloroplast expression vector pLDCtV2. The final construct was designated pLD-utr-pre-Tic40-His.

**Regeneration of Transplastomic Plants**

Tobacco (Nicotiana tabacum var Petit Havana) was grown aseptically on hormone-free Murashige and Skoog (MS) agar medium containing 30 g/L sucrose. Sterile young leaves from plants at the four- to six-leaf stages were bombarded using gold particles coated with vector pLD-utr-pre-Tic40-His, and transplastomic plants were regenerated as described previously (Daniell et al., 2004; Verma et al., 2008).

**Confirmation of Transgene Integration by PCR and DNA Gel Blot Analysis**

Plant genomic DNA was isolated using the Qiagen DNeasy plant mini kit from the spectinomycin-resistant primary shoots. PCR analysis was performed to confirm transgene integration into the inverted repeat regions of the chloroplast genome using two sets of primers: 3P (5’-AAAACCCGTCTCAGTTGAGATTG C-3‘)/3M (5’-CCGCGTTGTTTCATCAAGCCCTACG-3‘) and 5P (5’-CTGTAGAAGTACCATGTTGTGC-3‘)/2M (5’-TGACTGCCCAACCTGAGAGCGGACA-3‘). The PCR was performed as described previously (Verma et al., 2008). Leaves from the PCR-positive shoots were again cut into small pieces and transferred on Regeneration Medium of Plants (Verma et al., 2008) containing 500 mg/L spectinomycin for an additional round of selection and subsequently moved to MSO (MS salts without vitamins and growth hormones) (Verma et al., 2008) containing 500 mg/L spectinomycin for another round of selection to generate homoplasm.

The DNA gel blot analysis was performed according to laboratory protocol (Kumar and Daniell, 2004). In brief, total plant genomic DNA (1 to 2 µg) isolated from products of the third round of selection was digested with SmalI and separated on a 0.8% agarose gel before transfer to a nylon membrane. The chloroplast flanking sequence probe was made by digesting pUC-Ct vector (Verma et al., 2008) DNA with BamHI and BglIII, which generate a 0.81-kb probe (Figure 1A). After labeling the probe with [α-32P]dCTP,
the membrane was hybridized using the Stratagene Quick-Hyb hybridization solution and protocol. Seeds collected from the DNA gel blot–confirmed T0 seeds were germinated in vitro in spectinomycin-containing medium (500 mg/L). Finally, they were transferred to pots and moved to a greenhouse. Total DNA was isolated from the T1 plants, and DNA gel blot analysis was performed to track the inheritance of the transgenes using the same flanking probe used above.

**Phenotypic Differences between Wild-Type and Transgenic Lines**

To check the differences in phenotypes and fertility between wild-type and transgenic lines, plants were raised in the greenhouse. Floral parts were dissected from 16-week-old transgenic lines and wild-type plants. Leaf chlorophyll was quantified in 80% (v/v) acetone extracts by measuring the $A_{663}$ and $A_{645}$ and using the Arnon equations for total chlorophyll content (Arnon, 1949).

**Chloroplast Isolation, Fractionation, and Immunoblotting**

Chloroplast isolation was performed as described previously (Smith et al., 2002). Briefly, tobacco leaves were homogenized in 25 mM HEPES-KOH, pH 7.7, 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 0.25% (w/v) BSA, and 0.1% (w/v) ascorbic acid (GR buffer). The homogenate was used as the total leaf lysate. The homogenate was separated into pellet and soluble fractions by centrifugation at 15,000 g for 2 min. The pellet fraction was resuspended in GR buffer, and chloroplasts were isolated by Percoll gradient fractionation. Isolated chloroplasts were lysed by suspension in 50 mM HEPES-KOH, pH 7.5, and 330 mM sorbitol (HS buffer) to a concentration of 0.5 to 1 mg chlorophyll/mL and diluted with 5 volumes of 2 mM EDTA. The lysate was mixed vigorously and incubated on ice for 10 min. The samples were adjusted to 0.2 M NaCl, and the membrane fraction was collected by centrifugation at 18,000 g for 30 min at 4°C. For alkaline extraction, the membrane pellet was resuspended with a small volume of HS buffer and diluted with 20 volumes of 0.2 M Na$_2$CO$_3$, pH 12. The samples were homogenized with a Teflon homogenizer (Kontes Glass) and incubated at room temperature for 10 min, and the membrane fraction was collected by centrifugation at 100,000 g for 15 min. The soluble fractions were removed and concentrated by precipitation in 20% trichloroacetic acid. Protein concentrations were determined by the method of Bradford (1976).

