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Members of the Toc159 Import Receptor Family Represent Distinct Pathways for Protein Targeting to Plastids

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Plastids represent a diverse group of organelles that perform essential metabolic and signaling functions within all plant cells. The differentiation of specific plastid types relies on the import of selective sets of proteins from among the ~2500 nucleus-encoded plastid proteins. The Toc159 family of GTPases mediates the initial targeting of proteins to plastids. In *Arabidopsis thaliana*, the Toc159 family consists of four genes: *atTOC159*, *atTOC132*, *atTOC120*, and *atTOC90*. In vivo analysis of atToc159 function indicates that it is required specifically for the import of proteins necessary for chloroplast biogenesis. In this report, we demonstrate that atToc120 and atToc132 represent a structurally and functionally unique subclass of protein import receptors. Unlike atToc159, mutants lacking both atToc120 and atToc132 are inviable. Furthermore, atToc120 and atToc132 exhibit preprotein binding properties that are distinct from atToc159. These data indicate that the different members of the Toc159 family represent distinct pathways for protein targeting to plastids and are consistent with the hypothesis that separate pathways have evolved to ensure balanced import of essential proteins during plastid development.

INTRODUCTION

Plastids are a biochemically and morphologically diverse family of organelles that perform essential metabolic and signaling processes in all plant cells. The development and maintenance of specific plastid types in different tissues (e.g., chloroplasts in green tissues) relies on the import and assembly of several thousand different nucleus-encoded proteins (Soll and Tien, 1998; Chen and Schnell, 1999; Keegstra and Cline, 1999; Keegstra and Froehlich, 1999). This requires a remarkable flexibility in the import apparatus because the relative amounts and compositions of imported proteins vary considerably depending on the type and developmental stage of the particular plastid. This is exemplified by the ability of the import apparatus to accommodate the 1000-fold increase in the expression of key photosynthetic proteins while maintaining the import of essential housekeeping proteins during the transition from proplastids to chloroplasts in green tissues (Mache *et al.*, 1997).

The majority of proteins are targeted to plastids via an amino-terminal extension or transit peptide. Although there is no consensus for the length or sequence of transit peptides from different preproteins (von Heijne *et al.*, 1989; von Heijne and Nishikawa, 1991; Bruce, 2001), original studies of the mechanism of protein import led to the proposal that transit peptides are functionally interchangeable (Mishkind *et al.*, 1985; Van den Broeck *et al.*, 1985; de Boer *et al.*, 1991) and are recognized by a common, general protein import machinery (Soll and Tien, 1998). In chloroplasts, transit peptides are recognized by receptor components of the preprotein translocon at the outer envelope membrane of chloroplasts (Toc complex) (Hirsch *et al.*, 1994; Perry and Keegstra, 1994; Schnell *et al.*, 1994). Two families of Toc GTPases, Toc159 (Kessler *et al.*, 1994; Perry and Keegstra, 1994; Kouranov and Schnell, 1997) and Toc34 (Gutensohn *et al.*, 2000; Sveshnikova *et al.*, 2000; Jelic *et al.*, 2002), mediate preprotein recognition and initiate membrane transport via a GTPase cycle (Becker *et al.*, 2004; Smith *et al.*, 2004). Both proteins interact with transit peptides, but the basis for transit peptide recognition is not understood due in large part to the sequence diversity and disordered structures of transit peptides (von Heijne *et al.*, 1989; von Heijne and Nishikawa, 1991).

Recently, the concept of a general import machinery has been challenged by the discovery of families of genes in *Arabidopsis* and rice encoding related, but structurally distinct components of the protein import apparatus. Two differentially expressed genes of the Toc34 family are present in *Arabidopsis*, *atTOC33*, and *atTOC34* (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000), prompting the proposal that the proteins play specific roles during plastid development. atToc33 and atToc34 seem to exhibit some preferences for binding to transit peptides (Gutensohn *et al.*, 2000; Jelic *et al.*, 2003; Kubis *et al.*, 2003), suggesting that they might be involved in the import of distinct preproteins. However, ectopic expression of atToc34 can rescue the pale phenotype of an atToc33 null mutant (Jarvis *et al.*, 1998), *ppi1*, indicating that the GTPases play overlapping roles in plastid protein

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import. Therefore, atToc33 and atToc34 seem to function at distinct stages in development, but it remains to be demonstrated whether they represent functionally distinct import pathways.

The Toc159 family consists of four genes in *Arabidopsis*: *atTOC90*, *atTOC120*, *atTOC132*, and *atTOC159* (Bauer *et al.*, 2000, 2001; Jackson-Constan and Keegstra, 2001). A null mutant of *atTOC159*, *ppi2*, gives rise to a severe albino phenotype and is seedling lethal (Bauer *et al.*, 2000). Chloroplast biogenesis in *ppi2* is blocked at an early stage, resulting in the accumulation of undifferentiated proplastids in leaves. Remarkably, *ppi2* plants are viable when grown on sucrose, suggesting that atToc159 is specifically required for the import of chloroplast proteins but not other constitutively expressed plastid proteins involved in essential nonphotosynthetic processes. On the basis of these observations, we proposed that the *TOC159* gene family encodes a set of selective protein import receptors with distinct specificities (Bauer *et al.*, 2000). This hypothesis predicts that the other members of the Toc159 family are required for the import of different sets of preproteins and/or at different stages of plastid development.

In this report, we explore this hypothesis by examining the roles of atToc120 and atToc132 in plastid biogenesis. We provide evidence that atToc120 and atToc132 form separate Toc complexes from those containing atToc159. Although individual *Arabidopsis* T-DNA insertion mutations in *atTOC120* and *atTOC132* are phenotypically normal, double mutations in both atToc120 and atToc132 are lethal. Our studies indicate that atToc120 and atToc132 play essential roles distinct from atToc159 at critical stages in plastid biogenesis. These data demonstrate the existence of multiple, separate targeting pathways for the import of nucleus-encoded preproteins into plastids.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All experiments were performed with *A. thaliana* ecotype Wassilewskija. Plants were grown at 21°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% (wt/vol) phytagar (Invitrogen, Carlsbad, CA), containing 0.5 × Murashige and Skoog growth medium, 1% (wt/vol) sucrose and/or 50 μg/ml BASTA (glufosinate-ammonium; Sigma-Aldrich, St. Louis, MO) or 50 μg/ml kanamycin.

Identification of T-DNA Insertion Mutants

The *attoc120-1* and *attoc132-1* T-DNA insertion lines were identified by screening the new activation tagging BASTA (glufosinate)-resistant population (Weigel *et al.*, 2000) of the Arabidopsis Knockout Facility at the University of Wisconsin-Madison by a standard polymerase chain reaction (PCR) based strategy (Sussman *et al.*, 2000). The lines were identified using a primer specific for the left border of the T-DNA (JL-202), 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′, in combination with a gene specific primer to *atTOC132*, 5′-GGTGAGATCTGATGAAGTAAGGGATGA-3′ (forward) or *atTOC120*, 5′-TCTATTGTTCAGATTAGCACGAGCAAT-3′ (reverse). PCR products generated by positive hits in the screening reactions were sequenced to determine the location of the T-DNA insertions. After lines containing T-DNA within the coding region of *atTOC120* and *atTOC132* were isolated, plants homozygous for the T-DNA insertions were generated for further studies.

