The M Domain of atToc159 Plays an Essential Role in the Import of Proteins into Chloroplasts and Chloroplast Biogenesis*

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Toc159, a protein located in the outer envelope membrane and the cytosol, is an important component of the receptor complex for nuclear-encoded chloroplast proteins. We investigated the molecular mechanism of protein import into chloroplasts by atToc159 using the ppi2 mutant, which has a T-DNA insertion at atToc159, shows an albino phenotype, and does not survive beyond the seedling stage due to a defect in protein import into chloroplasts. First we established that transiently expressing atToc159 in protoplasts obtained from the white leaf tissues of ppi2 plants complements the protein import defect into chloroplasts. Using this transient expression approach and a series of deletion mutants, we demonstrated that the C-terminal membrane-anchored (M) domain is targeted to the chloroplast envelope membrane in ppi2 protoplasts, and is sufficient to complement the defect in protein import. The middle GTPase (G) domain plays an additional critical role in protein import: the atToc159[S/N] and atToc159[D/L] mutants, which have a mutation at the first and second GTP-binding motifs, respectively, do not support protein import into chloroplasts. Leaf cells of transgenic plants expressing the M domain in a ppi2 background contained nearly fully developed chloroplasts with respect to size and density of thylakoid membranes, and displayed about half as much chlorophyll as wild-type cells. In transgenic plants, the isolated M domain localized to the envelope membrane of chloroplasts but not the cytosol. Based on these results, we propose that the M domain is the minimal structure required to support protein import into chloroplasts, while the G domain plays a regulatory role.

The majority of chloroplast proteins are encoded by the nuclear genome and synthesized in the cytosol. Numerous studies have focused on the molecular mechanism of protein import into chloroplasts (1–8). The in vitro import assay system, which utilizes purified chloroplasts and in vitro-translated precursor protein, is a crucial tool in these studies (1). These analyses have indicated that the N-terminal transit peptide contains all the necessary information for the translocation of cargo proteins from the cytosol into chloroplasts. It has been proposed that following translation, soluble precursor proteins migrate by diffusion to receptor complexes located on the envelope membranes of chloroplasts (6, 7, 9). The precursor initially binds to lipids of the outer envelope membrane of the chloroplast (10, 11) and subsequently diffuses to the receptor complex. This allows specific interactions between the transit peptide and receptor complex. The precursor is subsequently translocated through a channel formed by subunits of the receptor complexes (12, 13), followed by cleavage of the transit peptide to produce mature protein within the chloroplast (14).

Components of the import complex, including proteins located at the outer and inner envelope membranes, have been identified and characterized at the molecular level (6, 13). However, the exact roles of these components have not yet been fully elucidated. Toc75 forms a channel at the outer envelope membrane for translocation of chloroplast proteins (15, 16). Toc34 and Toc159 may function as receptor components that bind transit peptides of precursor proteins. Recent studies show that these three Toc proteins form complexes at the outer envelope membrane (17). Another protein component, Toc64, may also bind to the transit peptide at early time points (18). However, the role of this protein needs to be further delineated. Using ppi2 plants, which do not have functional Toc159, Toc159 has been shown to be essential for protein import into chloroplasts as well as chloroplast biogenesis (8). Chloroplasts in ppi2 plant cells are in a proplastid state with almost no thylakoid membranes, which gives an albino phenotype to the leaf tissues (8). Toc132 and Toc120, two homologs of Toc159, have been identified in the Arabidopsis genome (8). However, these homologs do not complement the loss of Toc159 in ppi2 plants, implying that these proteins are either not expressed in the same cells or are functionally different from Toc159. ppi1 plants, which contain a mutation at Toc34, also have defective protein import into chloroplasts (19), but in contrast to Toc159, the loss of Toc34 in the ppi1 plant induces a mild phenotype. This may be due to compensation by the expression of Toc33, a close homolog of Toc34 (20). Toc33/34 and Toc159 contain GTP-binding domains; the GTP-binding domain of Toc159 plays a critical role in its targeting to the outer envelope membrane (21, 22). The GTP-binding domains of Toc159 and Toc34 are proposed to form a heterodimer for facilitating Toc159 targeting (21, 22). In addition to the Toc components, the translocon of inner envelope membrane components (Tic)1 subunits have been identified, including Tic115, Tic55, and Tic40 (23). However, these proteins are less well characterized with respect to their precise roles in protein import.

To clarify the role of Toc159, we examined whether Arabidopsis thaliana Toc159 (atToc159) supports protein import into chloroplasts when transiently expressed in ppi2 protoplasts. The atToc159 protein is composed of three different domains, the N-terminal acidic (A), middle GTPase (G), and C-terminal

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The abbreviations used are: Tic, translocon of inner envelope membrane components; atToc159, A. thaliana Toc159; GFP, green fluorescent protein; WT, wild type.
membrane-anchored (M) domains (8). We further characterized these domains and examined their roles in protein import into chloroplasts. Our data show that the C-terminal M domain is essential for protein import into chloroplasts in a transient expression system of protoplasts as well as transgenic plants.

