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The Molecular Basis for Distinct Pathways for Protein Import into *Arabidopsis* Chloroplasts [W]

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The translocons at the outer envelope membrane of chloroplasts (TOCs) initiate the import of thousands of nucleus-encoded proteins into the organelle. The identification of structurally and functionally distinct TOC complexes has led to the hypothesis that the translocons constitute different import pathways that are required to coordinate the import of sets of proteins whose expression varies in response to organelle biogenesis and physiological adaptation. To test this hypothesis, we examined the molecular basis for distinct TOC pathways by analyzing the functional diversification among the Toc159 family of TOC receptors. We demonstrate that the N-terminal A-domains of the Toc159 receptors regulate their selectivity for preprotein binding. Furthermore, the in vivo function of the two major Toc159 family members (atToc159 and atToc132) can be largely switched by swapping their A-domains in transgenic *Arabidopsis thaliana*. On the basis of these results, we propose that the A-domains of the Toc159 receptors are major determinants of distinct pathways for protein import into chloroplasts.

INTRODUCTION

Chloroplasts evolved into a diverse array of organelles, collectively known as plastids, that play central roles in the major metabolic processes within plants, including photosynthesis and carbohydrate partitioning, lipid and amino acid synthesis, and nitrogen and sulfur metabolism (Lopez-Juez and Pyke, 2005; Lopez-Juez, 2007; Reyes-Prieto et al., 2007). Plastid biogenesis and function are dependent on the coordinate expression and import of thousands of nucleus-encoded proteins (Jarvis, 2004). Transcription of nucleus-encoded plastid proteins is tightly regulated by a complex control network that mediates the differentiation of plastids into specific functional forms (e.g., chloroplasts, amyloplasts, chromoplasts, etc.) and responds to variations in metabolic, physiological, or stress conditions that impact organelle function (e.g., light and nutrient availability) (Lopez-Juez and Pyke, 2005; Lopez-Juez, 2007; Kakizaki et al., 2009). Recent studies suggest that multiple protein import pathways have evolved to mediate the import of specific groups of preproteins that are coordinately expressed in response to developmental or physiological cues (Jarvis et al., 1998; Bauer et al., 2000; Kubis et al., 2003, 2004; Ivanova et al., 2004; Kessler and Schnell, 2009). The distinct protein import pathways are proposed to compensate for fluctuations in the expressions of specific sets of genes (i.e., photosynthetic versus constitutive).

The majority of nucleus-encoded plastid proteins are synthesized as preproteins on cytoplasmic ribosomes and are imported into the organelle via the coordinated action of two protein translocation machineries within the chloroplast envelope, the TOC (for translocon at the outer envelope membrane of chloroplasts) and TIC (for translocon at the inner envelope membrane of chloroplasts) complexes (Inaba and Schnell, 2008; Jarvis, 2008). The initial targeting of preproteins to the chloroplast surface is mediated by the interaction of their intrinsic transit peptides with two GTPase receptor subunits of the TOC complexes, the Toc34 and Toc159 receptors (Agne and Kessler, 2009; Sommer and Schleiff, 2009). In vascular plants, the Toc34 and Toc159 receptors are encoded by small gene families, and studies in *Arabidopsis thaliana* demonstrate that different members of the receptor families combine to form functionally and structurally distinct TOC complexes (Kubis et al., 2003, 2004; Ivanova et al., 2004). Furthermore, mutations in genes encoding individual TOC receptor family members in *Arabidopsis* differentially affect the accumulation of various nucleus-encoded chloroplast proteins and cause disruptions in distinct stages of chloroplast biogenesis (Jarvis et al., 1998; Bauer et al., 2000; Kubis et al., 2003, 2004; Ivanova et al., 2004). These observations led to the hypothesis that different TOC receptors mediate the import of distinct groups of preproteins and therefore define distinct import pathways that control protein import in coordination with specific events in plastid development.

Whereas studies with the two Toc34 genes in *Arabidopsis*, *TOC33* and *TOC34*, suggest that their functions are significantly overlapping (Kubis et al., 2003; Constan et al., 2004), several members of the *Arabidopsis* Toc159 receptor family appear to be functionally distinct. The major type of Toc159 receptor in green tissues, Toc159, is required for chloroplast biogenesis in *Arabidopsis*. The Toc159 null mutant, *ppi2*, is albino and results in a seedling lethal phenotype (Bauer et al., 2000). The lethal phenotype can be partially rescued with sucrose, and other plastid types are less severely affected in *ppi2* plants (Yu and Li, 2001).

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The accumulation of photosynthetic proteins is dramatically reduced in *ppi2*, but the levels of other nonphotosynthetic chloroplast proteins are not reduced, suggesting that Toc159 functions primarily in the import of proteins involved in photosynthesis (Bauer et al., 2000). Two other Toc159 family members from *Arabidopsis*, Toc120 and Toc132, are structurally very similar and appear to be functionally overlapping (Ivanova et al., 2004; Kubis et al., 2004). However, double null mutants in Toc120 and Toc132 (*toc120-1 toc132-1*) exhibit phenotypes distinct from *ppi2* (Ivanova et al., 2004; Kubis et al., 2004). In vitro binding studies indicate that Toc159 and Toc132 possess distinct binding specificities, suggesting that the receptors represent different import pathways into the chloroplast for photosynthetic and constitutive proteins (Ivanova et al., 2004; Smith et al., 2004). Furthermore, overexpression of either Toc159 or Toc132 cannot complement the lethal phenotypes of the null mutations in the other genes (Ivanova et al., 2004; Kubis et al., 2004). These observations led to the proposal that the different Toc159 receptors are the major contributors to the selectivity of TOC complexes for distinct classes of nucleus-encoded preproteins.

To test the hypothesis that the Toc159 receptors define distinct import pathways, we examined the structural basis for the selective functions of at-Toc159 and at-Toc132. The Toc159 family members contain three structural domains: a C-terminal membrane anchor domain (M-domain), a central GTPase domain (G-domain), and a highly acidic N-terminal domain (A-domain). The G- and M-domains of Toc159 and Toc132 are ~50% identical, whereas the N-terminal A-domains of the receptors vary considerably in length and primary structure (~11% sequence identity) (Ivanova et al., 2004; Richardson et al., 2009). Chemical cross-linking and preprotein binding studies with Toc159 suggest that the G-domain of the receptor contains a transit peptide binding site (Smith et al., 2004). The A-domain of Toc159 does not appear to bind to preproteins with a measurable affinity, but the sequence divergence among the receptor A-domains led to the proposal that the A-domains might contribute to the functional distinction between the receptors through an unknown mechanism (Smith et al., 2004). To investigate the structural determinants for receptor and import pathway specificity, we analyzed a set of deletion-mutation and chimeric receptors for preprotein binding selectivity in vivo and in vitro. Our in vitro analyses indicate that the variable N-terminal A-domains of the receptors regulate their selectively for preprotein recognition. Furthermore, we demonstrate in transgenic plants that the distinct functions of the two major TOC receptors (Toc159 and Toc132) can be switched by swapping their A-domains. On the basis of these results, we propose that the A-domains of the Toc159 family members are major determinants of distinct pathways for protein import into plastids.

RESULTS

The A-Domain Contributes to the Transit Peptide Selectivity of Isolated Receptors

To facilitate our analyses, we generated a set of modular gene constructs in which the coding regions of the A-, G-, and M-domains of Toc159 and Toc132 from *Arabidopsis* were linked by unique restriction sites introduced by site-directed mutagenesis in the full-length cDNAs (see Supplemental Figure 1 online). C-myc epitope tags were also introduced into the constructs at their C termini. The Toc159 and Toc132 constructs were referred to as atToc159AGM$_{myc}$ and atToc132AGM$_{myc}$, respectively. The mutations resulted in conservative amino acid changes in most cases, potentially minimizing their effects on receptor function. Furthermore, the atToc159AGM$_{myc}$ construct was able to complement the *ppi2* null mutant in *Arabidopsis* (see Supplemental Figure 2 online), confirming that the mutations did not detectably affect receptor function.

As a first step, we examined whether deletion of the A-domains of Toc159 and Toc132 affect their selective binding to different preproteins. Previous studies demonstrated that Toc159 preferentially binds to the preproteins of several chloroplast-specific photosynthetic proteins, including the precursors to ferredoxin (pFd) and the small subunit of ribulose-1,5-bis-phosphate carboxylase/oxygenase (Ivanova et al., 2004; Smith et al., 2004; Lee et al., 2009a). By contrast, Toc132 binds preferentially to nonphotosynthetic preproteins that are expressed in green and nongreen tissues, including the precursor to the pyruvate dehydrogenase E1α subunit (pE1α) (Ivanova et al., 2004). We generated two deletion constructs, atToc159GM and atToc132GM, lacking the respective A-domains of the receptors. The binding of atToc159GM and atToc132GM was compared with full-length atToc159AGM$_{myc}$ and atToc132AGM$_{myc}$ in a solid phase binding assay (Smith et al., 2004).