Reverse Transcription (RT)-PCR

Total RNA was extracted from total above ground tissue or leaf tissue of mutant and wild-type plants of the same age grown on soil by using the RNeasy plant mini kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized using random hexamer primers and 1 μg of total RNA with the SuperScript first-strand synthesis system (Invitrogen). The gene-specific primer pairs to cDNA for atToc120 were 5′-AGGGTCTAAAACCTGAATCTCT-3′ (forward) and 5′-TCACCTGAAACAATTTTAACAC-3′ (reverse), for atToc132 were 5′-GATTCGGTTTCTGCGGGGTTG-3′ (forward) and 5′-AACCACAAACTCAGTCCCATC-3′ (reverse), and for atToc75 were 5′-TCCATCACAAGTCCATATGTAAACTTACTC-3′ (forward) and 5′-AAGTCAGTACAGAATGGAGTATTGTTC-3′ (reverse).

Quantitative PCR analysis of mRNA was performed by competitive PCR by using the PCR MIMIC construction kit according to the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). AtToc159, atToc132, and atToc120 were amplified with the following primers pairs, respectively: forward, CACAGTCTTGCTCTAGCTAGCCGGTTC, and reverse, GCTGTACTTGTCGTTCGTCGCTTC); forward, GATTCGGTTTCTGCGGGGTTG, and reverse, TCATTGTCCATATTGCGTTTG CGG); and forward, AATGCTGGGAAGGAATTAGCGTACACTA, and reverse, TCAGTGTCCATATTGCATTTGCTCAGG.

Construction of pKMB-atToc132 for Overexpression and Plant Transformation

The coding region of *atTOC132* (GenBank accession no. At2g16640) was amplified by PCR by using atToc132.*Xho*I (forward primer), 5′-TTACTCGAGAAGAAAGCCATGGGAGATGGGACTGAG-3′ and atToc132.*Sac*I (reverse primer) 5′-TTCAGAGCTCATTGTCCATATTGCGTTTGCGGGT-3′ and subcloned into the *Sac*I and *Xho*I sites of the binary pKMB vector (Mylne and Botella, 1998). The pKMB vector was a kind gift from Dr. Jose Ramon Botella (University of Queensland, Brisbane, Australia).

The pKMB-atToc132 plasmid was transformed into *Agrobacterium tumefaciens* (GV3101) by electroporation and was introduced into *Arabidopsis* plants by using the floral-dip protocol (Clough and Bent, 1998). Transformed plants were selected on 50 μg/ml kanamycin (a marker linked to the *ppi2* mutation) and 50 μg/ml BASTA (a marker linked to the *atTOC132* transgene). BASTA-resistant plants were confirmed for T-DNA presence by PCR of genomic DNA of the transformants (McKinney *et al.*, 1995). The confirmed transformants were grown to maturity under long-day conditions. Plants from the T1 seeds of the transformed plants were assumed to be derived from independent T-DNA insertion events for purposes of phenotype characterization.

Chlorophyll Extraction and Quantification

Chlorophyll was extracted from total aboveground tissue of *attoc132-1* homozygous plants carrying a single intact *atTOC120* allele and wild-type plants of the designated age by grinding in 80% acetone followed by centrifugation. The chlorophyll concentrations were determined by measuring absorbance at 652 nm (Arnon, 1949). The chlorophyll content was defined as follows: chlorophyll content (micrograms per milligram of fresh tissue) = $(OD_{652}/36) \times Ve$ (microliters)/W (milligrams), with OD_{652} being the absorbance of chlorophyll at 652 nm, Ve being the volume of the extraction solution, and W being the fresh weight of tissue. For each measurement, four samples were prepared and the mean of their chlorophyll contents was determined.

Protein Extraction and Immunoblotting

Protein was extracted directly in boiling SDS-PAGE sample buffer from total aboveground tissue of *attoc132-1* homozygous plants carrying a single intact *atTOC120* allele and wild-type plants of the same designated age (Bauer *et al.*, 2000). Samples corresponding to equivalent amounts of fresh tissue were resolved in SDS-PAGE gels, transferred to nitrocellulose membranes, and subjected to immunoblotting with antisera to atTic110, atToc33, atToc75, atToc120, atToc132, and atToc159. Immunoblotting was performed as described previously (Ma *et al.*, 1996), by using chemiluminescence detection. Quantitative immunoblots were performed by loading serial dilutions of the original atToc120, atToc132 or atToc159 antigens as standards on the SDS-PAGE gels. Chemiluminescence signals from *Arabidopsis* extracts and the standards were compared using a Kodak Digital DC290 EDAK digital camera system (Eastman Kodak, Rochester, NY), and the levels of the receptor proteins were quantified against the standard curve. Only signals falling within the linear range of the chemiluminescence signal from the standards were used.

Antisera to atToc120 and atToc132 were raised against amino acids 1–343 and 1–431, respectively. The antigens were expressed as fusions to C-terminal hexahistidine tags in *Escherichia coli* and purified by chromatography on Ni^{2+}-NTA matrix. Affinity-purified antibodies were prepared by chromatography on antigen coupled to Sepharose.

Antisera to atToc33, atToc34 were prepared as described previously (Schnell *et al.*, 1991). Anti-actin (#A4700) was purchased from Sigma-Aldrich. Antisera to atTic110, atToc75, and atToc159 were a kind gift from Dr. Felix Kessler (Université de Neuchâtel, Neuchâtel, Switzerland).

Immunoprecipitation Reactions

Immunoprecipitation reactions using affinity-purified anti-atToc120, anti-atToc132, and anti-atToc159 were performed as described previously (Kouranov *et al.*, 1998) from purified chloroplasts isolated from evacuolated protoplasts. The membrane fraction was generated from chloroplasts corresponding to 3 mg of chlorophyll by lysis and separation into membrane and soluble fractions by differential centrifugation. The membrane pellet was dissolved in 50 mM tricine-KOH, pH 7.5, 2 mM EDTA, 10% glycerol, 150 mM NaCl (TEGS buffer), containing 2% (wt/vol) Triton X-100 for 10 min on ice and clarified by centrifugation at 100,000 *g* for 30 min. The membrane extract

was applied to anti-atToc132, anti-atToc120, and anti-atToc159 IgG-Sepharose in the sequences indicated in Figure 3. The Sepharose was washed with 10 volumes of TEGS buffer containing 1% (wt/vol) Triton X-100 and eluted with 0.2 M glycine, pH 2.2. The eluates and unbound fractions were analyzed by SDS-PAGE and immunoblotting.

Arabidopsis Chloroplast Preparation

Intact chloroplasts were isolated from 2- to 3-wk-old plate-grown plants as described previously (Smith *et al.*, 2002a,b), with the following modification. Protoplasts were subjected to evacuolation (Newell *et al.*, 1998) to prevent proteolytic degradation of the atToc120, atToc132, and atToc159 receptors. For this purpose, protoplasts resuspended in 0.4 M mannitol, 1 mM $CaCl_2$, and 20 mM MES-KOH, pH 6.6, were layered onto 0.5 M mannitol, 20 mM MES-KOH, pH 6.8, containing 30% (vol/vol) Percoll and evacuolated by centrifugation for 30 min at 100,000 × *g* at 21°C in a SW41-Ti swing-out rotor (Beckman Coulter, Fullerton, CA).