**EXPERIMENTAL PROCEDURES**

**Growth of Plants—**Arabidopsis (ecotype Columbia) (wild type and mutants) were grown on B5 plates in a growth chamber. Leaf tissues were harvested from plants and immediately employed for protoplast isolation. Arabidopsis mutant seeds (seed line, CS11072, Arabidopsis Stock Center, Ohio State University, Columbus, OH) that have a T-DNA insertion at the atToc159 gene were planted on kanamycin plates.2 Plants that were kanamycin-resistant and had a wild-type appearance were transferred to soil to set seeds (8). ppi2 homozygote plants have an albino phenotype (plants with white leaf tissues, see Fig. 5a, panel b) and do not develop beyond the seedling stage with a few true leaves, indicating that ppi2 plants are seedling lethal. However, heterozygote plants (atto159/atact159) are normal and develop into mature plants that set seeds. Thus, the mutants were maintained as heterozygotes (atto159atact159). Homozygous ppi2 mutants (atto159attoct159) were selected from pools of plants on plates based on the albino phenotype and used for preparation of protoplasts.

**Construction of Plasmids—**A cDNA encoding atToc159 was isolated from a library by PCR, using the primers 5′-GCTCTAGAATG-GCTCTAGATGGCTAGCATGACTGGTGGACAGCAAATGGTTAGATCCCCGCCTCTC-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′. PCR amplification was performed, and the following primers were used: GCTCTAGATGGCTAGCATGACTGGTGGACAGCAAATGGTTAGATCCCCGCCTCTC-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ for atToc159-A, 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ for atToc159-G, 5′-GCTCTAGAATG-GCTCTAGAATG-GCTCTAGAATG-GCTCTAGAATG-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ for atToc159-M, 5′-GCTCTAGAATG-GCTCTAGAATG-GCTCTAGAATG-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ for atToc159-MC31. All PCR products were confirmed by nucleotide sequencing and placed into a pUC-based expression vector with the [35S]CaMV promoter and nos-terminator.

**Generation of Transgenic Plants and Analysis by PCR—**Transgenic Arabidopsis plants expressing various forms of T7-tagged atToc159 were generated using a binary expression vector with a hygromycin-resistance gene (pBI-Hyg) and the floral dip method (24). Transgenic plants resistant to both kanamycin and hygromycin were screened as (8). Plasmid DNA was isolated from transgenic plants selected for transgene expression using the alkaline lysis method (25). DNA fragments were isolated from agarose gels, purified, and used as templates for PCR amplification using the following primers: 5′-GCTCTAGATGGCTAGCATGACTGGTGGACAGCAAATGGTTAGATCCCCGCCTCTC-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′ as template, and the following primers: GCTCTAGATGGCTAGCATGACTGGTGGACAGCAAATGGTTAGATCCCCGCCTCTC-3′ and 5′-GAGGGTG-GGAAAAATGCTACTATAAATTCC-3′.

**Ultrastructural Analysis—**To obtain a detailed structure of chloroplast morphology, leaf tissues were fixed for 4 h with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (pH 7.2), rinsed with the same buffer, and postfixed with 1% osmium tetroxide in a cacodylate buffer for 1 h. After dehydration, specimens were embedded in London Resin White (London Resin Co., London, UK). Ultra-thin
sections (40–60 nm thick) were collected on uncoated nickel grids (300 mesh), stained with 4% uranyl acetate, and examined using a transmission electron microscope (Jeol 1200) at 60–80 kV.

**Protein Fractionation and Gel Blot Analysis**—Protein extracts were prepared as described previously (25). Whole-cell extracts were subjected to low-speed centrifugation (7000 × g) at 4 °C for 5 min to eliminate cellular debris. The low-speed supernatant was further fractionated by ultracentrifugation at 100,000 × g for 1 h. Whole-cell extracts or fractions were assayed by Western blotting using anti-GFP (Clontech, Inc) and anti-T7 (Novagen) antibodies. RbcS was detected with a polyclonal anti-Rbc antibody (26).

**Transient Expression and in Vivo Targeting of Reporter Proteins**—Plasmids were introduced by PEG-mediated transformation (25, 27) into Arabidopsis protoplasts prepared from leaf tissues. Expression of fusion constructs was monitored at various times after transformation, and images were captured with a cooled CCD camera and a Zeiss (Jena, Germany) Axiosplan fluorescence microscope (25).