Inner membrane and thylakoid membranes were separated from hypertonically lysed chloroplasts by linear sucrose gradients using the method of Keegstra and Yousif (1986). Membrane vesicles (1 mg protein/mL) were treated with thermolysin at a ratio of 200 mg thermolysin/mg protein for 30 min on ice in the presence or absence of 1% (w/v) Triton X-100. Proteolysis was stopped with the addition of an excess of ice-cold 10 mM EDTA. All samples were resolved by SDS-PAGE and transferred to nitrocellulose filters. Nitrocellulose filters were stained with 0.1% (w/v) imido black in water. Antisera to LSu, IEP37, Tic110 (Inaba et al., 2003), Toc159 (Kessler et al., 1994), and the triose phosphate-phosphate translocator (Schnell et al., 1991) were described previously. Anti-Hsp93 was a generous gift of Kenneth Keegstra. Mouse monoclonal anti-actin (clone C4) serum was obtained from Millipore. Antisera to 23-kD oxygen-evolving complex protein and light-harvesting complex protein were generous gifts from Kenneth Cline. All antisera were used at 1:5000 dilution. Horseradish peroxidase–conjugated goat anti-rabbit and goat anti-mouse sera (1:5000) were used as the secondary antibodies. Antibody binding was detected using chemiluminescence with luminol reagent (Sigma-Aldrich) and exposure to x-ray film. The in vitro translation of constructs encoding pre-Tic40-His, int-Tic40-His, and Tic40-His was performed as described previously (Li and Schnell, 2006) and imaged using a Storm 840 PhosphorImager (Molecular Dynamics) and ImageQuant version 1.2 software.

**Cross-Linking of Chloroplasts and Coimmunoprecipitation Experiments**

Isolated intact chloroplasts (200 µg of chlorophyll) were resuspended in HS buffer to a concentration of 1
mg/mL chlorophyll, and dithiobis(succinimidyl propionate) was added to a final concentration of 0.5 mM. The cross-linking reaction was incubated on ice in the dark for 15 min and quenched by adding Gly to 50 mM and continuing incubation for another 15 min. Cross-linked chloroplasts were reisolated through Percoll gradients, washed with HS buffer, and dissolved with dissolving buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, and 1% Triton X-100) with 1% protease inhibitor cocktail (Sigma-Aldrich) at 4°C for 30 min with constant gentle shaking. After a 30-min centrifugation at 18,000g, the supernatant was collected and incubated with 20 µg of anti-Tic110 antibody (Inaba et al., 2005) for 2 h. Packed protein A–agarose beads (20 µL; GE Healthcare) were added, and the incubation was continued overnight. The beads were recovered at 1000g for 2 min and washed with 500 µL of washing buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, and 0.1% protease inhibitor cocktail). Bound proteins were separated by SDS-PAGE and analyzed by immunoblotting as described above.

Immunofluorescence and Electron Microscopy

Immunofluorescence microscopy was performed as described previously (Schnell et al., 1991) with affinity-purified anti-His6 primary antibody (1:250 dilution) generated to the following peptide: LAAALGHHHHHH. Fluorescein-coupled goat anti-rabbit antibody (1:500 dilution; Sigma-Aldrich) was used as the secondary antibody. Samples were viewed with a Nikon E-600 epifluorescence microscope equipped with a fluorescein isothiocyanate–HYQ filter set (EX460-500, DM505, BA510-560). A Spot-RT camera system (Diagnostic Instruments) was used for image capture.

For electron microscopy, tissue samples were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4, under vacuum for 3 h and subsequently washed three times with 0.05 M sodium cacodylate, pH 7.4. Fixed samples were treated with 1% osmium tetroxide in 0.05 M sodium cacodylate, pH 7.4, for 2 h, and washed three times with 0.05 M sodium cacodylate, pH 7.4. The samples were dehydrated by the following treatments: incubation in 70% ethanol for 10 min, incubation in 100% ethanol for 10 min, and incubation twice in 100% propylene oxide for 15 min. EMbed812 embedding mixture (Electron Microscopic Sciences) was prepared according to the manufacturer's instructions. Dehydrated samples were infiltrated with one-third-concentrated EMbed812 (in propylene oxide) for 3 h, in two-thirds-concentrated EMbed812 overnight, and in 100% EMbed812 for 1.5 h before being embedded in EMbed812 by incubation at 60°C for 24 h. Seventy-nanometer sections of the samples were prepared, dried on 150-mesh copper grids, and poststained with uranyl acetate and lead citrate as described previously (Smith and Croft, 1991). The grids were dried and observed using a Philips-Tecnai 12 transmission electron microscope.