We performed an in vitro competition assay in which in vitro–translated [^{35}S]-labeled receptors were incubated with immobilized fusion proteins corresponding to hexahistidine-tagged staphylococcal protein A fused to pFd (pFd-protA$_{His}$) or the pE1α transit peptide (pE1α-protA$_{His}$). The binding reactions were performed in the presence or absence of soluble forms of pFd fused to staphylococcal protein A (pFd-protA) or pE1α fused to hexahistidine-tagged dihydrofolate reductase (pE1α-DHFR$_{His}$) as competitors. As controls, we tested competition with forms of the fusion proteins lacking transit peptides (Fd-protA and DHFR$_{His}$). The binding of full-length [^{35}S]atToc159AGM$_{myc}$ to immobilized pFd-protA$_{His}$ was competed by soluble pFd-protA. Maximal binding (19.2% $\pm$ 5.2% of added [^{35}S]atToc159AGM$_{myc}$) was reduced by 75% at the highest concentration of competitor tested (Figure 1B). Neither pE1α-DHFR$_{His}$ nor the Fd-protA or DHFR$_{His}$ controls had measurable effects on binding at the same concentrations (Figure 1B). Conversely, maximal binding of [^{35}S] atToc132AGM$_{myc}$ to immobilized pE1α-protA$_{His}$ (11.8% $\pm$ 0.1% of added [^{35}S]atToc132AGM$_{myc}$) was competed by soluble pE1α-DHFR$_{His}$ but not by pFd-protA, Fd-protA or DHFR$_{His}$ (Figure 1D). These results are consistent with the previously established selectivity of the receptors for preprotein binding (Ivanova et al., 2004; Smith et al., 2004).

By contrast, [^{35}S]atToc159GM and [^{35}S]atToc132GM did not exhibit selectivity toward binding to pFd-protA$_{His}$ or pE1α-protA$_{His}$. Although the maximal binding of [^{35}S]atToc159GM to immobilized pFd-protA$_{His}$ (19.7% $\pm$ 0.6% of added [^{35}S]atToc159GM) and [^{35}S]atToc132GM to immobilized pE1α-protA$_{His}$ (12.6% $\pm$ 2.2% of added [^{35}S]atToc132GM) was indistinguishable from the full-length receptors, the binding of either receptor

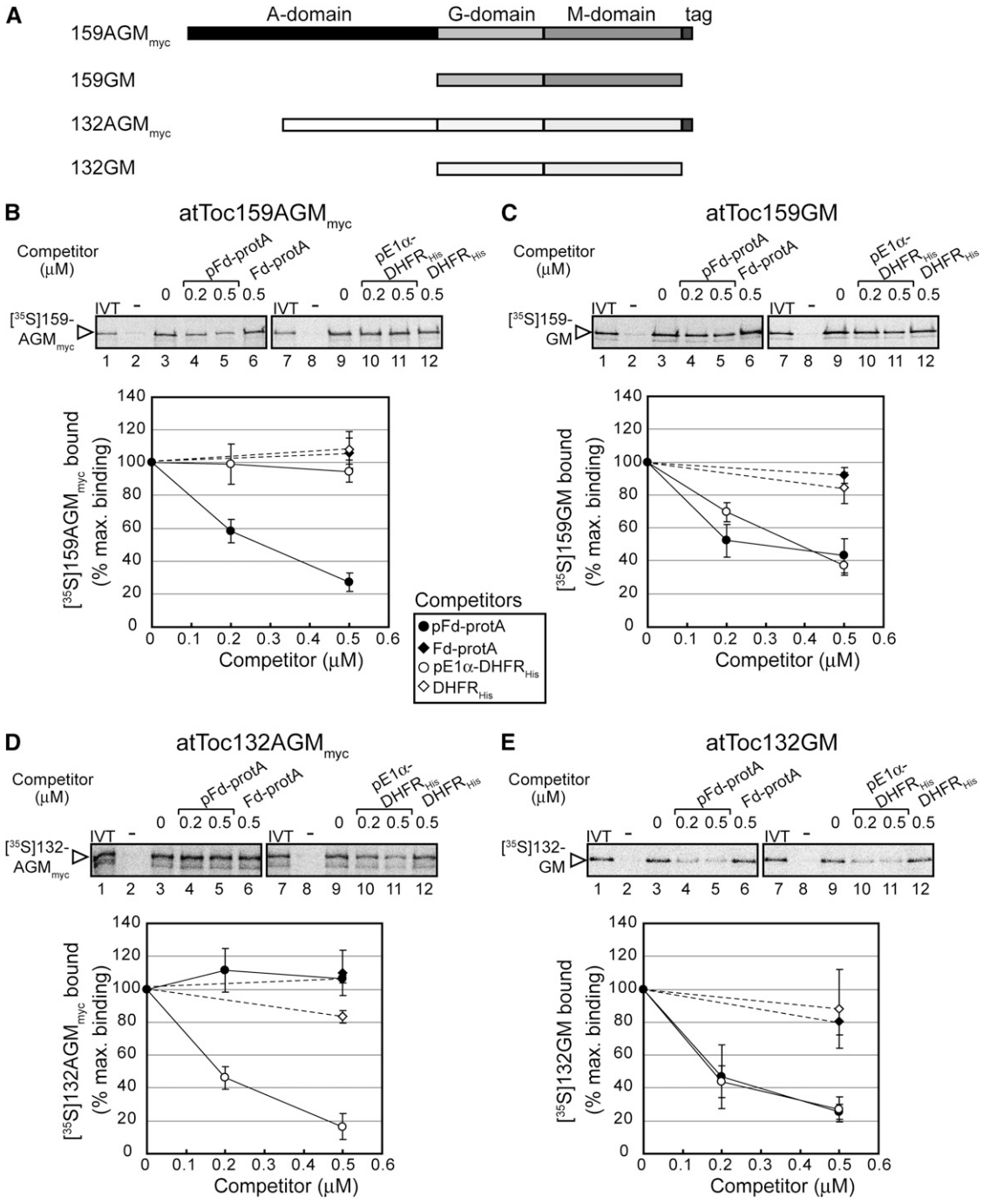

Figure 1. The A-Domain Is Required for Selective Binding of Toc159 and Toc132 to Different Preproteins.

(A) Schematic representation of the Toc159 and Toc132 constructs used in the binding studies.

(B) and **(C)** 25 pmol Ni-NTA–immobilized pFd-protA$_{His}$ (lanes 3 to 6) or IgG-Sepharose–immobilized pFd-protA$_{His}$ (lanes 9 to 12) was incubated with in vitro–translated [^{35}S]atToc159AGM$_{myc}$ **(B)** or [^{35}S]atToc159GM **(C)** in the absence or presence of increasing concentrations of soluble pFd-protA, Fd-protA, pE1α-DHFR$_{His}$, or DHFR$_{His}$ as indicated.

(D) and **(E)** 25 pmol Ni-NTA–immobilized pE1α-protA$_{His}$ (lanes 3 to 6) or IgG-Sepharose–immobilized pE1α-protA$_{His}$ (lanes 9 to 12) was incubated with in vitro–translated [^{35}S]atToc132AGM$_{myc}$ **(D)** or [^{35}S]atToc132GM **(E)** in the absence or presence of increasing concentrations of soluble pFd-protA, Fd-protA, pE1α-DHFR$_{His}$, or DHFR$_{His}$ as indicated. Bound protein was analyzed by SDS-PAGE and phosphor imaging. Lanes 1 and 7 in each panel contain 10% of the in vitro translation product added to each reaction. Lanes 2 and 8 contain in vitro translation products that bound to the Ni-NTA or IgG-Sepharose in the absence of immobilized fusion protein. The graphs present quantitative analysis of data from triplicate experiments, including the representative data shown in each panel. Binding is presented as the percentage of maximal binding of in vitro translation products observed in the absence of competitor. Error bars represent SE (n = 3) of the mean.

lacking its A-domain was competed by both soluble pFd-protA and pE1α-DHFR$_{His}$ at similar concentrations (Figures 1C and 1E). Fd-protA and DHFR$_{His}$ exhibited very modest effects on binding in both cases at the highest concentrations tested, demonstrating that preprotein binding to the receptors was dependent upon the presence of the transit peptide. These data confirm that the G- and M-domains of the receptors are sufficient for transit peptide recognition but suggest that these domains are insufficient to confer selectivity of Toc159 and Toc132 for different classes of transit peptides.

Deletion of the A-Domain Results in Chloroplasts with Less Selective TOC Complexes

The results in Figure 1 prompted us to examine whether the A-domains of the Toc159 family might define the selectively of different TOC complexes and therefore contribute to distinct import pathways in chloroplasts. Previous studies have shown that Toc159 constructs lacking the A-domain (Lee et al., 2003) or containing an epitope tag in place of the A-domain can complement the seedling lethal phenotype of *ppi2* (Agne et al., 2009), indicating that the deletion mutant retains sufficient activity to function in protein import. To investigate whether atToc159GM would alter protein import selectivity, we transformed *ppi2* with atToc159GM$_{His}$ under control of the cauliflower mosaic virus 35S promotor (CaMV 35S) and selected a line expressing the transgene at levels comparable to Toc159 in wild-type plants (see Supplemental Figure 2B online). As expected, atToc159GM$_{His}$ complemented the lethal phenotype of *ppi2* (see Supplemental Figure 2A online) and restored chlorophyll to levels indistinguishable from wild-type plants or plants complemented with full-length atToc159AGM$_{myc}$ (see Supplemental Figure 2C online).