Electron Microscopy

Cotyledons and true leaves from *attoc132-1* homozygous plants carrying a single intact *atTOC120* allele and wild-type plants were fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4, under vacuum for 3 h and subsequently washed three times with 0.05 M sodium cacodylate, pH 7.4. Fixed samples were treated with 1% osmium tetroxide in 0.05 M sodium cacodylate, pH 7.4, for 2 h, and washed three times with 0.05 M sodium cacodylate, pH 7.4. The samples were dehydrated by the following treatments: incubation in 70% ethanol for 10 min, incubation in 100% ethanol for 10 min, and incubation twice in 100% propylene oxide for 15 min. EMbed812 embedding mixture (Electron Microscopic Sciences, Fort Washington, PA) was prepared according to the manufacturer's instructions. Dehydrated samples were infiltrated with one-third concentrated EMbed812 (in propylene oxide) for 3 h, two-thirds concentrated Embed812 overnight, and 100% Embed812 for 1.5 h before being embedded in EMbed812 by incubation at 60°C for 24 h. Sections (70 nm) of the samples were prepared and dried on 150-mesh copper grids and poststained with uranyl acetate and lead citrate as described previously (Smith and Croft, 1991). The grids were dried and observed using a Philips-Tecnai 12 transmission electron microscope.

DNA Constructs

Plasmids encoding atToc159, atToc132, atToc120 (Bauer *et al.*, 2000), atToc33G, atToc34G (Weibel *et al.*, 2003), pSSU-$DHFR_{His}$, pE1α-$DHFR_{His}$, $DHFR_{His}$ (Smith *et al.*, 2004), and pSSU-protA (Schnell and Blobel, 1993) have been described previously. The coding sequence for the transit peptide of the E1α subunit of plastid pyruvate dehydrogenase (pE1α) was amplified from *A. thaliana* cDNA and fused in-frame with the coding sequence of staphylococcal protein A containing a C-terminal hexahistidine tag to generate pET21a-pE1α-$protA_{His}$.

In Vitro Translation and Protein Expression in E. coli

[^{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, Madison, WI).

Bacterial expression of all constructs was performed in *E. coli* BL21(DE3) by using 0.4 mM isopropyl β-D-thiogalactoside for 3 h at 37°C. pE1α-$DHFR_{His}$, pSSU-$DHFR_{His}$ and $DHFR_{His}$ were purified from the insoluble fraction of *E. coli* lysates under denaturing conditions in the presence of 6 M urea by using Ni^{2+}-NTA chromatography (Novagen, Madison, WI). atToc33G_{His}, atToc34$_{His}$, and pE1α-$protA_{His}$ were purified from the soluble fraction of cleared *E. coli* lysates under native conditions by using Ni^{2+}-NTA chromatography (Novagen). pSSU-protA without a C-terminal hexahistidine tag was purified from *E. coli* lysate by using IgG-Sepharose chromatography as described previously (Schnell and Blobel, 1993).

Solid Phase Binding Assays

Direct interactions between atToc159, atToc132, or atToc120 and the GTPase domains of atToc33 or atToc34 were measured using solid phase binding assays as described previously (Smith *et al.*, 2002b; Wallas *et al.*, 2003). Briefly, increasing amounts of atToc33G_{His} or atToc34G_{His} were immobilized on ~7 μl of packed Ni^{2+}-NTA resin in the presence of 0.1 mM GTP and incubated with equivalent amounts (1–4 μl) of in vitro-translated [^{35}S]atToc159, [^{35}S]atToc132, or [^{35}S]atToc120. In vitro pull-down and competition assays to test binding of transit peptide fusion proteins to [^{35}S]atToc132 were performed as described previously (Smith *et al.*, 2004).

RESULTS

AtToc120, atToc132, and atToc159 Exhibit Distinct Expression Patterns

AtToc120, atToc132, and atToc159 share a common tripartite domain structure consisting of a carboxy-terminal membrane anchor domain (M-domain), a central GTPase domain (G-domain), and a highly acidic amino-terminal domain (A-domain) (Figure 1A). Although all three proteins exhibit significant sequence identity (Figure 1B), atToc132 and atToc120 are more closely related to each other than to atToc159. The G- and M-domains of atToc132 and atToc120 are ~90% identical to each other and only ~50% identical to atToc159. This observation suggests that atToc132 and atToc120 represent a subgroup of receptors within the Toc159 family (Hiltbrunner *et al.*, 2001a). A fourth member of the family, atToc90, is more distantly related to atToc120 and atToc132 because it possesses a truncated A-domain (Figure 1A) and exhibits significantly less sequence identity with these putative receptors than to atToc159 (Figure 1B).

To confirm the expression and localization of atToc120 and atToc132, we generated antisera to each protein by using their relatively divergent A-domains as the antigens. Immunoblotting of *E. coli*-expressed A-domains of each protein demonstrates that the antisera are highly specific for their corresponding antigens and exhibit no detectable cross-reactivity with the unrelated A-domains at any of the levels of antigen tested (Figure 1C). The antisera were used to probe extracts of chloroplast and soluble proteins from *Arabidopsis* plants. Figure 1D demonstrates that atToc120 and atToc132 are detected in the soluble fraction that contains cytosolic proteins (lane 2) and the chloroplast fraction (lane 3) in a distribution identical to that observed for atToc159. In contrast, the integral membrane Toc component atToc75 localizes exclusively to the chloroplast fraction (compare lanes 2 and 3). These data confirm that atToc120 and atToc132 are expressed and localized to chloroplasts. Furthermore, the immunoblots indicate that they also exist in soluble, cytosolic forms similar to atToc159 (Hiltbrunner *et al.*, 2001b).

The detection of all three putative receptors in isolated chloroplasts suggests that they have overlapping expression patterns. Immunoblots of protein extracts from the aboveground tissues of plants at different developmental stages indicate that atToc120, atToc132, and atToc159 exhibit very similar expression patterns in green tissues (Figure 2A). The receptors are expressed predominantly during the early stages of plant growth, peaking at ~18 d after germination. This pattern is similar to the expression of atToc75 (Figure 2A) and correlates with the high rates of chloroplast proliferation that occur during cell division and leaf expansion.

We previously reported that atToc159 mRNA was predominantly expressed in green tissues at more than fivefold higher levels than atToc120 and atToc132 (Bauer *et al.*, 2000). However, a subsequent study of the mRNA levels for the three receptors suggested that all three proteins were present at similar levels in all tissues examined (Yu and Li, 2001). To confirm the abundance and distribution of the three receptors in various tissues, we quantified the levels of their respective mRNAs by using quantitative RT-PCR and the levels of each protein in tissue extracts by using a series of known concentrations of the original antigens as standards for our immunoblots. As shown in Figure 2, all three receptors are detected at the mRNA (Figure 2B) and protein levels (Figure 2C) in all tissues examined. The relative levels of atToc132 and atToc120 proteins are similar in all tissues with the greatest difference observed in green plants where atToc132 protein is expressed at approximately twofold higher levels than atToc120 (Figure 2C). In contrast, the atToc159 protein exhibits significant variability in expression. Although it is detected at similar levels to the other receptors in etiolated and root tissue, it is expressed at four- to eightfold higher levels in green plants than atToc132 and