**Immunohistochemistry**—For immunohistochemistry, transformed protoplasts were prepared as described previously (28). Fixed cells were incubated with a mouse monoclonal anti-T7 antibody at 4 °C overnight and washed three times with TSW buffer. Subsequently, the cells were incubated with a fluorescein isothiocyanate-conjugated anti-mouse IgG (Zymed Laboratories Inc.) secondary antibody. Images were captured as described above.

**RESULTS**

**Transient Expression of atToc159 Supports Protein Import into Chloroplasts in ppi2 Protoplasts**—We investigated the role of atToc159 in protein import into chloroplasts using protoplasts derived from Arabidopsis leaf tissues as an experimental system. In this system, DNA constructs encoding reporter proteins destined for the chloroplasts were introduced into protoplasts by the polyethylene glycol-mediated transformation method (25, 27), and expression of the encoded proteins was monitored at various subsequent time points. We employed two different approaches to analyze the expression and targeting of the proteins. One is based on expressing fusion proteins including green or red fluorescent proteins (GFP or RFP), followed by image analysis using a fluorescent microscope. The other is based on Western blot analysis using antibodies to the expressed proteins. We employed a GFP fusion protein with the N-terminal transit peptide of the small subunit of rubisco complex (RbcS) as a reporter protein (Fig. 1A), as described previously (27). Fluorescence microscopy analyses revealed that RbcS-nt:GFP is efficiently imported into chloroplasts in protoplasts derived from leaf tissues of wild-type plants (Fig. 1B, panels d–f). In contrast, GFP alone was observed as a diffuse pattern in the cytosol (Fig. 1B, panels a–c). To further confirm the localization of RbcS-nt:GFP, we performed Western blotting using proteins obtained from RbcS-nt:GFP-transformed protoplasts. As shown in Fig. 1D, the majority of RbcS-nt:GFP was detected at 31 kDa (lane 3). Furthermore, the 31-kDa band

**Fig. 2**. Transiently expressed atToc159 supports protein import into chloroplasts in protoplasts. A, localization of RbcS-nt:GFP. ppi2 protoplasts were transformed with RbcS-nt:GFP alone or together with T7:atToc159, and localization of RbcS-nt:GFP was examined. Chlorophyll, autofluorescence of chlorophyll. Arrows indicate the punctate staining pattern of GFP signals overlapping the red autofluorescence of chlorophyll. V, vacuole. Bar = 20 μm. B, Western blot analysis. Protein extracts were prepared from transformed protoplasts and used for Western blotting. RbcS-nt:GFP (top panel) and T7:atToc159 (bottom panel) were detected with anti-GFP and anti-T7 antibodies, respectively. Lane 1, protein extracts obtained from non-transformed ppi2 protoplasts; lane 2, protein extracts obtained from ppi2 protoplasts transformed with RbcS-nt:GFP alone; lane 3, protein extracts obtained from ppi2 protoplasts transformed with RbcS-nt:GFP plus T7:atToc159. P, precursor form; M, mature form; M’, a proteolytic product of the mature form.
co-purified with chloroplasts in a Percoll gradient (lane 4), indicating the presence of the protein within this organelle. A very weak band was sometimes detected at 39 kDa (the expected size of full-length RbcS-nt:GFP) in total protein extracts (lane 3) but not in chloroplast extracts (lane 4); this likely corresponds to the precursor RbcS-nt:GFP protein. The 31-kDa band may correspond to the mature protein because the N-terminal transit peptide of chloroplast proteins is proteolytically processed during import (1, 2). A band that is smaller than 31 kDa was also observed in the protein extracts, which may be a product of additional proteolytic processing in chloroplasts. Control GFP was detected at 29 kDa, as expected (Fig. 1D, lane 2).

Next we examined the import of RbcS-nt:GFP into chloroplasts in protoplasts derived from leaf tissues of ppi2 plants. We examined whether proteins can be imported into the undeveloped chloroplasts in ppi2 plants (8). ppi2 protoplasts prepared from the white leaf tissues of ppi2 plants were transformed with RbcS-nt:GFP, and the reporter protein was localized by observing its green fluorescence under a fluorescent microscope. As shown in Fig. 1C, a diffuse pattern of green fluorescent signals from RbcS-nt:GFP (panels d–f) was detected, and was similar to that of GFP alone (panels a and c) except for a few punctate stains (indicated by arrows). The narrow strip patterns of GFP signals observed in these protoplasts are due to the cytosol that is confined between the large central vacuole and the plasma membrane. A similar result was obtained with other reporter proteins such as RA-nt:GFP (a GFP fusion protein with the transit peptide of rubisco activase) (data not shown). Interestingly, the GFP signals of RbcS-nt:GFP at the few punctate stains closely overlapped the red autofluorescence of chlorophyll, as depicted by the yellow signals (indicated by arrows, Fig. 1C, panel f). This suggests that although RbcS-nt:GFP is not efficiently imported into chloroplasts, some portion of RbcS-nt:GFP expressed in ppi2 protoplasts may be imported into the undeveloped chloroplasts. To further confirm the GFP pattern of RbcS-nt:GFP in ppi2 protoplasts, protein extracts were prepared from transformed ppi2 protoplasts and examined by Western blot analysis using an anti-GFP antibody. RbcS-nt:GFP was detected as two strong bands at 31 and 39 kDa (Fig. 1D, lane 5). In ppi2 protoplasts, the 39-kDa band was 40% (±5%, n = 6) of total expressed RbcS-nt:GFP and the 31-kDa band was 60% (±5%, n = 6), indicating that the amount of the precursor form is greatly increased in ppi2 protoplasts compared with the wild-type protoplasts. These results are in agreement with the results obtained from the image analysis.