To explore the selectivity of Toc complexes containing the atToc159GM $_{His}$ receptor, we isolated chloroplasts from atToc159GM$_{His}$/*ppi2*, atToc159AGM$_{myc}$/*ppi2*, and wild-type plants and performed import competition assays using [^{35}S]pFd-protA$_{His}$ as the import substrate and pFd-protA$_{His}$ and pE1α-DHFR$_{His}$ as competitors. Consistent with a previous report, the levels of import of [^{35}S]pFd-protA$_{His}$ into chloroplasts from wild-type (18.1% ± 1.6% of added [^{35}S]pFd-protA$_{His}$), atToc159AGM$_{myc}$/*ppi2* (16.2% ± 3.5% of added [^{35}S]pFd-protA$_{His}$), and atToc159GM$_{His}$/*ppi2* (15.4% ± 2.1% of added [^{35}S]pFd-protA$_{His}$) plants were indistinguishable, demonstrating that the deletion of the A-domain of atToc159 had little effect on the import capacity of these complexes (Figure 2A). In the presence

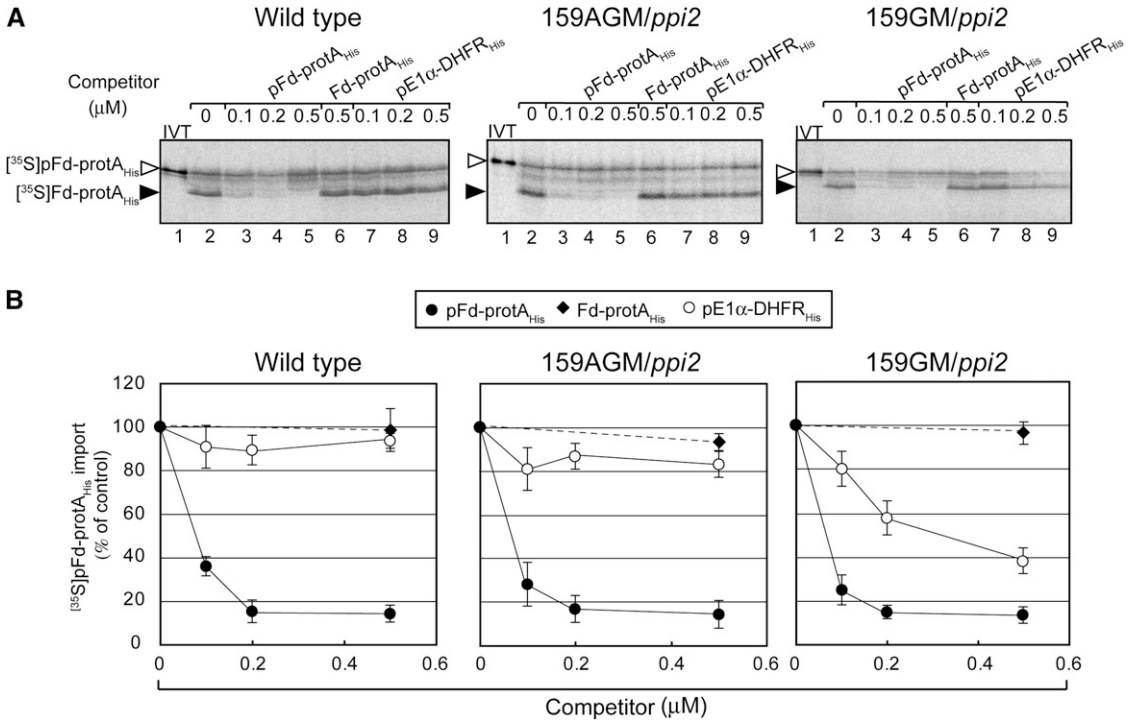

Figure 2. atToc159GM$_{His}$/*ppi2* Chloroplasts Exhibit Reduced Selectivity for the Import of a Model Toc159 Substrate.

(A) Isolated chloroplasts from wild-type, atToc159AGM$_{myc}$/*ppi2* (159AGM/*ppi2*), and atToc159GM$_{His}$/*ppi2* (159GM/*ppi2*) plants were incubated with in vitro–translated [^{35}S]pFd-protA$_{His}$ in the presence or absence of increasing concentrations of soluble pFd-protA$_{His}$, Fd-protA$_{His}$, or pE1α-DHFR$_{His}$ under import conditions. Lane 1 contains 10% of the in vitro translation product added to each reaction. [^{35}S]pFd-protA$_{His}$ import was analyzed by SDS-PAGE and phosphor imaging.

(B) Quantification of the data from replicate experiments including the representative data presented in **(A)**. Import is presented as the percentage of maximal import of [^{35}S]pFd-protA$_{His}$ in the absence of competitor. Error bars represent SE (n = 3) of the mean.

of increasing concentrations of recombinant pFd-protA$_{His}$ as the competitor, the import of in vitro–translated [^{35}S]pFd-protA$_{His}$ into chloroplasts from wild-type or atToc159AGM$_{myc}$/*ppi2* plants was reduced to ~15 to 20% of control levels at the highest concentration tested (Figure 2B). The presence of recombinant pE1α-DHFR$_{His}$ or Fd-protA$_{His}$ lacking a transit peptide had no effect on import (Figure 2B). By contrast, [^{35}S]pFd-protA$_{His}$ import into chloroplasts from atToc159GM$_{His}$/*ppi2* plants was competed by both pFd-protA$_{His}$ and pE1α-DHFR$_{His}$ (Figure 2A). Import was reduced to 15 and 40% of control levels at the highest concentration of pFd-protA$_{His}$ and pE1α-DHFR$_{His}$ tested, respectively (Figure 2B). Fd-protA$_{His}$ did not affect import, indicating that competition occurs at the level of transit peptide interactions with TOC complexes. These data demonstrate that the selectivity of receptor binding contributed by the A-domain of Toc159 also acts as a major determinant of TOC complex specificity in chloroplasts.

To identify the stage of import at which the A-domain exerts its influence, we examined the effect of competitor on [^{35}S]pFd-protA$_{His}$ binding to chloroplasts under different energy conditions. Chloroplasts from atToc159AGM$_{myc}$/*ppi2* or atToc159GM$_{His}$/*ppi2* plants were incubated with competitor in the absence of added nucleoside triphosphates to assay for initial receptor binding or in the presence of low levels of GTP (0.1 mM) and ATP (0.05 mM) to assay for the partial translocation of the preprotein across the outer envelope membrane (early import intermediate) (Schnell and Blobel, 1993; Ma et al., 1996; Inoue and Akita, 2008). pFd-protA$_{His}$ was an effective competitor of initial binding (Figures 3A and 3B) and outer membrane translocation (Figures 3A and 3C) in both atToc159AGM$_{myc}$/*ppi2* and atToc159GM$_{His}$/*ppi2* chloroplasts, consistent with the role of at-Toc159 in the initial recognition of preproteins at the Toc complex (Perry and Keegstra, 1994; Ma et al., 1996; Chen et al., 2000; Wang et al., 2008; Agne et al.,

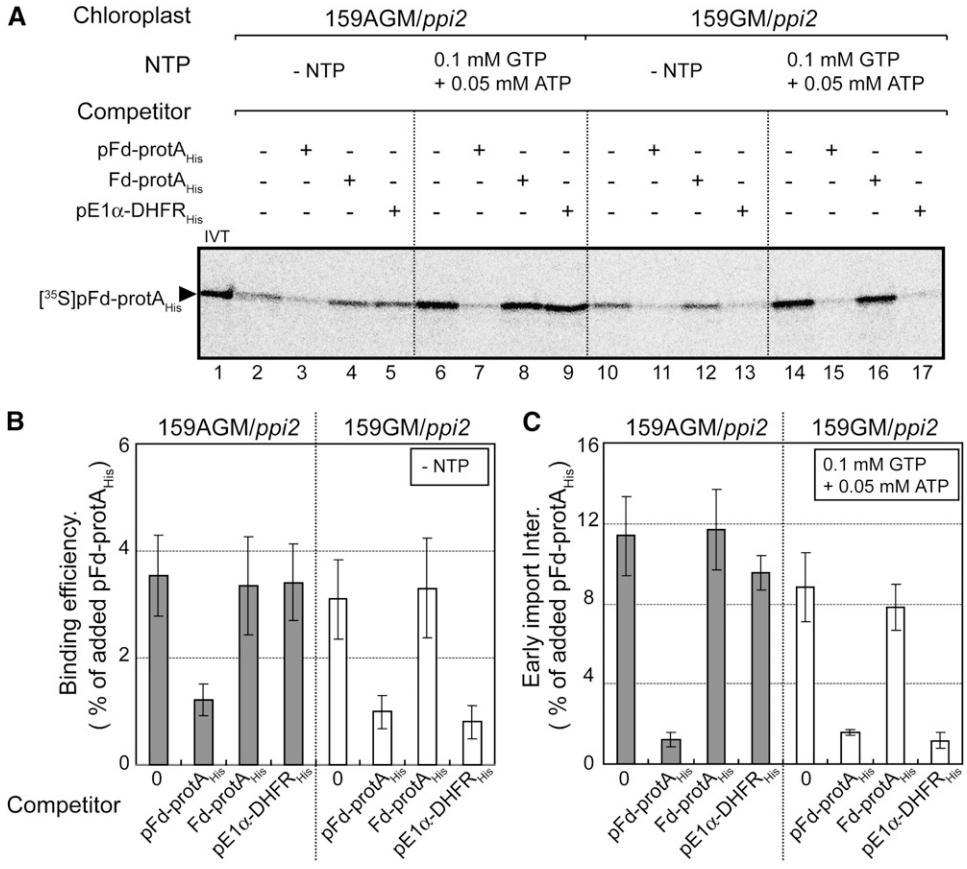

Figure 3. The Reduced Selectivity of atToc159GM$_{His}$/*ppi2* Chloroplasts Occurs at the Initial Stages of Preprotein Binding at TOC Complexes.