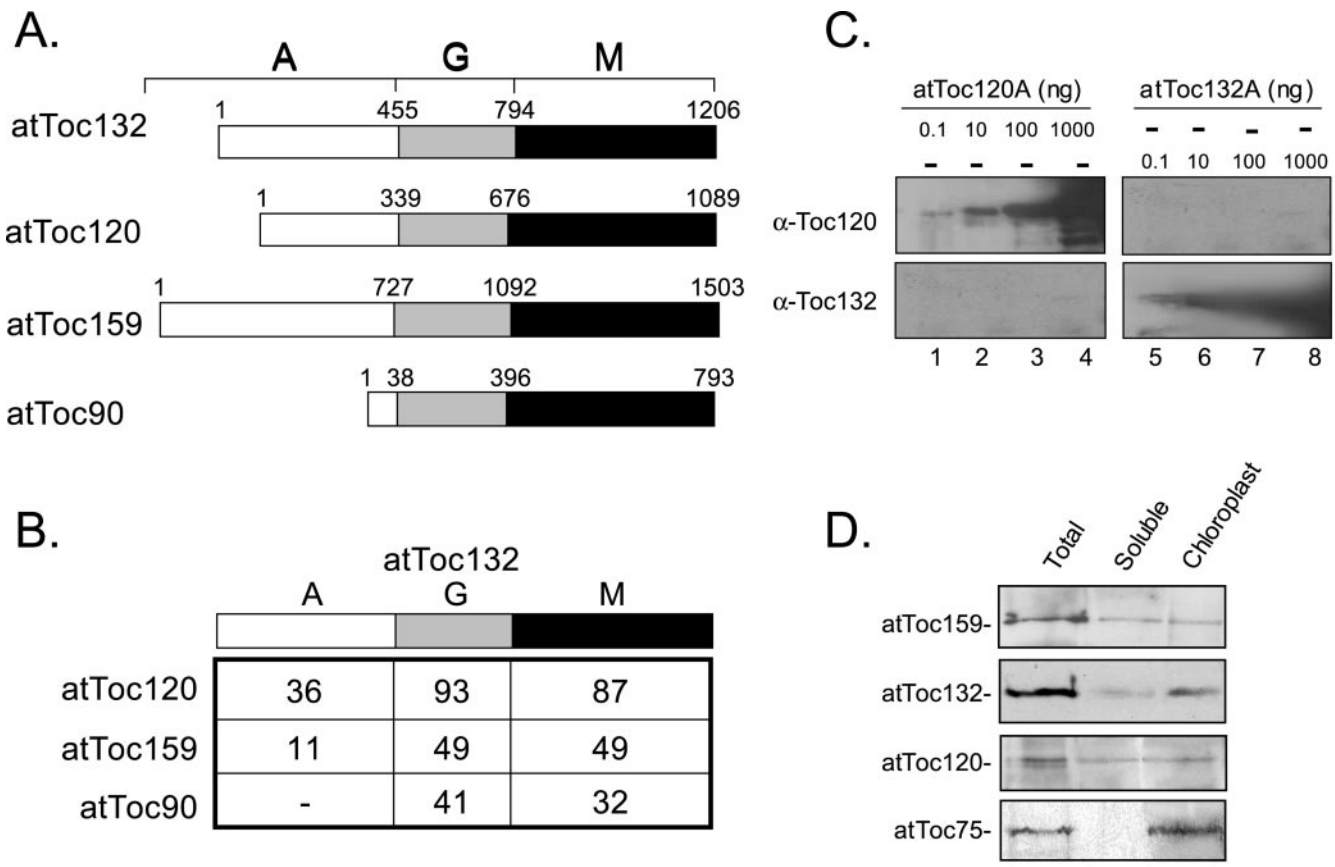


Figure 1. Structural comparison of the members of the Toc159 import receptor family. (A) Alignment of linear representations of the four members of the Toc159 family. The positions of the acidic domains (A, white boxes), the GTPase domains (G, gray boxes), and the membrane anchor domains (G, black boxes) are shown. The amino acid numbers above each protein indicate the borders of each domain. (B) Comparison of the amino acid sequence identity between the Toc159 family members. The table presents the percentage of identity between the domains of each protein relative to atToc132. (C) Immunoblot titration of anti-atToc120 (α-Toc120) and anti-Toc132 (α-Toc132) serum against increasing amounts of the A-domain antigens (atToc120A or atToc132A) that were used to generate each antiserum. (D) Immunoblots of *Arabidopsis* total protein extracts (Total), soluble protein extracts (Soluble), and chloroplast protein extracts (Chloroplast) with antiserum to the proteins indicated by the labels at the left of the panels. Each lane contains 50 μg of protein.

atToc120, respectively (Figure 2, B and C). These data confirm that the expression of atToc159 is highest in green tissues, consistent with its proposed central role in chloroplast biogenesis (Bauer *et al*., 2000). The expression of atToc120 and atToc132 is not strictly tissue dependent, making it unlikely that their functions are restricted only to specific plastid types.

AtToc120, atToc132, and atToc159 Form Distinct Toc Complexes at the Chloroplast Envelope

The presence of essential plastid metabolic functions in the *ppi2* mutant lacking atToc159 led us to propose that atToc120 and atToc132 mediate the import of a set of proteins required for constitutive plastid activities (Bauer *et al*., 2000). This observation and the overlapping expression patterns of the three receptors raises the question of whether they assemble into the same or distinct Toc complexes. To examine the association of the receptors with other Toc components, we subjected detergent extracts of total chloroplast membranes to sequential immunoaffinity chromatography on Sepharose coupled to anti-atToc132, anti-atToc120, and anti-atToc159 IgGs. The chromatography was performed in the sequence anti-atToc132, anti-atToc120, anti-atToc159 (Figure 3A), or anti-atToc120, anti-atToc132, anti-atToc159 (Figure 3B). As shown in Figure 3A, atToc132 antibodies quantitatively precipitate atToc132 (lane 2) and ~50% of atToc120 (compare lanes 2 and 3), indicating that these two Toc components partially associate in the outer membrane. Remarkably, atToc159 is not coimmunoprecipitated with the atToc132–atToc120 complex or the fraction of atToc120 that is not associated with atToc132 (Figure 3, lanes 2 and 3). Furthermore, no atToc132 or atToc120 was present in anti-atToc159 immunoprecipitates (Figure 3, lane 4). These data demonstrate that atToc120 and atToc132 are not components of atToc159 Toc complexes. The putative translocon channel component atToc75 is detected in anti-atToc120, anti-atToc132, and anti-atToc159 immunoprecipitates (Figure 3, lanes 2–4), confirming that all three receptors form authentic Toc complexes. AtToc75 is encoded by a single gene and therefore is predicted to be a common component of all Toc complexes (Keegstra and Cline, 1999; Bauer *et al*., 2001; Jackson-Constan and Keegstra, 2001). These observations and the high degree of structural similarity between atToc120 and atToc132 are consistent with the proposal that the two putative receptors form distinct Toc complexes from those containing atToc159. Reversing the order of the anti-atToc132 and anti-atToc120 immunoaffinity columns gave similar results (Figure 3B, lanes 6–8).