Next, we examined whether transient expression of atToc159 complements the loss of atToc159 with respect to protein import into chloroplasts in ppi2 protoplasts. We generated atToc159 tagged with a small epitope (T7) at the N terminus for detection by Western blot. Protoplasts obtained from ppi2
plants were co-transformed with two plasmids, RbcS-nt:GFP and T7:atToc159, and localization of the reporter protein was examined. When co-transformed with T7:atToc159, the green fluorescence of RbcS-nt:GFP was detected as punctate stains with no diffuse signals (Fig. 2A, panels e and g), in contrast to what was seen in ppi2 protoplasts expressing RbcS-nt:GFP alone (panels a and c). Furthermore, these green punctate stains closely overlapped the red autofluorescence of chlorophyll. To further verify protein import in the presence of co-expressed atToc159, Western blotting was performed using proteins prepared from transformed protoplasts and employed to detect expression of deletion mutants by Western blotting with an anti-T7 antibody. The deletion mutants were present at nearly equal levels in the protein extracts prepared from transformed protoplasts together with T7:atToc159, the amount of precursor protein was 15% of the total RbcS-nt:GFP expressed, implying that the ability of this mutant to support protein import into chloroplasts was hampered but still functional. However, in protoplasts expressing atToc159-M (lane 5), the amount of precursor RbcS-nt:GFP was equivalent to that in protoplasts transformed with vector alone, indicating that the M domain is most critical for protein import into chloroplasts.

Expression of these mutant atToc159 proteins in ppi2 protoplasts was analyzed by Western blotting using the anti-T7 antibody. The deletion mutants were present at nearly equal levels in the protein extracts prepared from transformed protoplasts (Fig. 3D), confirming their expression in ppi2 protoplasts. atToc159-A (lane 3) and atToc159-M (lane 6) were detected at the expected positions based on their calculated molecular weights, whereas the atToc159 (lane 2), atToc159-G (lane 4), and atToc159-M (lane 5) bands were much larger than their expected sizes. AtToc159 has previously been detected at 230 kDa instead of 159 kDa (the calculated molecular weight of atToc159) (29). The migration patterns of these deletion mutants strongly suggest that the abnormal mobility of atToc159 is due to the A domain.

To further define the domain necessary for protein import into chloroplasts, we generated additional mutants, atToc159-MN78 and atToc159-MC31 (Fig. 4A). The atToc159-MN78 mutant underwent a 78-amino acid deletion from the N-terminal side of the M domain, while atToc159-MC31 has a 31-amino acid deletion from the C terminus of the M domain. The deletion mutants were introduced into ppi2 protoplasts together with RbcS-nt:GFP. Protein extracts were prepared and RbcS-nt:GFP import was examined by Western blot. As depicted in Fig. 4, B and C, ~29% of RbcS-nt:GFP was detected as the precursor form upon co-expression with atToc159-MN78 (lane 3), and 24% was detected as precursor when RbcS-nt:GFP was

![Fig. 4. An intact M domain is critical for protein import into chloroplasts. A, schematics of constructs. B, protein import assay. Protoplasts were transformed with RbcS-nt:GFP alone (lane 1) or together with atToc159 (lane 2), atToc159-MN78 (lane 3), or atToc159-MC31 (lane 4). Protein extracts obtained from transformed protoplasts were analyzed as described for Fig. 3B. C, quantification of protein import was performed as described for Fig. 3C. Columns 1–4 are the same as those in B. D, expression of deletion mutants. Protein extracts were prepared from transformed protoplasts and employed to detect expression of deletion mutants by Western blotting with an anti-T7 antibody. Lanes 1–4 are the same as in B.](image-url)
co-expressed with atToc159-MC31 (lane 4). In the control protoplasts transformed with RbcS-nt:GFP alone, 35% of the RbcS-nt:GFP was in the precursor form. Expression of the atToc159-MN78 and atToc159-MC31 mutant proteins was confirmed by Western blotting using the anti-T7 antibody (Fig. 4D). Our results strongly suggest that the intact M domain is
necessary to support protein import into chloroplasts.