(A) Isolated energy-depleted chloroplasts from atToc159AGM$_{myc}$/*ppi2* (159AGM/*ppi2*) and atToc159GM$_{His}$/*ppi2* (159GM/*ppi2*) plants were incubated with energy-depleted in vitro–translated [^{35}S]pFd-protA$_{His}$ under preprotein binding (−NTP) or early import intermediate (0.1 mM GTP + 0.05 mM ATP) conditions. The reactions were performed in the presence (+) or absence (−) of 0.5 μM soluble pFd-protA$_{His}$, Fd-protA$_{His}$, or pE1α-DHFR$_{His}$ competitor as indicated. Chloroplast-associated [^{35}S]pFd-protA$_{His}$ was analyzed by SDS-PAGE and phosphor imaging. Lane 1 contains 10% of the in vitro translation product added to each reaction.

(B) and **(C)** Quantification of the data from replicate experiments including those presented in **(A)** for the assays of preprotein binding **(B)** or early import intermediate formation **(C)**. The data are presented as the percentage of added [^{35}S]pFd-protA$_{His}$ that was recovered with the chloroplasts at the end of the reaction. Error bars represent SE (n = 3) of the mean.

2009). By contrast, pE1α-DHFR$_{His}$ competes with [^{35}S]pFd-protA$_{His}$ binding (Figure 3B) and TOC translocation (Figure 3C) only in the atToc159GM$_{His}$:*ppi2* chloroplasts. The reduction of initial binding by pE1α-DHFR$_{His}$ in atToc159GM$_{His}$ chloroplasts suggests that the A-domain of Toc159 participates in the selective recognition of preproteins at the earliest stages of receptor–preprotein interactions. Taken together, our results support the hypothesis that the A-domain of Toc159 is a major determinant of the selectively of distinct TOC complexes. However, the observation that the import efficiency of atToc159AGM$_{myc}$/*ppi2* or atToc159GM$_{His}$/*ppi2* chloroplasts is indistinguishable (Figure 2) suggests that the A-domain does not play a significant role in the overall import capacity of TOC complexes.

The A-Domain Contributes to the Functional Distinction between Toc132 and Toc159 Complexes in Vivo

Previous studies demonstrated that overexpression of full-length Toc132 could not rescue the albino seedling-lethal phenotype of the *ppi2* (Ivanova et al., 2004; Kubis et al., 2004). These data support the hypothesis that Toc132 and Toc159 are functionally distinct. On the basis of the reduced selectivity of the atToc132GM receptor (Figure 1), we speculated that TOC complexes containing this receptor might have preprotein binding characteristics that partially overlap those of Toc159 complexes. To test this possibility, we overexpressed the atToc132GM construct in *ppi2* plants under control of the CaMV 35S promoter at levels approximately fivefold to sevenfold higher than the expression of endogenous Toc132 in wild-type plants (Figure 4C). Remarkably, atToc132GM/*ppi2* plants were viable when grown on soil under standard growth conditions (Figure 4B). The plants remained pale, with a chlorophyll content that was ~20% of control plants at 28 d after germination (Figure 4D). Although the plants remained small, they were fully fertile.

We estimated the relative changes in the levels of a set of representative plastid proteins in the atToc132GM/*ppi2* plants by immunoblotting total seedling extracts and comparing them to the abundance in wild-type, atToc159AGM/*ppi2*, atToc159GM/*ppi2*, and *ppi2* plants (Figure 5). The amounts of protein extracts used in the immunoblots were titrated to ensure a linear chemiluminescence signal for each protein (see Supplemental Figure 3 online), and all images used for quantification were not saturated black. The changes in protein levels were measured by comparing the immunoblot signals to the immunoblot signals of cytoplasmic actin. Two other components of TOC complexes, Toc75 and Toc33, accumulated to similar levels as the wild type in all plants examined (Figure 5B). The constitutively expressed protein, pyruvate dehydrogenase E1α, was unchanged in wild-type and atToc132GM/*ppi2* plants (Figure 5C). Consistent with previous observations (Bauer et al., 2000), *ppi2* plants contained 20% of the levels of ribulose-1,5-bis-phosphate carboxylase/oxygenase small subunit (SSU) and 25% of the levels of light-harvesting complex protein (LHCP) relative to the wild type (Figure 5C). Both SSU and LHCP are major photosynthetic proteins. The levels of SSU and LHCP increased to 40 to 55% of the wild type in the atToc132GM/*ppi2* plants (Figure 5C), indicating that the import and accumulation of these proteins was partially recovered by expression of

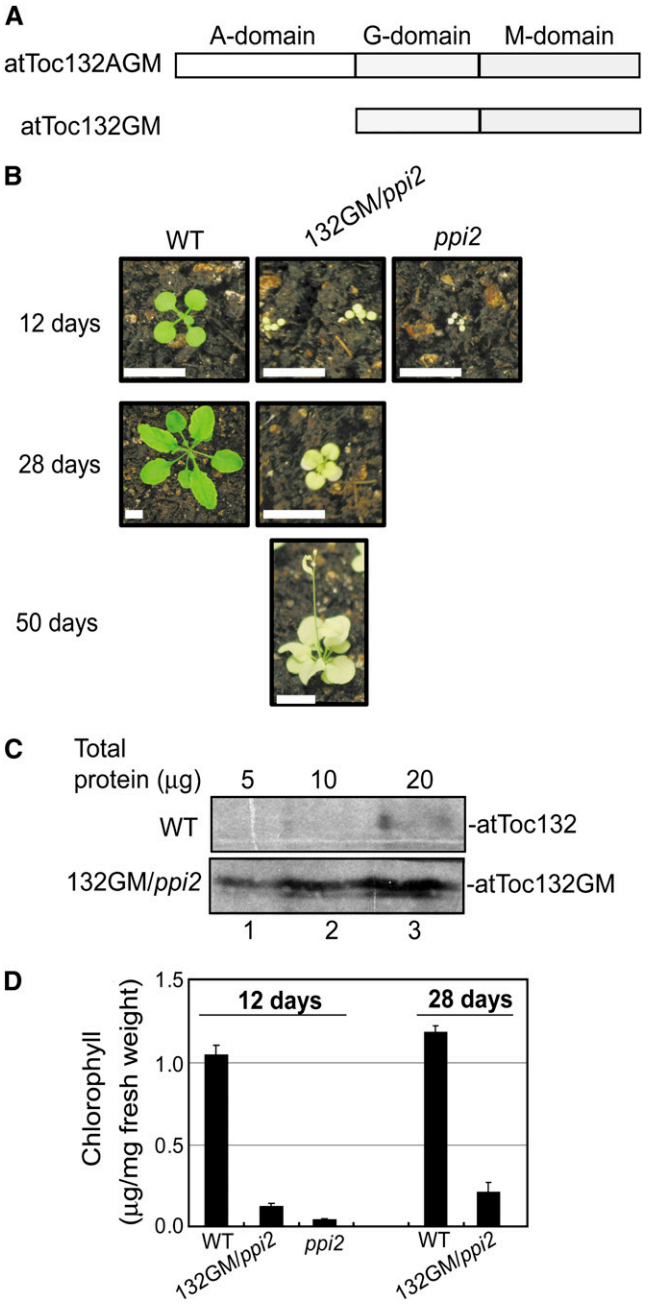

Figure 4. atToc132GM Is Able to Complement Partially the *ppi2* Mutant.

(A) Schematic representation of the atToc132GM construct used in the transformations.

(B) Visible phenotypes of wild-type (WT), atToc132GM/*ppi2* (132GM/*ppi2*), and *ppi2* plants at 12, 28, and 50 d after germination. Bars = 1 cm.

(C) Immunoblot analysis of total protein extracts from wild-type and atToc132GM/*ppi2* (132GM/*ppi2*) plants with antisera recognizing the Toc132 M-domain.

(D) Chlorophyll content of wild-type, atToc132GM/*ppi2* (132GM/*ppi2*), and *ppi2* seedlings at 12 and 28 d after germination. Error bars represent SE (n = 3) of the mean.

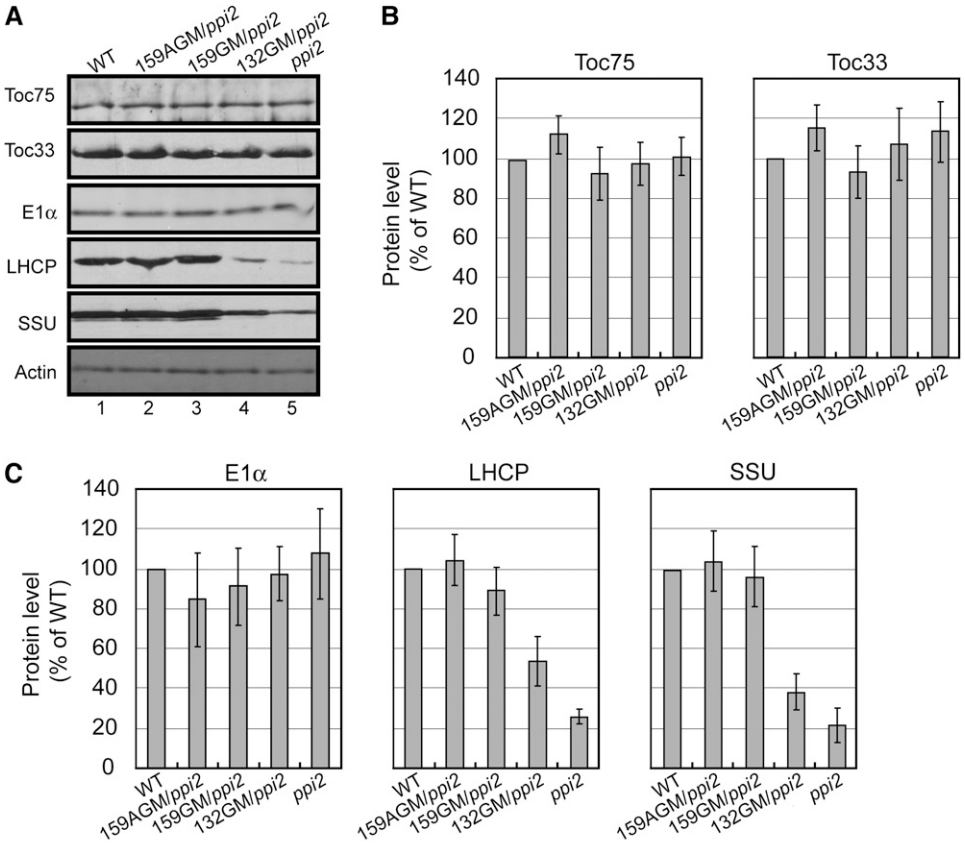

Figure 5. The Accumulation of Photosynthesis-Related Proteins Increases in atToc132GM/*ppi2* Plants.