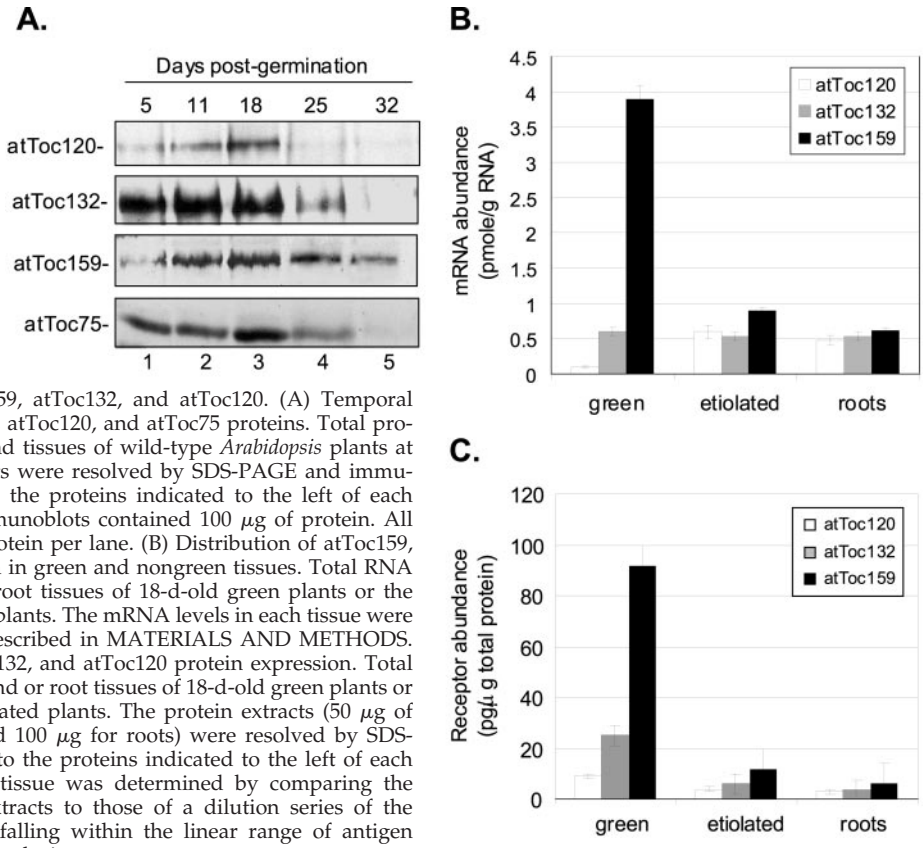


Figure 2. Expression profiles of atToc159, atToc132, and atToc120. (A) Temporal expression patterns of atToc159, atToc132, atToc120, and atToc75 proteins. Total proteins were extracted from the aboveground tissues of wild-type *Arabidopsis* plants at the ages indicated. Samples of the extracts were resolved by SDS-PAGE and immunoblotted with antisera corresponding to the proteins indicated to the left of each panel. Each sample for anti-atToc120 immunoblots contained 100 μg of protein. All other immunoblots contained 50 μg of protein per lane. (B) Distribution of atToc159, atToc132, and atToc120 mRNA expression in green and nongreen tissues. Total RNA was extracted from the aboveground or root tissues of 18-d-old green plants or the aboveground tissues of 18-d-old etiolated plants. The mRNA levels in each tissue were determined by quantitative RT-PCR as described in MATERIALS AND METHODS. (C) Tissue distribution of atToc159, atToc132, and atToc120 protein expression. Total protein was extracted from the aboveground or root tissues of 18-d-old green plants or the aboveground tissues of 18-d-old etiolated plants. The protein extracts (50 μg of protein for green and etiolated tissue and 100 μg for roots) were resolved by SDS-PAGE and immunoblotted with antisera to the proteins indicated to the left of each panel. The receptor abundance in each tissue was determined by comparing the chemiluminescence signal from tissue extracts to those of a dilution series of the antigen on the same gels. Only signals falling within the linear range of antigen detection were used for the quantitative analysis.

We recently demonstrated that targeting of soluble atToc159 to Toc complexes is mediated by interactions between its GTPase domain and the related GTPase domain of atToc33 (Bauer *et al.*, 2002; Smith *et al.*, 2002b; Wallas *et al.*, 2003). This raises the possibility that the distinct receptor complexes observed in Figure 3 result from preferential association of the atToc159 family members with atToc33 or atToc34, the two members of the *Arabidopsis* Toc34 family. Immunoblots of the immunoprecipitates in Figure 3A with an antiserum that reacts with atToc34 and atToc33 confirms that the two small GTPases exhibit differential association with atToc120/132 and atToc159 complexes. AtToc33 is preferentially associated with atToc159 complexes (Figure 3A, lane 4), whereas atToc120 or mixed atToc120/132 complexes are preferentially associated with atToc34 (Figure 3A, lanes 2 and 3). Interestingly, the association of the Toc GTPases does not seem to be exclusive because minor amounts of atToc33 and atToc34 are found in atToc120/132 and atToc159 immunoprecipitates, respectively. This observation is consistent with genetic analyses demonstrating that atToc34 can complement an atToc33 null mutant when overexpressed at high levels, indicating that these two Toc components have overlapping functions (Jarvis *et al.*, 1998).

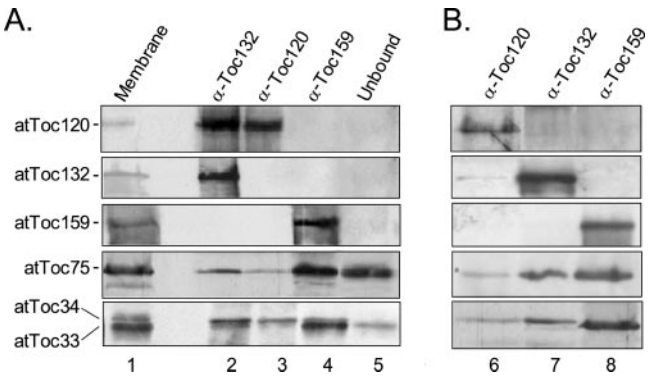


Figure 3. Association of atToc120, atToc132, and atToc159 in the chloroplast envelope membrane. Total chloroplast membranes corresponding to 3 mg of chlorophyll were dissolved in buffer containing Triton X-100 and subjected to sequential immunoaffinity chromatography on anti-atToc132 Sepharose (α-Toc132), anti-atToc120 Sepharose (α-Toc120), and anti-atToc159 Sepharose (α-Toc159) (A) or a similar series in which the order of the anti-atToc132 Sepharose and anti-atToc120 Sepharose was reversed (B). The eluates were resolved by SDS-PAGE and immunoblotted with antisera to the proteins indicated at the left of each panel. Lane 1 contains 2% of the membrane fraction loaded onto the column (Membrane). Lane 5 contains 2% of the unbound membrane fraction (Unbound) after sequential immunoaffinity chromatography.