The M Domain Alone Is Targeted to Chloroplasts—Earlier studies have shown that Toc159 is present in both membrane-associated and soluble forms (29), and that the G domain plays a critical role in the targeting of the protein to the outer envelope membrane (21, 22). However, the fact that the M domain is sufficient to support protein import into chloroplasts in ppi2 protoplasts raises the possibility that the isolated M domain may also be targeted to chloroplasts. Thus, we examined the localization of atToc159 and the M domain. We generated GFP-tagged atToc159 by inserting the GFP coding region between the A and G domains; the resulting construct was introduced into protoplasts of wild-type plants. As shown in Fig. 5A (panels e–g), atToc159-GFP localized to chloroplasts with a ring pattern, an indication of localization in the envelope membrane. In addition, the GFP signal was detected in the cytosol (the area where the red autofluorescent signals of chlorophyll are not detected) as a diffuse pattern, signifying that a part of atToc159-GFP is also present in the cytosol, consistent with previous data (21, 22). GFP alone gave a diffuse pattern (Fig. 5A, panels a–c).

Next we examined the targeting of atToc159-GFP to chloroplasts in ppi2 protoplasts. Protoplasts were transformed with atToc159:GFP together with AtOEPT-RFP, a fusion protein of the outer envelope membrane protein AtOEPT and RFP that is targeted to the outer envelope membrane of chloroplasts (30). As shown in Fig. 5B (panels i and l), atToc159:GFP again displayed a diffuse pattern together with a ring pattern. The ring pattern of the atToc159-GFP signal closely overlapped that of the AtOEPT-RFP signal (panels i, j, and l). Furthermore, GFP and RFP signals surrounded the autofluorescence signals of chlorophyll (panels i–l). These results collectively suggest that atToc159-GFP localizes to both the cytosol and to the undeveloped chloroplasts in ppi2 protoplasts. As a control, we transformed ppi2 protoplasts with GFP and AtOEPT-RFP and examined the localization of these proteins. GFP on its own gave a diffuse pattern and did not overlap the red fluorescent signals of AtOEPT-RFP (Fig. 5B, panels a–d). However, as expected, the red fluorescent signals of AtOEPT-RFP surrounded the autofluorescent signals of chlorophyll. As another control for the localization of atToc159 to the chloroplast envelope membrane, we transformed ppi2 protoplasts with atToc159-GFP with F1-ATPase-γ-RFP, a mitochondrial marker generated by fusion of the transit peptide of mitochondrial F1-ATPase-γ with RFP (31). As expected, the red signals of F1-ATPase-γ-RFP gave a punctate staining pattern that did not overlap green signals of atToc159:GFP (Fig. 5B, panels e–h), although the red punctate stains of the mitochondria were concentrated around the chloroplasts, as often occurs in plant cells. To determine whether the GFP domain affects atToc159 activity, we performed a complementation assay in ppi2 protoplasts using atToc159:GFP. atToc159:GFP supported the import of RbcS-atGFP as efficiently as atToc159 (Fig. 5, C and D, lanes 2 and 3), confirming that the GFP domain insertion into atToc159 does not affect the function of the protein.

We then investigated the localization of the M domain using a form with a GFP tag at the N terminus. The atToc159:GFP fusion protein was not targeted to chloroplasts, but formed large aggregates for reasons that are unknown at present (data not shown). We also examined the localization of the T7-tagged M domain, T7:atToc159M, by immunohistochemical analysis using the T7 antibody. The green fluorescence of atToc159M was observed as a ring pattern surrounding the red autofluorescence of chlorophyll in ppi2 protoplasts (Fig. 6A, panels c and d), suggesting that atToc159M is targeted to the envelope membranes of chloroplasts. In control protoplasts transformed with vector alone (Fig. 6A, panels a and b), no green signal was observed, confirming the specificity of the anti-T7 antibody. To further establish the localization of atToc159M, protein extracts prepared from ppi2 protoplasts co-transformed with T7: atToc159M and GFP were fractionated into membrane and soluble fractions. Co-expressed GFP was used as a control for soluble proteins in the subcellular fractionation. As shown in Fig. 6B, T7:atToc159M was detected in the membrane fraction, but not the soluble fraction, whereas GFP alone was present in the soluble fraction.

The GTPase Domain Is Critical for Protein Import into Chloroplasts—We next assessed the role of the GTPase domain (G domain) in protein import into chloroplasts. The G domain plays an essential role in the targeting of Toc159 to the outer membrane of chloroplasts (21, 22). Mutations were introduced in the first and second GTP-binding motifs to generate atToc159[S/N] and atToc159[D/L], respectively, and were tagged with T7 at the N terminus (Fig. 7A). The substitution of serine (S) with asparagine (N), atToc159[S/N], at the first GTP-binding motif of GTP-binding proteins produces a GDP-bound form, whereas the substitution of aspartic acid (D) with leucine (L), atToc159[D/L], at the second GTP-binding motif generates...
The GTPase-defective mutant form (32). The mutants, T7:Toc159[S/N] and T7:Toc159[D/L], were introduced into ppi2 protoplasts along with RbcS-nt:GFP, and targeting of RbcS-nt:GFP was examined. In the presence of both atToc159[S/N] and atToc159[D/L], 30 to 35% of RbcS-nt:GFP was detected as the precursor form (Fig. 7, B and C), similar to that in control protoplasts. This result is in accordance to previous studies that showed that the G domain is critical for targeting of Toc159 to chloroplasts (21, 22).