(A) Immunoblot analysis of 10 μg of total protein extracts from 12-d-old wild type (WT), atToc159AGM$_{myc}$/*ppi2* (159AGM/*ppi2*), atToc159GM$_{His}$/*ppi2* (159GM/*ppi2*), atToc132GM/*ppi2* (132GM/*ppi2*), and *ppi2* plants with antisera toward the proteins indicated at the left of the figure.

(B) Measurement of the relative levels of chloroplast proteins as a ratio to cytosolic actin from replicate immunoblots including those shown in **(A)**. The levels of each protein are presented as a percentage of that observed in wild-type extracts. Error bars represent SE (n = 3) of the mean.

atToc132GM. The relative fragility of the atToc132GM/*ppi2* plants prevented us from isolating chloroplasts from this line for a direct analysis of protein import. Nonetheless, we conclude that removal of the A-domain of Toc132 reduces its binding selectivity, thereby generating a receptor with specificity that partially overlaps that of Toc159. In this way, atToc132GM translocons could catalyze the import of sufficient levels of chloroplast specific proteins to complement partially the *ppi2* mutation.

Our next goal was to investigate whether we could further affect receptor selectivity by adding the A-domain of Toc159 to atToc132GM. To this end, we fused the Toc159 A-domain to the Toc132 GM-domains to generate atToc159A132GM$_{myc}$ and tested the selectivity of the receptor for binding to pFd-protA$_{His}$ in the solid phase competition assay (Figure 6). Interestingly, atToc159A132GM$_{myc}$ bound to pFd-protA$_{His}$ with similar specificity to that of atToc159AGM$_{myc}$ (cf. Figures 6B and 1B). atToc159A132GM$_{myc}$ binding to immobilized pFd-protA$_{His}$ was effectively competed with soluble pFd-protA but not pE1α-DHFR$_{His}$. Maximal binding of [^{35}S]atToc159A132GM$_{myc}$ to pFd-protA$_{His}$ was 25% ± 1.7% of added receptor (Figure 6B), levels similar to the binding of [^{35}S]atToc159AGM$_{myc}$ (Figure 1). Receptor specificity for transit peptides was not altered in the chimerical receptor: competitors lacking transit peptides (Fd-protA and DHFR$_{His}$) did not compete for atToc159A132GM$_{myc}$ binding. These data suggest that the A-domain of Toc159 confers selectivity on the chimerical receptor that is similar to that of Toc159 and further supports the hypothesis that the A-domain is the major determinant of the ability of receptors to distinguish between different transit peptides.

To test the function of atToc159A132GM$_{myc}$ in chloroplasts, we transformed the gene into *ppi2* under control of the 35S CaMV promoter (Figure 7). As before, we selected a transformed line (atToc159A132GM$_{myc}$/*ppi2*) that expressed the transgene at levels comparable to those of Toc159 in wild-type plants (Figure 7B). The atToc159A132GM$_{myc}$ construct rescued the lethal *ppi2* phenotype, although the plants were somewhat paler than wild-type plants (Figure 7A). Chlorophyll levels of

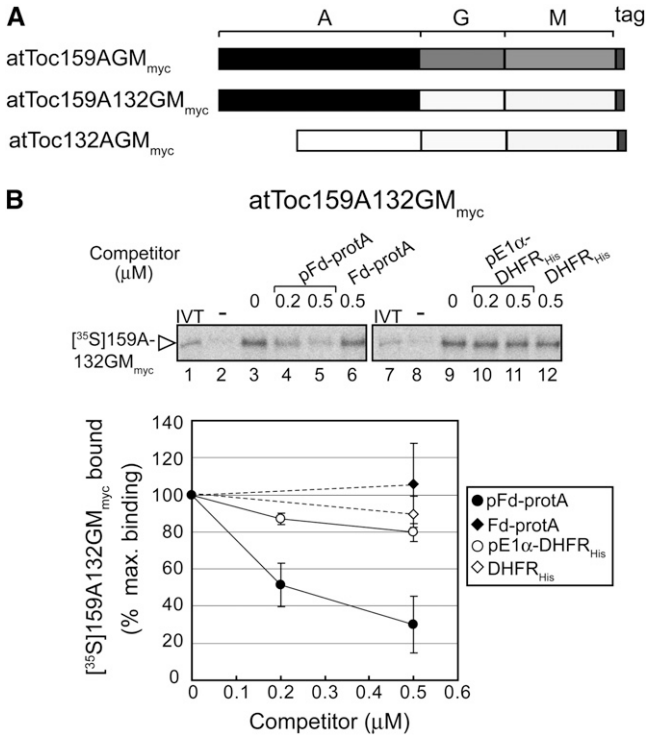

Figure 6. atToc159A132GM Exhibits a Similar Selectivity for Preprotein Binding as Toc159.

(A) Schematic representation of the atToc159A132GM$_{myc}$ construct used in the binding studies.

(B) 25 pmol Ni-NTA–immobilized pFd-protA$_{His}$ (lanes 3 to 6) or IgG-Sepharose–immobilized pFd-protA$_{His}$ (lanes 9 to 12) was incubated with in vitro–translated [^{35}S]atToc159A132GM$_{myc}$ in the absence or presence of increasing concentrations of soluble pFd-protA, Fd-protA, pE1α-DHFR$_{His}$, or DHFR$_{His}$ as indicated. Lanes 1 and 7 contain 10% of the in vitro translation products added to each reaction. Lanes 2 and 8 contain in vitro translation products that bound to the Ni-NTA or IgG-Sepharose in the absence of immobilized fusion protein. Binding is presented as the percentage of maximal binding of in vitro translation products observed in the absence of competitor. Error bars represent SE (n = 3) of the mean.

atToc159A132GM$_{myc}$/*ppi2* were ~50% of those of wild-type plants at both 12 and 28 d after germination compared with nearly undetectable levels of chlorophyll in *ppi2* seedlings (Figure 7C). Furthermore, immunoblotting of seedling extracts indicated that the levels of SSU and LHCP increased to >90% of normal wild-type levels in atToc159A132GM$_{myc}$/*ppi2* plants (Figures 7D and 7E). These results demonstrate that expression of atToc159A132GM$_{myc}$ was more effective at complementing *ppi2* than was atToc132GM, indicating that the A-domain of Toc159 confers selectivity on atToc159A132GM$_{myc}$ TOC complexes that is similar to that of Toc159.

We isolated chloroplasts from atToc159A132GM$_{myc}$/*ppi2* plants and examined the selectivity of protein import with the import competition assay (Figure 8). The pFd-protA$_{His}$ competitor reduced import to ~20% of control levels in both wild-type and atToc159A132GM$_{myc}$/*ppi2* chloroplasts at the highest concentration tested (Figure 8B). pE1α-DHFR$_{His}$ was unable to compete with the import of [^{35}S]pFd-protA$_{His}$ (Figure 8B), indicating that atToc159A132GM$_{myc}$/*ppi2* chloroplasts exhibited a similar selectivity for import compared with atToc159AGM$_{myc}$/*ppi2* chloroplasts. These results provide further evidence that the A-domain of the receptors is a major determinant of receptor selectivity toward distinct transit peptides.

Finally, we examine whether Toc33 and Toc34 might contribute to the selectively of different TOC complexes. Previous in vitro binding and in vitro chloroplast import studies suggest that Toc33/34 receptors exhibit differential substrate specificity (Gutensohn et al., 2000; Jelic et al., 2003; Kubis et al., 2003). To explore the selectivity of Toc complexes in the absence of Toc34 or Toc33, we isolated chloroplasts from mutants lacking Toc34 expression, *ppi3* (Constan et al., 2004), or Toc33 expression, *ppi1* (Jarvis et al., 1998), and performed import competition assays (Figure 9). We chose the single concentration of competitor (0.5 μM) that was sufficient to achieve maximal competition in wild-type chloroplasts (Figure 2). As previously reported, the level of import into chloroplasts from *ppi1* plants was reduced to ~50% of that of wild-type chloroplasts (Figure 9, compare lanes 2 and 10), whereas import into chloroplasts from *ppi3* plants was indistinguishable from that in wild-type chloroplasts (Figure 9, compare lanes 2 and 6) (Kubis et al., 2003; Constan et al., 2004; Lee et al., 2009b). However, both *ppi1* and *ppi3* chloroplasts exhibited a wild-type selectivity for the import of [^{35}S]pFd-protA$_{His}$. In the presence of the pFd-protA$_{His}$ competitor, import of [^{35}S]pFd-protA$_{His}$ was reduced to ~10% of control levels in wild-type, *ppi1*, and *ppi3* chloroplasts (Figure 9B). pE1α-DHFR$_{His}$ was unable to compete the import of [^{35}S]pFd-protA$_{His}$ (Figure 9B). Taken together, these results provide further evidence that the A-domain of the Toc159 family of receptors is a major determinant of TOC complex selectivity toward distinct transit peptides.