To directly examine the possibility that members of the Toc159 family differentially bind to atToc33 and atToc34, we used a solid phase binding assay that was used previously to study the association of atToc159 and atToc33 (Smith *et al.*, 2002b; Wallas *et al.*, 2003). Recombinant forms of atToc33 (atToc33G) and atToc34 (atToc34G) lacking their transmembrane segments and containing carboxyl-terminal hexahistidine tags were expressed in *E. coli* and immobilized on a Ni^{2+}-NTA matrix. Samples of the immobilized atToc33G or atToc34G were incubated with equal amounts of in vitro-translated, ^{35}S-labeled atToc120, atToc132, or atToc159, and

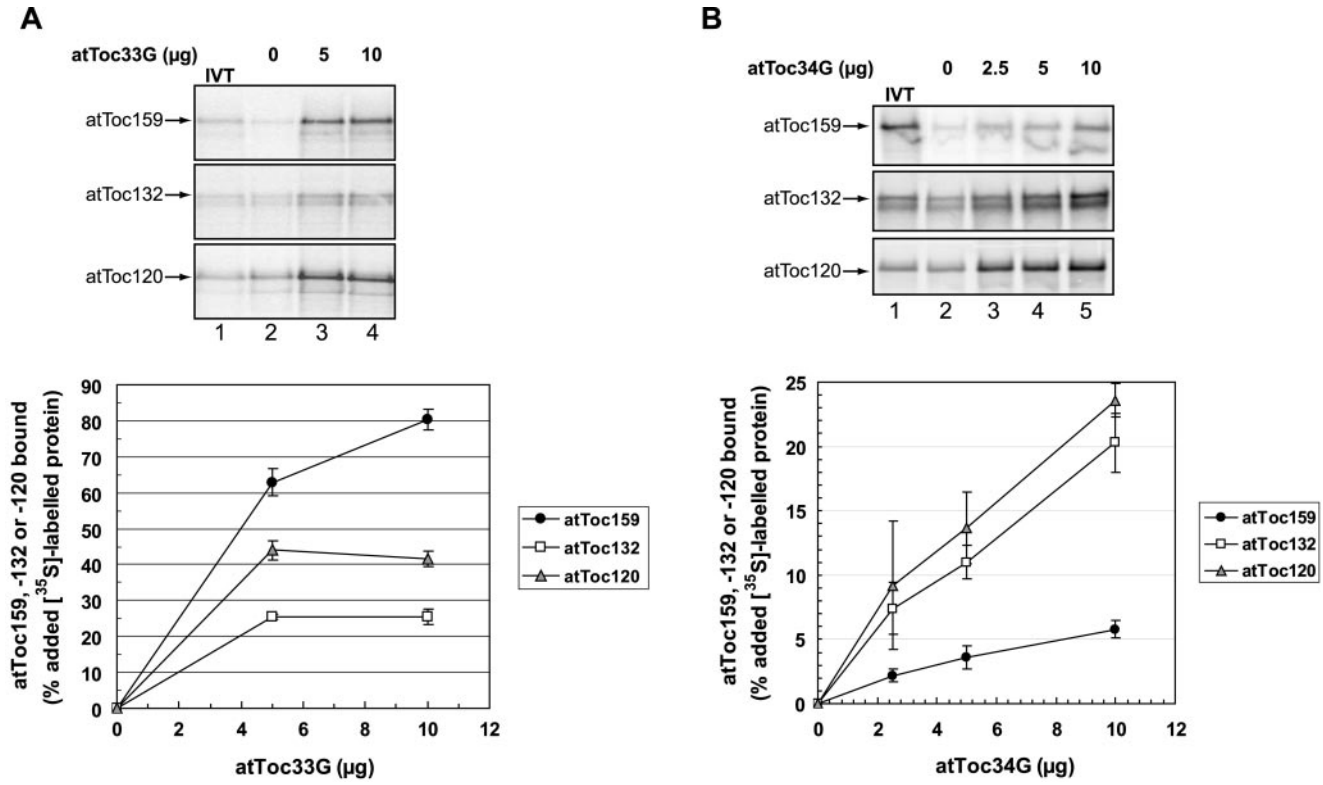


Figure 4. Direct binding of atToc120, atToc132, and atToc159 to the GTPase domains of atToc33 and atToc34. Equal amounts of in vitro translated [^{35}S]atToc120, [^{35}S]atToc132, or [^{35}S]atToc159 were incubated in the presence of GTP with the indicated amounts of immobilized hexahistidine-tagged atToc33G (A) or atToc34G (B). Bound proteins were eluted and separated by SDS-PAGE and analyzed using a PhosphorImager. Top panels present a representative experiment out of triplicates. Lane 1 in each panel contains 10% of the in vitro translation product added to each reaction. The graphs present quantitative analysis of the triplicate binding experiments with SE bars.

the amount of bound receptor was determined by SDS-PAGE and PhosphorImager analysis. Figure 4 demonstrates atToc159 binds atToc33G at levels that are twofold and threefold higher than atToc132 and atToc120, respectively (Figure 4A). In contrast, atToc120 and atToc132 bind to atToc34 at fourfold higher levels compared with atToc159 (Figure 4B). These data demonstrate that atToc159 preferentially associates with atToc33. Interestingly, atToc120 and atToc132 do not seem to exhibit a strong binding preference to either protein (compare the levels of binding in Figure 4, A and B). Therefore, the differential binding of the Toc159 receptor family members to atToc33/34 observed in the coimmunoprecipitation studies (Figure 3) could result simply from a high-affinity interaction between atToc159 and atToc33 that excludes the association of atToc34 with atToc159 or atToc33 with the other two receptors. This interpretation is consistent with the observation that small amounts of atToc33 coimmunoprecipitate with atToc120 and atToc132 in Toc complexes (Figure 3).

Identification of atTOC120 and atTOC132 T-DNA Insertion Mutants

The detection of structurally distinct Toc complexes supports the existence of different pathways for protein targeting to chloroplasts. As a first step in examining the possibility that atToc120, atToc132, and atToc159 play distinct functions, we identified *Arabidopsis* lines from the University of Wisconsin Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/Arabidopsis) containing T-DNA insertions in each gene by using a standard genomic DNA PCR strategy. A single T-DNA insertion line for each gene was identified. Sequencing of PCR products spanning the borders of the T-DNAs indicated that the insertions occurred at nucleotide 364 and 3294 of *atTOC120* and *atTOC132*, respectively (Figure 5A). PCR of genomic DNA with primers specific for each gene and the T-DNA confirm the gene insertions (Figure 5B). Both genes lack introns and therefore the insertions interrupt their coding regions. We designate the *atTOC120* and *atTOC132* insertion mutants *attoc120-1* and *attoc132-1*, respectively.

RT-PCR analyses of the expression of atToc120 and atToc132 in homozygous *attoc120-1* and *attoc132-1* plants demonstrate that the insertions disrupt the expression of the putative receptors (Figure 5C). In addition, atToc120 and atToc132 are not detected in immunoblots of total protein extracts from the lines, confirming the absence of functional protein (Figure 5D). The expression levels of atToc159, atToc75, and atToc33/34 in the T-DNA lines are similar to wild-type plants (Figure 5E), indicating that the inactivation of *atTOC120* or *atTOC132* does not lead to a general disruption of the expression of TOC genes.

attoc120-1 or *attoc132-1* homozygous plants failed to exhibit noticeable growth or developmental differences from wild-type plants of the same ecotype when grown under a variety of conditions (Figure 6A). Furthermore, the plants were not visibly or quantitatively paler than wild-type plants as has been observed for other plastid protein import mutants. Therefore, we conclude that the loss of either gene individually does not have a significant effect on plastid development. These results subsequently were confirmed by