To further investigate the failure of atToc159[S/N] and atToc159[D/L] to support protein import into chloroplasts in ppi2 protoplasts, we examined the localization of these mu-
Role of the M Domain of atToc159

The M domain complements ppi2 mutants in transgenic plants. A. phenotype of wild-type (a) and ppi2 (b) plants. B. phenotype of transgenic plants. Transgenic plants were generated with various deletion constructs as specified below. Transgenic plants expressing the indicated proteins in the ppi2 homozygote background were selected from the T2 generation. Plants were grown on MS plates for 10 days. a, pBI122; b, atToc159; c, atToc159-A; d, atToc159-G; e, atToc159-M; f, atToc159M. All plants displayed a ppi2 background, as confirmed below in C. D, genotype analysis of transgenic plants. Genomic DNA samples were obtained from rosette leaves of individual plants and used for PCR amplification as depicted in E. ppi2, ppi2 homozygote; atToc159/atToc159, atToc159, heterozygotes harboring the transgene T7/atToc159. Panels b–f are the same in B. The numbers 1–4 are depicted in E. E, location of PCR primers for PCR. A, G, and M are the A, G, and M domains, respectively. UTR, 5’-untranslated region of atToc159; LB, left border; T7, T7 tag. The numbers 1–4 indicate the lanes in D.

Table I
Phenotype analysis of the T2 generation of transgenic plants

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<tr>
<th>Constructs introduced</th>
<th>Phenotype of plants</th>
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<tr>
<td></td>
<td>Green</td>
</tr>
<tr>
<td>Vector (pBI122)</td>
<td>93</td>
</tr>
<tr>
<td>T7:atToc159</td>
<td>134</td>
</tr>
<tr>
<td>T7:atToc159-A</td>
<td>128</td>
</tr>
<tr>
<td>T7:atToc159-G</td>
<td>85</td>
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<tr>
<td>T7:atToc159-M</td>
<td>112</td>
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<td>T7:atToc159M</td>
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Transformants. Again, the GFP coding region was inserted between the A and G domains and the resulting constructs were introduced into wild-type protoplasts. Both atToc159[S/N];GFP (panels d–f) and atToc159[D/L];GFP (panels g–i) were observed only as diffuse patterns in the cytosol (Fig. 7E). Our data indicate that the G domain is critical for targeting to the outer envelope membrane as observed previously (21, 22). Thus it is possible that the mutant G domain may mask the targeting signal present at the M domain, which in turn results in failure to support protein import into chloroplasts.

Chloroplasts Are Well Developed in Transgenic Plants Expressing the M Domain in ppi2 Mutant Backgrounds—To confirm the results obtained with the transient expression approach in ppi2 protoplasts, we generated transgenic plants expressing various deletion mutants in a ppi2 background. The ppi2 mutant was maintained as heterozygotes, since homozygotes are seedling lethal (Fig. 8B, panel b) (8). Transformation was performed using heterozygote plants at the atToc159 locus. Heterozygotes were similar to wild types in appearance and were selected from a pool of seeds obtained from heterozygous plants based on wild-type appearance and kanamycin resistance. Transformants were selected using both kanamycin and hygromycin resistance, since deletion mutants were introduced into plants together with the hygromycin-resistance gene. We scored the phenotype and segregation of transgenic plants at the T2 generation on plates containing both kanamycin and hygromycin. A summary of the segregation ratios from transgenic plants is shown in Table I. As expected from transgenic plants transformed with the vector (pBI-Hyg), all the plants resistant to both kanamycin and hygromycin were nearly identical to ppi2 homozygotes (Fig. 8B, panel a). When atToc159 and atToc159-A were introduced into ppi2 heterozygotes, most of plants resistant to both kanamycin and hygromycin were wild-type plants (Fig. 8B, panels b and e), indicating that these proteins complement the mutation. Moreover, atToc159-A transgenic plants (Fig. 8B, panel e) were nearly identical to wild-type plants (Fig. 8A, panel a), suggesting that the A domain is dispensable. Upon introduction of atToc159-G, we observed yellowish (Fig. 8B, panel d) and wild-type plants (data...
not shown), but no albino plants among plants that were resistant to both kanamycin and hygromycin. The ratio of yellowish to wild-type plants was nearly 1 to 2, suggesting that the yellow plants represent homozygous pp12 plants expressing atToc159-G, which weakly complements the loss of atToc159.