DISCUSSION

In this study, we investigated the molecular basis for the functional distinctions between multiple pathways for protein import into chloroplasts. Previous genetic and preprotein binding studies suggested that members of the Toc159 family of TOC receptors formed structurally and functionally distinct TOC complexes (Bauer et al., 2000; Ivanova et al., 2004; Kubis et al., 2004). Studies with two classes of *Arabidopsis* Toc159 receptors, Toc159 and Toc132/Toc120, indicated that they exhibit distinct binding selectivities for different nucleus-encoded preproteins (Smith et al., 2004). This led to the hypothesis that different TOC complexes catalyze the import of specific classes of differentially expressed preproteins that were essential for constitutive and specialized plastid functions. Here, we show that the variable N-terminal A-domains of Toc159 and Toc132 influence their selectivities for different nucleus-encoded preproteins. In vitro binding competition assays demonstrated that deletion of the A-domains of Toc159 and Toc132 reduced their selectivity for binding to different types of preproteins, generating receptors with similar binding properties. Consistent

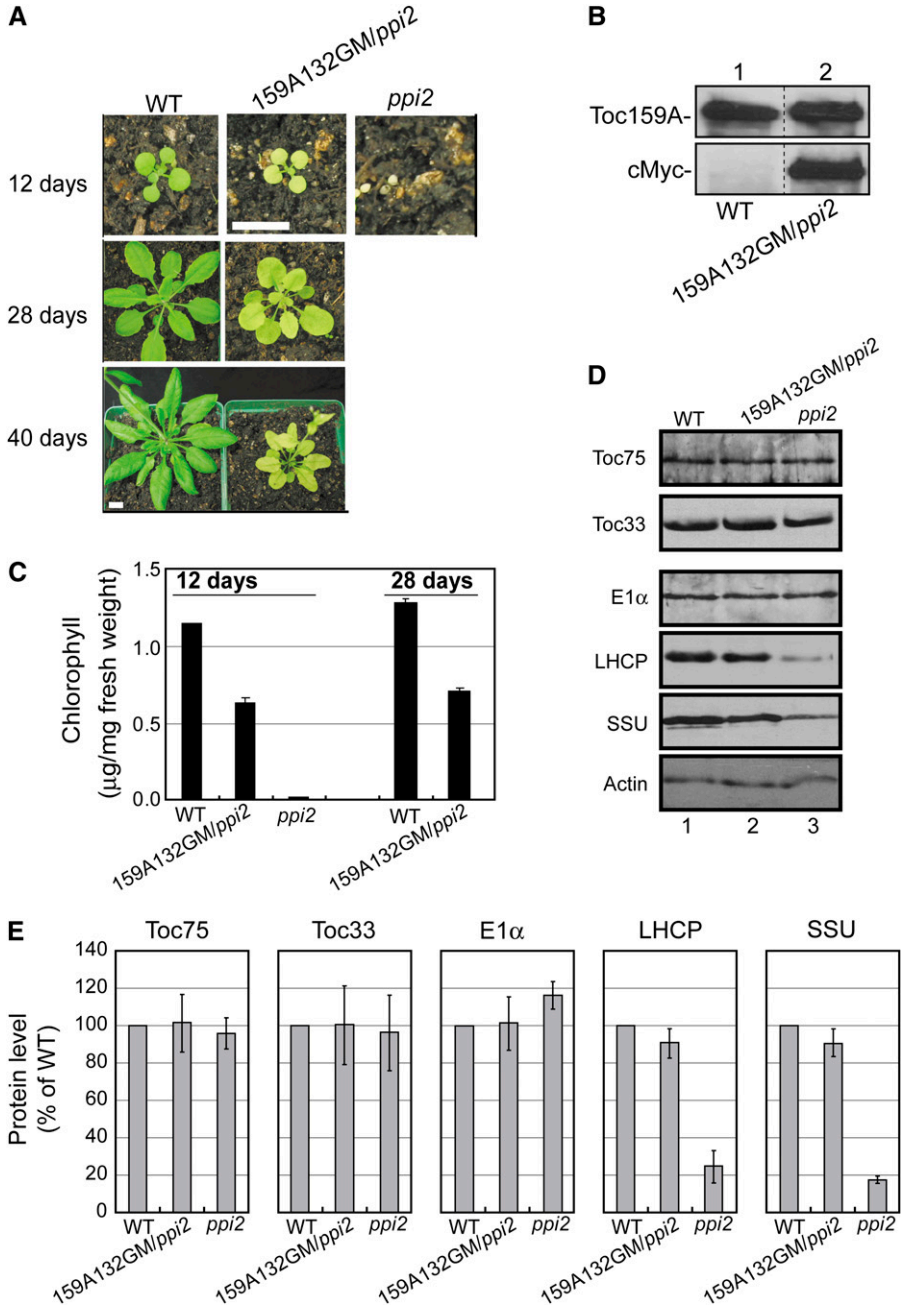

Figure 7. atToc159A132GM$_{myc}$ Largely Complements the *ppi2* Mutant.

(A) Visible phenotypes of wild-type (WT), atToc159A132GM$_{myc}$/*ppi2* (159A132GM/*ppi2*), and *ppi2* plants at 12, 28, and 40 d after germination. Bars = 1 cm.

(B) Immunoblot analysis of total protein extracts from 12-d-old wild-type and atToc159A132GM$_{myc}$/*ppi2* (159A132GM/*ppi2*) plants with antisera raised against the atToc159 A-domain or the c-Myc epitope.

(C) Chlorophyll content of wild-type, atToc159A132GM$_{myc}$/*ppi2* (159A132GM/*ppi2*), and *ppi2* seedlings at 12 and 28 d after germination. Error bars represent SE (n = 3) of the mean.

(D) Immunoblot analysis of 10 µg of total protein extracts from wild-type, atToc159A132GM$_{myc}$/*ppi2* (159A132GM/*ppi2*), and *ppi2* seedlings with antisera toward the proteins indicated at the left of the figure.

(E) Measurement of the relative levels of chloroplast proteins as a ratio to cytosolic actin from replicate immunoblots including those shown in **(D)**. The levels of each protein are presented as a percentage of that observed in wild-type extracts. Error bars represent SE (n = 3) of the mean.

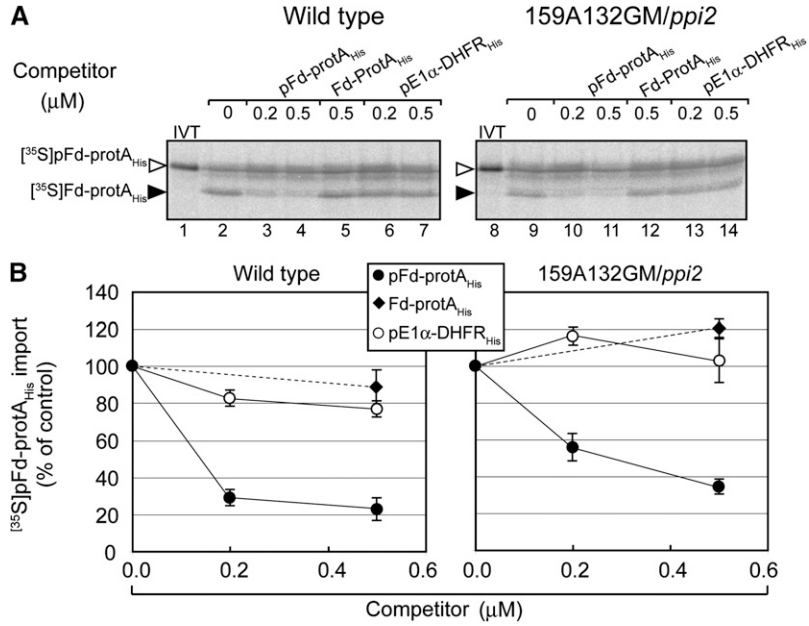

Figure 8. atToc159A132GM$_{myc}$/*ppi2* Chloroplasts Exhibit a Similar Selectivity for the Import of a Model Toc159 Substrate as Wild-Type Chloroplasts.

(A) Isolated chloroplasts from wild-type and atToc159A132GM$_{myc}$/*ppi2* (159A132GM/*ppi2*) plants were incubated with in vitro–translated [^{35}S]pFd-protA$_{His}$ in the presence or absence of increasing concentrations of soluble pFd-protA$_{His}$, Fd-protA$_{His}$, or pE1α-DHFR$_{His}$ under import conditions. Lane 1 contains 10% of the in vitro translation product added to each reaction. [^{35}S]pFd-protA$_{His}$ import was analyzed by SDS-PAGE and phosphor imaging.
(B) Quantification of the data from replicate experiments including the representative data presented in **(A)**. Import is presented as the percentage of maximal import of [^{35}S]pFd-protA$_{His}$ in the absence of competitor. Error bars represent SE (n = 3) of the mean.

with these results, we showed that protein import into chloroplasts isolated from plants expressing the Toc159 A-domain deletion construct, atToc159GM, exhibited reduced selectivity for the import of a model Toc159 substrate (pFd-protA$_{His}$) relative to a model Toc132 substrate (pE1α-DHFR$_{His}$). The A-domain was shown to affect the earliest stage of preprotein binding to chloroplasts, indicating that it functions during the initial recognition of preproteins by TOC complexes.