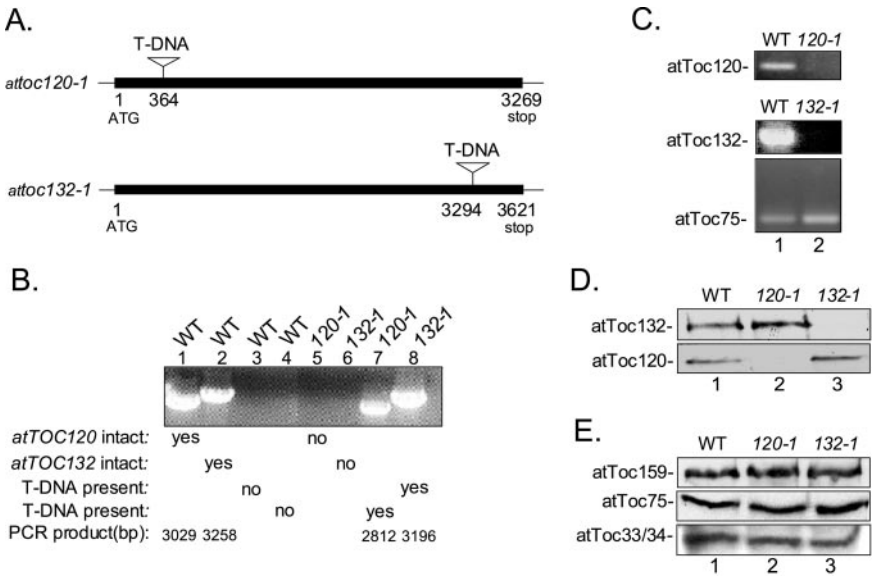


Figure 5. Identification of T-DNA insertion mutants in *atTOC120* and *atTOC132* genes. (A) Schematic representation of the position of the T-DNA insertions in the *atTOC120* and *atTOC132* genes. The positions of the insertions are indicated in nucleotides with position 1 corresponding to the first nucleotide of the start codon. (B) Confirmation of homozygous insertional mutations in the *atTOC120* and *atTOC132* genes. PCR analysis of genomic DNA from wild-type plants and plants homozygous for the T-DNA insertions in *atTOC120* (*attoc120-1*) and *atTOC132* (*attoc132-1*). PCR primers specific for each gene and the T-DNA insertion were used to confirm the presence of intact or T-DNA disrupted genes. (C) Confirmation of the lack of atToc132 and atToc120 mRNA expression in the *attoc120-1* and *attoc132-1* homozygous mutants, respectively. The presence or absence of mRNA for atToc120, atToc132 and atToc75 was assayed by RT-PCR using primers specific for each corresponding cDNA. (D) Confirmation of the lack of atToc132 and atToc120 protein expression in the homozygous *attoc120-1* and *attoc132-1* mutants, respectively. Total protein extracts from 18-d-old wild-type (50 μg of protein) and homozygous *attoc120-1* (100 μg of protein) and *attoc132-1* (50 μg of protein) plants were resolved by SDS-PAGE and immunoblotted with anti-atToc132 or anti-atToc120 sera. (E) Expression of Toc components in homozygous *attoc120-1* and *attoc132-1* mutants. Total protein extracts (50 μg of protein) from 18-d-old wild-type and homozygous *attoc120-1* and *attoc132-1* plants were resolved by SDS-PAGE and immunoblotted with antisera corresponding to the proteins indicated at the left of each panel.

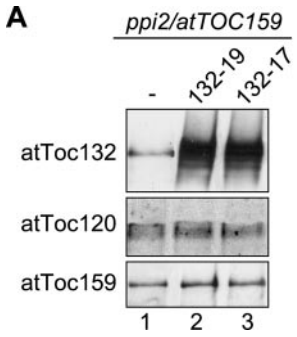
the analysis of two additional T-DNA insertion lines in the *atTOC120* and *atTOC132* genes that were identified in the Salk Institute Genome Analysis Laboratory (La Jolla, CA) (Alonso *et al.*, 2003) and Syngenta Arabidopsis Insertion Library (our unpublished data). These lines were not studied in further detail.

The high degree of similarity between the two genes and the association of atToc120 and atToc132 in the same Toc complexes suggested that the lack of phenotypes in the individual mutants might result from redundant or overlapping functions. To examine this possibility, we generated double mutants by crossing homozygous *attoc120-1* and *attoc132-1* plants. Two groups with distinct phenotypes were observed in the analysis of 263 BASTA-resistant F2 progeny corresponding to plants carrying at least one T-DNA insertion in each gene (Figure 6B). Eighty-eight percent of the population was phenotypically normal. PCR of genomic DNA from individual plants of this group indicated that they were of the *attoc120-1/attoc120-1/attoc132-1/atTOC132*, *attoc120-1/attoc120-1/atTOC132/atTOC132*, and *atTOC120/atTOC120/attoc132-1/attoc132-1* genotypes. The second group, corresponding to 12% of the plants, exhibited a striking variegated phenotype and a pronounced growth defect compared with wild-type plants (see below). These plants all were confirmed as the *attoc120-1/atTOC120/attoc132-1/attoc132-1* genotype (Figure 6B). We failed to identify homozygous double mutant plants (*attoc120-1/attoc120-1/attoc132-1/attoc132-1*) in the progeny of self-crossed *attoc120-1/atTOC120/attoc132-1/atTOC132* (Figure 6B) plants, regardless of whether the plants were grown on soil or agar plates supplemented with nutrient salts and sucrose. A low frequency of empty or aborted seeds was observed in the siliques of the

B

Genotype	Phenotype	
	Green	Variegated
120-1/120-1/132-1/132 *120-1/120-1/132/132* *120/120/132-1/132-1*	231	0
120-1/120/132-1/132-1	0	32
120-1/120-1/132-1/132-1	0	0

Figure 6. Phenotypic analysis of *attoc120-1* and *attoc132-1* single and double mutants. (A) Visual phenotypes of 18-d-old wild-type (WT), *attoc120-1*, and *attoc132-1* homozygous single mutant plants. (B) Genotypes and numbers of plants exhibiting the indicated phenotypes in the progeny of self-crossed *attoc120-1/atTOC120/attoc132-1/atTOC132* plants.



B

Genotype of parental plants	Phenotype of progeny	
	Green	Albino
ppi2/atTOC159	102 (71%)	41(29%)
ppi2/atTOC159:atTOC132-19	172 (71%)	71(29%)

Figure 7. Overexpression of atToc132 in *ppi2* mutant plants. (A) Immunoblots of protein extracts from heterozygous *ppi2* plants (*ppi2/atTOC159*) transformed with *atTOC132* under control of the 35S CaMV promoter (132-19 and 132-17) or untransformed control plants (−). The extracts (50 μg of protein) were resolved by SDS-PAGE and immunoblotted with antisera corresponding to the protein indicated at the left of the figure. (B) Segregation analysis of 18-d-old F1 progeny of self-crossed heterozygous *ppi2* plants (*ppi2/atTOC159*) or heterozygous *ppi2* plants transformed with *atTOC132* under control of the 35S CaMV promoter (*ppi2/atTOC159:atTOC132-19*). The numbers correspond to the absolute numbers and percentages (in parentheses) of progeny exhibiting the indicated phenotype when selected on kanamycin (*ppi2* marker) and BASTA (*atTOC132-19* marker).

self-crossed plants (our unpublished data). In addition, a small proportion of seeds that did form failed to germinate or develop beyond the emergence of cotyledons. Although we were unable to confirm the genotypes of the aborted and inviable seeds, the frequency of their occurrence corresponded to the expected frequency of homozygous knockouts. Therefore, we conclude that at least one intact allele of either *atTOC120* or *atTOC132* is required for viability even in the background of two intact *atTOC159* genes.