When we examined transgenic plants obtained with at-Toc159-M, albino (Fig. 8B, panel e) and wild-type plants (data not shown) were observed, as is the case in plants obtained with the vector alone. Furthermore, the segregation ratio of T2 progeny was 1 to 2 (albino to wild-type plants), indicating that atToc159-M does not complement the loss of atToc159 in pp12. Among atToc159M transgenic plants that were resistant to both kanamycin and hygromycin, greenish (Fig. 8B, panel f) or wild-type plants (but no albino plants) were observed. The ratio of greenish to wild-type plants was 1 to 2, indicating that greenish plants are pp12 homozygotes with atToc159M. This finding strongly suggests that atToc159M functionally complements the loss of atToc159 at a slightly reduced efficiency compared with wild-type atToc159.

To further confirm the complementation, we examined the genotypes of individual plants (Fig. 8A) by polymerase chain reaction with specific primers (Fig. 8E). As a control, PCR products obtained from homozygotes, heterozygotes harboring a copy of atToc159, and wild-type plants were compared. As expected, pp12 homozygote plants displayed no atToc159 band with 5'- and 3'-primers but produced a T-DNA-specific band (800 bp) with LB and 5'-specific primers (lane 3) (Fig. 8B, panel pp12). Heterozygotes harboring T7:atToc159 (atToc159/attoc159/T7:atToc159) exhibited atToc159 (lane 1), T7:atToc159 (lane 2), and T-DNA-specific (lane 3) bands at 4.6 kb, 4.5 kb, and 800 bp, respectively. In contrast, wild-type plants (WT) displayed only the atToc159 band (lane 1, 4.6 kb), but no T-DNA band, as expected. Next, we examined the genotypes of transgenic plants harboring the various forms of atToc159. We selected plants resistant to both hygromycin and kanamycin from transgenic lines harboring the various deletion constructs (Fig. 8B). As shown in Fig. 8C, plants with a wild-type appearance transformed with atToc159 (panel b) or atToc159-A (panel c) displayed both T-DNA-specific (800 bp) and transgene-specific bands, but not the endogenous atToc159 band (Fig. 8D).
Kanamycin and hygromycin-resistant albino plants obtained from transgenic plants transformed with atToc159-M also exhibited T-DNA and transgene-specific bands, but not the endogenous atToc159 band (panel e). Finally, yellowish (panel d) and greenish (panel f) plants also contained T-DNA-specific and transgene-specific bands but not the endogenous atToc159 band, confirming that these phenotypes are a result of expression of atToc159-G and atToc159M, respectively, in a homozygous ppi2 background. Thus, the various deletion mutants have different abilities to complement the loss of atToc159 in ppi2 plants; specifically, atToc159 and atToc159-A have the capacity for full complementation, atToc159-M does not complement the loss of atToc159, atToc159-G provides only very weak complementation, and atToc159M has the capacity for nearly full complementation.

We examined the structures of ultra-thin sections of leaf tissues using transmission electron microscopy. We observed that albino plants obtained from transgenic lines harboring the vector alone had undeveloped chloroplasts with numerous lipid bodies but no thylakoid membranes (Fig. 9A, panel a), in accordance with previous data (8). However, as expected from the phenotype, chloroplasts in transgenic plants harboring atToc159 (panel b) and atToc159-A (panel c) were fully developed, with numerous stacks of thylakoid membranes. In contrast, chloroplasts in transgenic plants harboring atToc159-G were not fully developed and contained only a few strands of thylakoid membranes (panel d). In transgenic plants harboring atToc159-M, chloroplast morphology was similar to that observed in ppi2 plants (panel e). In transgenic plants with atToc159M, chloroplasts were well developed, as judged by density of thylakoid membranes (panel f), which were similar to those in transgenic plants with atToc159 or atToc159-A. However, careful analyses revealed that the density of thylakoid membranes was slightly lower in transgenic plants with atToc159M than in those with atToc159. These results confirm the phenotypic results in whole plants.

To obtain independent evidence of the degree of complementation by the deletion mutants of atToc159, we measured the chlorophyll content as a marker for complementation. ppi2 plants expressing atToc159 or atToc159-A displayed nearly identical chlorophyll content (Fig. 9B). Transgenic plants with atToc159-M were nearly identical to ppi2 with regard to chlorophyll content. Transgenic plants with atToc159-G and atToc159M displayed 10 and 50% of the chlorophyll content observed in wild-type plants, respectively. These results are in agreement with the data obtained from the experiments described above.

Next, we performed Western blotting with protein extracts obtained from transgenic plants using an anti-T7 antibody to confirm the expression of the various forms of atToc159 in transgenic plants. Similar levels of protein were expressed by the deletion mutants and wild-type atToc159 (Fig. 9C).