The role of the A-domain in determining the selectivity of TOC complexes for different classes of preproteins was supported by our observation that an A-domain deletion of Toc132, atToc132GM, could partially complement the Toc159 null mutant, *ppi2*. Furthermore, replacement of the A-domain of Toc132 with that of Toc159 resulted in a chimerical receptor, atToc159A132GM, with binding properties similar to those of Toc159. atToc159A132GM expression in transgenic plants was more effective at complementing *ppi2* than was the atToc132GM deletion construct, indicating that the addition of the Toc159 A-domain conferred additional Toc159-like functionality on the receptor. Previous studies demonstrated that full-length, authentic Toc132 could not complement *ppi2* (Ivanova et al., 2004; Kubis et al., 2004). Taken together, these data support the hypothesis that the A-domains of the Toc159 receptors are major determinants of the selectivity of TOC complexes for distinct preproteins.

Previous biochemical studies using the Toc159GM fragment from pea (*Pisum sativum*) chloroplasts (Kessler et al., 1994; Bolter et al., 1998; Chen et al., 2000) and in vivo expression studies using atToc159GM in *Arabidopsis* (Lee et al., 2003) suggested that the A-domain of this receptor was dispensable for function. Our results are of particular significance because they demonstrate that the A-domain plays a direct role in the ability of the receptor and TOC complexes to discriminate between different classes of preprotein transit peptides. The mechanism by which the A-domain influences transit peptide recognition remains to be determined. Previous studies with isolated at-Toc159 A-domain failed to demonstrate measurable binding of the domain to preproteins (Smith et al., 2004). Furthermore, direct binding and covalent cross-linking studies indicate that the G-domain of at-Toc159 contains a preprotein binding site that is specific for transit peptides (Perry and Keegstra, 1994; Ma et al., 1996; Kouranov and Schnell, 1997; Smith et al., 2004). These observations are consistent with in vivo expression studies in which the atToc159GM deletion mutant was shown to complement the *ppi2* mutant (Lee et al., 2003; Agne et al., 2009). Thus, atToc159GM appears to be functional as a transit peptide receptor, but it lacks the ability to discriminate between different types of transit peptides. Although we cannot eliminate the possibility that the A-domain transiently interacts with transit peptides, our results and those of previous studies suggest that the A-domain functions in modulating transit peptide binding by the GM portion of the receptor rather than directly mediating binding. We also envision that the A-domain could modulate binding by affecting the activity of

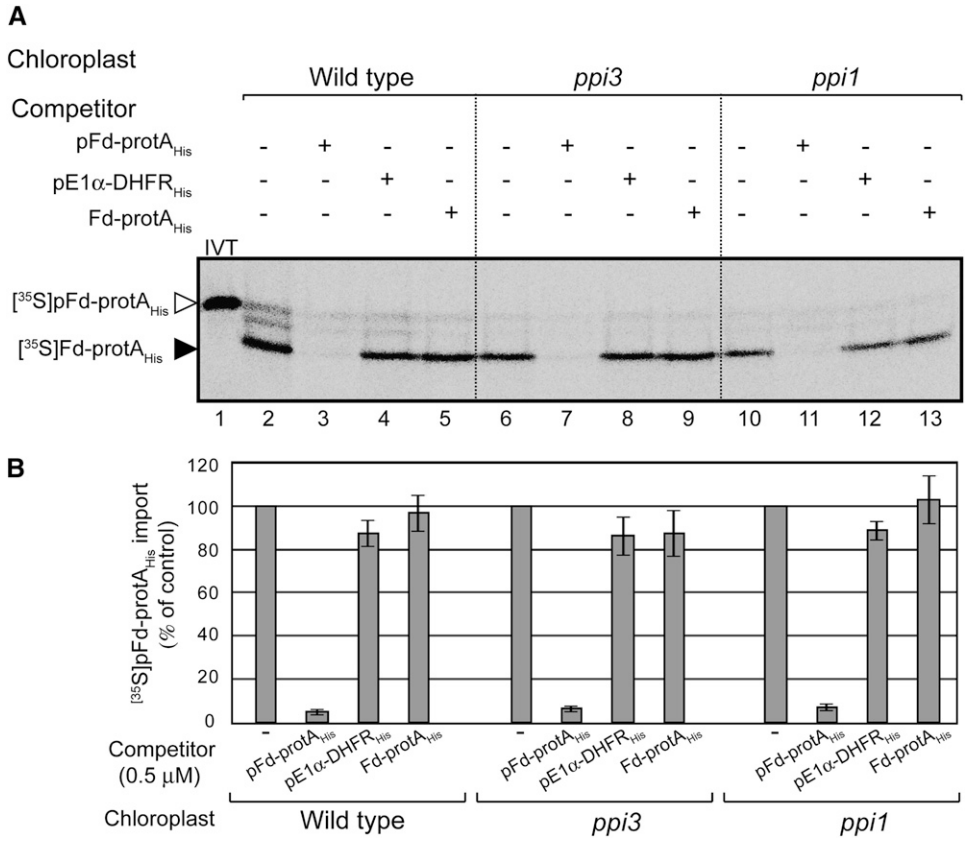

Figure 9. *ppi1* and *ppi3* Chloroplasts Exhibit a Similar Preprotein Import Selectivity as Wild-Type Chloroplasts.

(A) Isolated chloroplasts from wild-type, *ppi3*, and *ppi1* plants were incubated with in vitro–translated [^{35}S]pFd-protA$_{His}$ in the presence or absence of increasing concentrations of soluble pFd-protA$_{His}$, Fd-protA$_{His}$, or pE1α-DHFR$_{His}$ under import conditions. Lane 1 contains 10% of the in vitro translation product added to each reaction. [^{35}S]pFd-protA$_{His}$ import was analyzed by SDS-PAGE and phosphor imaging.

(B) Quantification of the data from replicate experiments including the representative data presented in **(A)**. Import is presented as the percentage of maximal import of [^{35}S]pFd-protA$_{His}$ in the absence of competitor. Error bars represent SE (n = 3) of the mean.

Toc33, the coreceptor that functions with Toc159 during preprotein recognition at the chloroplast surface (Becker et al., 2004; Oreb et al., 2008; Lee et al., 2009b; Rahim et al., 2009). This could be accomplished by a direct conformational effect on the receptor or by influencing the interactions between Toc159 and Toc33 within TOC complexes during the initial docking of the preprotein.

Our studies with the *ppi1* and *ppi3* mutants (Figure 9) indicated that the selectivity of TOC complexes is maintained in chloroplasts lacking Toc33 or Toc34. Previous preprotein binding and import studies demonstrated that Toc33 and Toc34 preferentially bind to different preproteins (Jelic et al., 2003; Kubis et al., 2003; Constan et al., 2004). Furthermore, Toc159 and Toc132 were shown to differentially assemble with Toc33 and Toc34 (Ivanova et al., 2004). Together, these data led to the hypothesis that these receptors also contribute to translocon selectivity. Our data suggest that the selectivity of Toc33 or Toc34 alone is insufficient to confer selectivity on TOC complexes in the absence of the Toc159 A-domain. This observation is consistent with genetic evidence indicating that the functions of Toc33 and Toc34 are overlapping in vivo. Alternatively, the selectivity of Toc33 and Toc34 could function at steps in import subsequent to the initial binding of preproteins at the chloroplast surface. In this way, Toc33 and Toc34 might contribute an additional level of selectivity in combination with Toc159 and Toc132.

The abilities of atToc159GM and atToc132GM to complement *ppi2* demonstrate that the GM fragments are capable of mediating preprotein recognition. Although we propose that atToc159GM and atToc132GM exhibit a degenerate specificity that allows recognition and import of a broader spectrum of preproteins, the inability of atToc132GM to fully complement *ppi2* (Figure 4) indicates that the GM domains of the two receptors are not fully interchangeable.

Our data provide additional evidence that the diverse members of the Toc159 family of import receptors play key roles in coordinating the activities of the protein import apparatus in conjunction with the expression of nucleus-encoded plastid proteins. The levels and profiles of several classes of nucleus-encoded plastid genes, particularly those associated with photosynthesis and chloroplast biogenesis, vary dramatically in

response to complex signals, including light (Jiao et al., 2007), diurnal/circadian rhythms (Kloppstech et al., 1991), and developmental events (Sawchuk et al., 2008). In the case of photosynthesis-related genes, such as SSU and LHCP, expression levels can increase from nearly undetectable levels to >25% of total nuclear transcript levels during plant greening and photomorphogenesis (Jiao et al., 2007). The current hypothesis proposes that diversification of TOC translocons to mediate the import of specific classes of proteins would accommodate the dramatic differences in import flux of these proteins by avoiding competition between proteins that are expressed at very different levels (i.e., highly expressed photosynthesis related proteins versus constitutively or lowly expressed proteins). This would ensure organelle homeostasis even as gene expression responds to environmental, developmental, and physiological changes.

Our results suggest that modifying the selectivity of a single pathway by deleting the A-domain of Toc159 or Toc132 has minimal consequences under ideal growth conditions. This is likely explained by the fact that the selectivity of the alternative pathways is maintained, thereby preventing competition for import between the different classes of preproteins. Future studies are aimed at providing additional evidence for the physiological role of the distinct TOC translocons by simultaneously eliminating the selectivity of multiple import pathways in transgenic plants.