Accession Number

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession number AAP31939 (Arabidopsis Tic40).

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NOTES

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Henry Daniell
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FIGURES AND TABLES
Figure 1.

(A) and (B) Schematic representations of the chloroplast flanking sequences used for homologous recombination, probe DNA sequence (0.81 kb), primer annealing sites (3P/3M and 5P/2M), and expected products of wild-type and transgenic lines with pLD-utr-pre-Tic40-His (when digested with SmaI). UTR, untranslated region.

(C) First round of selection and primary transplastomic shoots.

(D) Second round of selection.

(E) Regenerated shoots on rooting medium for the third round of selection; all rounds of selection contained spectinomycin (500 mg/L).

(F) and (G) PCR analysis of the transgenic plants using primer pairs 3P/3M and 5P/2M for evaluation of site-specific integration of the transgene cassette into the chloroplast genome. T1 to T4, transplastomic lines; P, positive control; M, 1-kb plus DNA ladder.
Confirmation of Homoplasmy and Maternal Inheritance.

**(A)** DNA gel blot hybridized with the flanking sequence probe showing homoplasmy. T1 to T4, T0 transplastomic lines.

**(B)** DNA gel blot analysis of T1 transplastomic lines (T1 and T2).

**(C)** Phenotypes of transgenic (TC59) and wild-type 12-week-old plants grown in a greenhouse.

**(D)** and **(E)** Wild-type and transgenic (TC59) seeds germinated on MSO containing spectinomycin (500 mg/L) showing maternal inheritance.
Figure 3.

Phenotypes and Fertility of Wild-Type and Transplastomic Lines.

(A) Transplastomic plants showing normal biomass and flowering.

(B) Stamens.

(C) Stigmas.

(D) to (F) Longitudinal sections of the flowers. TC42 and TC59, transplastomic lines.

(G) to (I) Transverse sections of the ovaries showing normal development.

(J) and (K) Wild-type and transplastomic (TC59) plants showing normal fertilization and pod development. The boxed region of each panel is shown at higher magnification at right.
Table 1.

Chlorophyll Content from Leaves of Tobacco Wild-Type and Tic40 Transplastomic Lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chlorophyll A (mg/g fresh weight ± SD)</th>
<th>Chlorophyll B (mg/g fresh weight ± SD)</th>
<th>Total Chlorophyll (mg/g fresh weight ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.517 ± 0.085</td>
<td>0.498 ± 0.095</td>
<td>1.01 ± 0.190</td>
</tr>
<tr>
<td>TC42</td>
<td>0.583 ± 0.051</td>
<td>0.548 ± 0.046</td>
<td>1.117 ± 0.090</td>
</tr>
<tr>
<td>TC59</td>
<td>0.537 ± 0.051</td>
<td>0.496 ± 0.048</td>
<td>1.02 ± 0.096</td>
</tr>
</tbody>
</table>

TC42 and TC59, Tic40 transplastomic lines.
Figure 4.
Expression and Localization of pre-Tic40-His in Transgenic Tobacco.

Leaf total lysates (10 µg of protein) from wild-type and transgenic tobacco (TC42 and TC59) lines were resolved by SDS-PAGE and transferred to nitrocellulose membrane. The positions of molecular size standards are shown at left.

(A) Imido black–stained nitrocellulose filter of protein samples. The arrow indicates the position of Tic40-His. Ly, total leaf lysate; M, lysate pellet fraction containing organelles and membranes; S, lysate soluble fraction.

(B) Immunoblot of the filter in (A) with anti-His, anti-LSu, and anti-IEP37 antibodies.
Pre-Tic40 Is Localized to Chloroplast Membranes.