Finally, we examined the localization of atToc159M with immunohistochemistry using a monoclonal anti-T7 antibody, with a view to elucidating the mechanism of complementation by the atToc159M mutant. As depicted in Fig. 10A (panels d and e), atToc159M was detected as a ring pattern around chloroplasts, indicating that it is targeted to chloroplasts. In contrast, no signal was detected from wild-type plants, confirming the specificity of the anti-T7 antibody. To confirm the presence of atToc159M at the envelope membrane, protein extracts obtained from transgenic plants with atToc159M were fractionated into membrane and soluble fractions by ultracentrifugation. The presence of atToc159M in these fractions was determined by Western blot analysis with the anti-T7 antibody. atToc159M was detected in the membrane, but not the soluble fraction (Fig. 10B, top panel). As a control, we also used an anti-RbcS antibody and found that RbcS is specifically detected in the soluble fraction (Fig. 10B, bottom panel).

**DISCUSSION**

In this study we established that the loss of protein import into chloroplasts due to the absence of atToc159 in ppi2 mutants can be complemented by transiently expressing atToc159 in protoplasts. This transient expression system in protoplasts has been shown to be a convenient way to study protein import into chloroplasts (27), despite certain potential limitations, such as possible mislocalization of proteins due to overexpression and alterations in responses due to the absence of the cell wall (33). In ppi2 protoplasts, RbcS-nt:GFP was partially imported into chloroplasts, as expected due to the loss of atToc159. The import of RbcS-nt:GFP in ppi2 protoplasts despite the loss of atToc159 may be due to the presence of other atToc159 homologs, such as atToc132, atToc120, and atToc90 (8, 34). However, the amount of imported RbcS-nt:GFP was 60% of the total amount of RbcS-nt:GFP expressed in protoplasts. This seems to contradict a previous study, which showed only a small amount of RbcS is present in ppi2 plants (8). This may be due to a difference in expression between endogenous RbcS and our RbcS-nt:GFP construct, which is under the control of the strong CaMV promoter. By this logic, the small amount of RbcS accumulated in ppi2 mutants may not directly reflect the protein import capacity per se in ppi2 plants. Rather, the defect in protein import into chloroplasts may also cause a defect in the expression of RbcS in ppi2 plants, possibly due to a lack of a positive feedback signaling from the chloroplast to the nucleus (35). In contrast, expression of RbcS-nt:GFP derived from the strong CaMV 35S-promoter in ppi2 protoplasts is not likely to be affected by the lack of chloroplast development.

From our experiments using transient and transgenic ex-
pression of various deletion mutants of atToc159 in ppi2 protoplasts, we conclude that the M domain alone fully complements the loss of atToc159 with respect to protein import into chloroplasts. In transgenic plants expressing atToc159M in a ppi2 background, atToc159M nearly fully complemented the loss of atToc159 with respect to the density of the thylakoid membrane. However, measurement of the degree of complementation by chlorophyll content revealed that transgenic plants with atToc159M in a ppi2 background displayed 50% of the chlorophyll content of wild types, suggesting that the M domain complements the loss of atToc159 with reduced efficiency compared with the full-length protein.

Previous findings suggest that the G domain is essential for the targeting of Toc159 (21, 22). We also confirmed the importance of the G domain for atToc159 targeting as well as the ability of the protein to support protein import into chloroplasts. When atToc159[S/N] and atToc159[D/L] were expressed transiently in ppi2 protoplasts, mutant proteins were observed in the cytosol and did not support protein import into chloroplasts. The A domain, in contrast, is dispensable for complementation with respect to protein import into chloroplasts in protoplasts as well as in transgenic plants with the ppi2 background. Interestingly, in both ppi2 protoplasts and transgenic plants, the isolated M domain was targeted to the chloroplast envelope membrane and co-fractionated with the membrane. This result appears to be inconsistent with previous studies (21, 22) and with the data obtained with atToc159[S/N] and atToc159[D/L] in this study. One possible explanation for this discrepancy is that the M domain itself contains the information to target Toc159 to chloroplasts, but targeting of atToc159 to the chloroplast envelope membrane is regulated by the G domain. A possible mechanism for this process is that the targeting signal, a part of the M domain, is masked by the G domain. A possible mechanism for this process is that the targeting signal, a part of the M domain, is masked by the G domain. An alternative mechanism is that the chloroplast envelope membrane would occur when the targeting signal is hidden by the G domain. Thus, deletion of both the A and G domains may result in the exposure of targeting signal, allowing the isolated M domain to be targeted to the chloroplast envelope membrane.

In conclusion, we have demonstrated that the M domain plays a regulatory role in the targeting of atToc159 to chloroplasts or support the function of atToc159 in the cytosol.

REFERENCES