METHODS

Plant Material and Growth Conditions

Experiments were performed with *Arabidopsis thaliana* ecotype Wassilewskija or Columbia-0 as indicated. Plants were grown at 22°C under long-day conditions (16 h of light/8 h of dark) on soil or on agar plates. For growth on plates, plants were grown on 0.8% (w/v) phytoagar containing half-strength Murashige and Skoog growth medium and 1% (w/v) sucrose.

DNA Constructs and Transgenic Plants

Plasmids encoding pFd-protA, Fd-protA, pFd-protA$_{His}$, Fd-protA$_{His}$, pE1α-protA$_{His}$, pE1α-DHFR$_{His}$, and DHFR$_{His}$ have been described previously (Ma et al., 1996; Ivanova et al., 2004; Smith et al., 2004). To prepare the Toc159 and Toc132 deletion and chimeric constructs, each domain of Toc132 and Toc159 was amplified individually using PCR with primers that included a *Bsi*WI site at the A/G domain junction and a *Cla*I site at the G/M domain junction and subsequently cloned into pCR2.1 TOPO (Invitrogen) (see Supplemental Figure 1 online). Oligonucleotides corresponding to a c-myc tag and stop codon were inserted into the 3′ end of DNA fragments corresponding to the M-domains with *Sal*I and *Sac*I sites in the pCR2.1 TOPO constructs. Both constructs were inserted into pET21d (Novagen) to generate pET21d:atToc159AGM$_{myc}$ and pET21d:atToc159A132GM$_{myc}$. Finally, these constructs were subcloned into pKMB-CaMV35S (Mylne and Botella, 1998) using *Xba*I and *Sac*I sites to generate pKMB-CaMV35S:atToc159AGM$_{myc}$ and pKMB- CaMV35S:atToc159A132GM$_{myc}$, respectively.

The plasmid encoding atToc159GM$_{His}$ (amino acids 728 to 1503) containing a C-terminal 6-histidine tag was described previously (Smith et al., 2002). atToc159GM lacking the 6-histidine tag was generated using PCR and inserted into the *Nco*I and *Hin*dIII sites of pET21d to generate pET21d:atToc159GM. The atToc159GM$_{His}$ fragment was subcloned into pSMB-CaMV35S (Mylne and Botella, 1998) to generate pSMB-CaMV35S:atToc159GM$_{His}$.

A DNA fragment corresponding to atToc132GM (encoding amino acids 472 to 1206) was generated using PCR with primers that introduced a 5′ *Nde*I and 3′ *Sac*I sites and inserted into the *Nde*I and *Sac*I sites of pET21a (Invitrogen) to generate pET21a:atToc132GM. The pET21a:atToc132GM plasmid was digested with *Xba*I and *Sac*I and subcloned into the *Xba*I and *Sac*I sites of pKMB-CaMV35S (Mylne and Botella, 1998) to generate pKMB-CaMV35S:atToc132GM. All of the constructs were confirmed by DNA sequencing.

pKMB-CaMV35S:atToc159AGM$_{myc}$, pSMB-CaMV35S:atToc159GM$_{His}$, pKMB- CaMV35S:atToc159A132GM$_{myc}$, and pKMB-CaMV35S:atToc132GM were introduced into transgenic plants using the *Agrobacterium tumefaciens*–mediated floral dip method (Clough and Bent, 1998). The presence of the transgenes and genetic background of transformed *ppi2* plants were confirmed by PCR of genomic DNA using transgene-specific primers as described previously (Wang et al., 2008). All transgenic plants were in the Wassilewskija ecotype with the exception of atToc159A132GM$_{myc}$/*ppi2*, which was in the Columbia-0 ecotype. Chlorophyll was quantified by extraction from total above ground tissue of transgenic and wild-type plants as described previously (Ivanova et al., 2004; Wang et al., 2008).

Arabidopsis Protein Extraction and Immunoblotting

Total protein extracts from 12-d-old plate-grown *Arabidopsis* seedlings were obtained by directly homogenizing leaves in SDS-PAGE sample buffer unless specified otherwise. To avoid proteolytic degradation, the extraction buffer was supplemented with 1000-fold diluted protease inhibitor cocktail for plant cell extracts (Sigma-Aldrich). Samples corresponding to equivalent amounts of total protein were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting with antisera to the indicated proteins. Immunoblotting was performed as described previously using chemiluminescence detection. The amounts of protein extracts used for each antibody were titrated to ensure a linear range of chemilumenescence signal (see Supplemental Figure 3 online), and the relative changes in levels of the immunoblotted proteins was determined using cytoplasmic actin as an internal control. Antisera to at-Toc159A, at-Toc75, at-Toc33, and SSU were described previously (Ivanova et al., 2004). The light-harvesting complex protein antibody was a generous gift of Kenneth Cline (University of Florida, Gainesville, FL) (Cline et al., 1989). The pyruvate dehydrogenase E1α antibody was a generous gift of Douglas Randall (University of Missouri, Columbia, MO) (Johnston et al., 1997). Anti-actin (A4700) was purchased from Sigma-Aldrich. The antiserum recognizing both at-Toc120M and at-Toc132M was raised against amino acids 751 to 1076 of at-Toc120. Chemiluminescence signals were compared using Multi Gauge V2.02 software.

In Vitro Translation and Protein Expression in *Escherichia coli*

All [^{35}S]methionine-labeled in vitro translation products were generated in a coupled transcription–translation system containing reticulocyte lysate according to the manufacturer's instructions (Promega) with the addition of RNase inhibitor. Where noted, the mixture was depleted of free nucleotides by gel filtration as described previously (Chen and Schnell, 1997). pFd-protA$_{His}$, Fd-protA$_{His}$, pE1α-DHFR$_{His}$, and DHFR$_{His}$ for competition experiments were expressed in *E. coli* BL21 (DE3) using 0.4 mM isopropyl β-D-1-thiogalactopyranoside for 3 h at 37°C. pE1α-protA$_{His}$ was expressed in *E. coli* Rosetta (DE3) for 3 h at room temperature. Hexahistidine-tagged proteins were purified using Ni-NTA resin under denaturing conditions as described previously (Ivanova et al., 2004; Smith

et al., 2004). pFd-protA and Fd-protA without a C-terminal hexahistidine tag were purified using IgG-Sepharose chromatography as described previously (Schnell and Blobel, 1993).

In Vitro Solid Phase Competition Assays

In vitro solid phase competition assays to test binding of transit peptide fusion proteins to the receptors were performed as described previously (Ivanova et al., 2004; Smith et al., 2004). Proteins bound to IgG-Sepharose or Ni-NTA resin were eluted with 0.2 M glycine, pH 2 to 3, or 0.5 M imidazole, respectively. All proteins were resolved using SDS-PAGE, and radiolabeled proteins were detected in dried gels using a phosphor imager (Fuji Fla-5000) and quantified with Multi Gauge V2.02 software. Binding was quantified as the percentage of total radiolabeled protein bound to the Ni-NTA resin. The variation between replicates is expressed as standard error. The comparisons of quantitative binding data are presented as percentages of maximal binding observed with each translation product.

Chloroplast Isolation and Protein Import Assays

Intact chloroplasts were isolated from 12- to 14-d-old plate-grown plants as described previously (Wang et al., 2008). Chloroplast import reactions were performed using [^{35}S] pFd-protA_{His} and chloroplasts corresponding to 20 μg of chlorophyll in the presence of 5 mM ATP in a total volume of 100 μL of import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 7.5, 25 mM KOAc, and 5 mM MgOAc) for 20 min at 26°C.

For preprotein binding reactions and early import intermediate formation, the in vitro–translated [^{35}S]pFd-protA_{His} was gel filtered to remove nucleotides. Chloroplasts were depleted of internal ATP by incubation for 30 min in the dark prior to initiating binding reactions. To uncouple ATP generation in chloroplasts, nigericin (final concentration, 400 nM) was added to the isolated chloroplasts (Theg et al., 1989; Akita and Inoue, 2009). Energy-depleted chloroplasts corresponding to 30 μg of chlorophyll and [^{35}S]pFd-protA_{His} were incubated in the absence or presence of 0.1 mM GTP and 0.05 mM ATP for 5 min at 26°C. Chloroplasts were recovered by isolation over a 35% Percoll cushion and washed once with import buffer.

Import, binding, or early import intermediate competition assays were performed using [^{35}S]pFd-protA_{His} and various concentrations of 8 M urea-denatured and purified pFd-protA_{His}, Fd-protA_{His}, or pE1α-$DHFR_{His}$. All samples including the control contained a final concentration of 0.12 M urea after dilution of the competitors. This concentration of urea previously has been shown not to affect preprotein binding or import (Schnell and Blobel, 1993; Inoue and Akita, 2008; Wang et al., 2008; Lee et al., 2009b). All samples were resolved by SDS-PAGE and analyzed by phosphor imaging (Fuji Fla-5000). Equivalent amounts of chloroplasts based on chlorophyll content were loaded in all lanes. Multi Gauge V2.02 software was used for quantification. Where necessary, radioactivity from in vitro translation products was normalized to reflect differing number of Met residues.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: TOC159 (At4g02510), TOC132 (At2g16640), TOC33 (At1g02280), TOC34 (At5g05000), pE1α (At1g01090), and pFd (P04669)

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Schematic Representation of the atTocAGM$_{myc}$ and atToc132AGM$_{myc}$ Constructs Used in This Study.

Supplemental Figure 2. Complementation of *ppi2* with the atToc159AGM$_{myc}$ or atToc159GM$_{myc}$ Constructs.

Supplemental Figure 3. Titration of Total Protein Extracts from Wild-Type Plants to Determine Linear Chemiluminescence Signals for Immunoblotting.

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