Chloroplasts were isolated from leaves of transgenic tobacco line TC59. Purified chloroplasts were extracted with alkaline carbonate to separate soluble and membrane components. Protein samples (10 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Top panels show imido black-stained nitrocellulose filter of fractions from transgenic tobacco (TC59) leaves. Ly, total leaf lysate; Pl, membrane pellet fraction; Sn, soluble fraction; T, total chloroplast fraction; M, alkaline-extracted chloroplast membrane fraction; S, alkaline-extracted chloroplast soluble fraction. Lane 1 contains a phosphorimage of in vitro translated (IVT) pre-Tic40-His, int-Tic40-His, and Tic40-His. The positions of molecular size standards are shown at right. Bottom panels show immunoblots of the samples at top with the
indicated antisera. Anti-His\textsubscript{6} was used to detect the expressed Tic40-His.
Figure 6.

(A) Tic40-His fractionates with chloroplast inner membrane vesicles. Chloroplast membranes were separated by sucrose gradient centrifugation, and fractions corresponding to IM and thylakoid membranes (Thyl; 2.5 µg of protein) were resolved by SDS-PAGE and immunoblotted with antisera to the proteins indicated at left.

(B) Tic40-His attains the correct topology in the inner membrane. Isolated inside-out IM vesicles were treated with thermolysin (T-lysin) in the presence or absence of Triton X-100 for the times indicated. The presence of Tic40-His,

C  anti-Tic110 immunoprecipitation

Functional Assembly of Tic40-His at the Chloroplast Inner Envelope in Transgenic Tobacco.

(A) Tic40-His fractionates with chloroplast inner membrane vesicles. Chloroplast membranes were separated by sucrose gradient centrifugation, and fractions corresponding to IM and thylakoid membranes (Thyl; 2.5 µg of protein) were resolved by SDS-PAGE and immunoblotted with antisera to the proteins indicated at left.

(B) Tic40-His attains the correct topology in the inner membrane. Isolated inside-out IM vesicles were treated with thermolysin (T-lysin) in the presence or absence of Triton X-100 for the times indicated. The presence of Tic40-His,
Tic110, and IEP37 was detected by immunoblotting.

(C) Tic40-His associates with other Tic components. Detergent-soluble chloroplast extracts from wild-type, TC42, and TC59 tobacco were immunoprecipitated with anti-Tic110 serum. Immunoblots of total chloroplast extract (St), unbound fraction (Ft), and immunoprecipitates (El) with anti-Tic110, anti-Hsp93, anti-His_{6} sera are shown.
Figure 7.

The Expression of Endogenous IM Proteins Is Upregulated in Tic40-His Transgenic Plants.

Immunoblots of serially diluted protein extracts from wild-type and TC59 transgenic chloroplasts. Protein extracts of the amounts indicated at top were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antisera to the proteins indicated at left. Toc159, outer envelope component of the TOC protein import complex; Hsp93, chloroplast stromal homolog of the 93-kD heat shock protein/molecular chaperone; Cpn60, chloroplast stromal homolog of the 60-kD heat shock protein/molecular chaperone; Tic110, 110-kD component of the inner envelope TIC complex; IEP37, 37-kD inner envelope protein (S-adenosyl methionine–dependent methyl transferase); PPT, inner envelope triose phosphate-phosphate translocator; LHCP, thylakoid light-harvesting complex protein; OE23, 23-kD component of the oxygen-evolving complex of the thylakoid lumen.
Localization of Tic40-His at the Chloroplast Inner Envelope in Transgenic Tobacco.

Immunofluorescence microscopic analyses of wild-type and TC59 chloroplasts. Isolated chloroplasts were imaged with anti-His₆ antiserum and goat anti-rabbit fluorescein IgG to detect Tic40-His. Chlorophyll autofluorescence is shown as an indicator of the positions of the thylakoid membranes. The bottom panels show the merged images of the two signals, and the top panels show differential interference contrast (DIC) images of the chloroplasts used for immunofluorescence labeling. The arrows indicate protruded areas of envelope membranes containing Tic40-His in transgenic chloroplasts. Bars = 5.0 µm.
Proliferation of the Inner Membrane in Tic40-His Transgenic Tobacco

Samples of intact leaf tissue from wild-type and pre-Tic40-His transgenic lines TC42 and TC59 were fixed, stained, and observed by transmission electron microscopy. The center four panels show chloroplasts from regions of leaf tissue from wild-type and transgenic lines TC59 and TC42 (bars = 1.0 µm). The side panels show higher magnifications of the boxed regions (bars = 0.1 µm). The positions of the outer envelope membrane (OM), the IM, the thylakoids (Th), the stroma (St), a plastoglobule (PG), the cytoplasm (Cy), and a mitochondrion (Mt) are labeled.