To confirm that either atToc120 or atToc132 was required for viability, we genotyped the progeny from self-crossed plants of the *attoc120-1/atTOC120/attoc132-1/attoc132-1* or *attoc120- 1/attoc120-1/attoc132-1/atTOC132* genotypes. All 122 progeny of the *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants and 40 progeny of the *attoc120-1/attoc120-1/attoc132-1/atTOC132* plants carried at least one intact *atTOC120* or *atTOC132* allele (our unpublished data). On the basis of these data, we propose that atToc120 and atToc132 form a unique receptor subgroup that is required for plastid biogenesis in general and therefore is essential for plant viability.

Overexpression of atToc132 Fails to Rescue the ppi2 *Phenotype*

The combined results from the analyses of the *attoc120-1/attoc132-1* double mutant plants and the detection of discrete Toc complexes suggest that the atToc120 and atToc132 receptor subgroup is functionally distinct from atToc159. To directly assess the functional overlap between these receptors and eliminate the possibility that expression differences are responsible for the *attoc120-1/attoc132-1* double mutant phenotype, we attempted to rescue the albino phenotype of the atToc159 null mutant, *ppi2*, by overexpressing the *atTOC132* gene. We selected *atTOC132* for the complementation studies because *attoc120-1/attoc132-1* double mutants with the *attoc120-1/attoc120-1/attoc132-1/atTOC132* genotype were phenotypically normal (Figure 6B), indicating that atToc132 alone was sufficient to fulfill the function of both atToc132 and atToc120.

Ppi2 heterozygous plants were transformed with a binary vector carrying the coding region for *atTOC132* under control of the constitutive 35S cauliflower mosaic virus promoter. Transformed plants were selected on kanamycin (a marker linked to the *ppi2* mutation) and BASTA (a marker linked to the *atTOC132* transgene). We focused on two *ppi2* heterozygous lines that were homozygous for the *atTOC132* transgene, 132-19 and 132-17 (Figure 7A). These plants exhibited an approximately fivefold overexpression of atToc132 as determined by semiquantitative immunoblotting (Figure 7A). These levels of atToc132 expression are comparable with those observed for atToc159 in the green tissues of wild-type plants (Figure 2C) and therefore should be sufficient to complement *ppi2* if atToc132 and atToc159 are functionally redundant. The lines were self-crossed to generate *ppi2* homozygous plants expressing the high levels of atToc132. The frequency of homozygous *ppi2* plants exhibiting the characteristic albino phenotype in the progeny of the transformants was indistinguishable from that observed in the progeny of self-crossed heterozygous *ppi2* plants that were not overexpressing atToc132 (Figure 7B). Furthermore, all green plants genotyped from the crosses were confirmed to be heterozygous for *ppi2* (Figure 7B). Therefore, we conclude that overexpression of atToc132 cannot rescue the *ppi2* phenotype. These results provide additional evidence that the receptor subgroups represented by atToc159 and atToc120/132 form structurally and functionally distinct Toc complexes for protein targeting into chloroplasts.

Underexpression of the atToc120/atToc132 Receptor Subgroup Affects Plastid Biogenesis

We invariably observed a variegated phenotype in *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants (Figure 8A). These plants presumably exhibit a phenotype due to the overall underexpression of the atToc120/132 receptors as a result of possessing a single intact *atTOC120* allele. The level of atToc120 expression from this single gene apparently represents the minimal level of atToc120/132 receptor required for plant viability. This conclusion is consistent with the fact that atToc120 is expressed at only one-half the level of atToc132 in green tissues.

To investigate the nature of the defect resulting from receptor underexpression, we examined these plants in more detail. The plants exhibited uniformly pale cotyledons and variegated true leaves throughout their life cycle (Figure 8A). The phenotype resulted in retarded growth on soil with mature plants reaching the flowering stage 6–8 d later than wild-type plants and achieving only 20–25% of the fresh weight of wild-type plants at maturity. The defects could not be reversed by supplementing the plants with sucrose, although the variegation was less pronounced (our unpublished data). Therefore, the growth defects are not restricted to a compromise of photosynthetic activity. Although the variegation persisted throughout the life cycle of these plants, the pale sectors became greener as the plants matured. This is illustrated by the observation that overall chlorophyll content increased from ~30% of the levels of wild-type plants at the cotyledon stage of development to ~75% of the levels of wild-type plants at maturity (Figure 8B). These observations suggest that a deficiency in atToc132

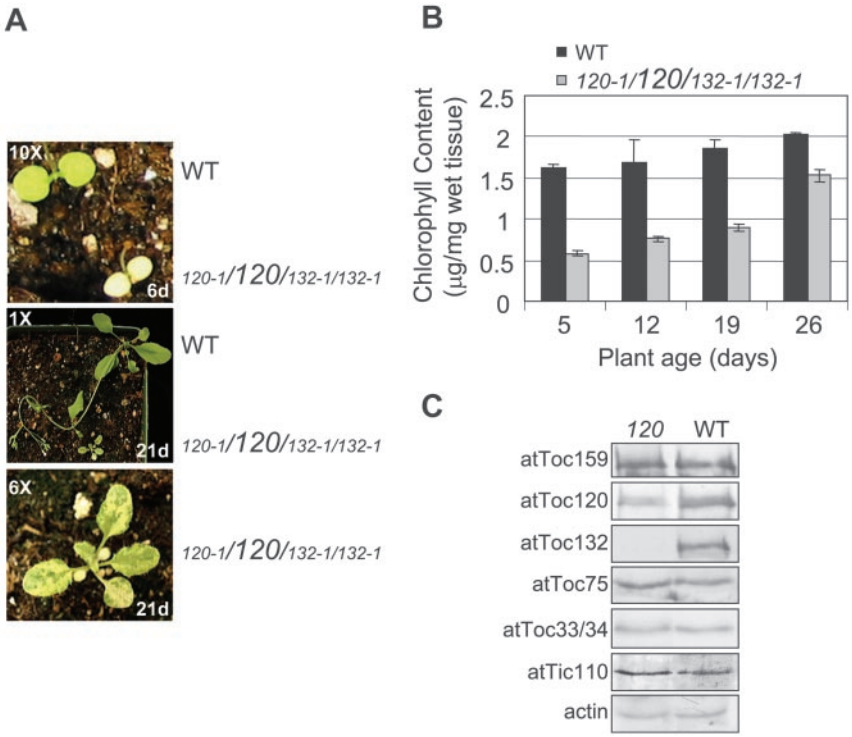


Figure 8. Phenotype of *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants. (A) Visual phenotypes of 6- (6d) and 21 (21d)-d-old wild-type plants (WT) and *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants (*120-1/120/132-1/132-1*). The top and bottom panels correspond to a 6× magnification of the mutant plant shown in the middle panel. (B) Chlorophyll content of wild-type (WT) and *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants. Chlorophyll content was determined from the aboveground tissues of plants at the ages indicated in MATERIALS AND METHODS. (C) Immunoblots of total plant extracts from *attoc120-1/atTOC120/attoc132-1/attoc132-1* (120) and wild-type (WT) plants with antisera to the representative Toc and Tic components indicated at the left of the figure. Immunodetection of actin was used as a loading control.

and atToc120 has a significant effect on plastid biogenesis at all stages of development, but it is particularly severe in young, rapidly growing plants. Furthermore, the fact that the defects could not be reversed by supplementation with sucrose indicates that the functions of atToc120/132 are not restricted to biogenesis of the photosynthetic apparatus as is observed for atToc159. No significant changes in the levels of representative Toc and Tic components were apparent (Figure 8C). These observations support the conclusion that the reduction in atToc120/132 disrupts plastid biogenesis, resulting in defects in chloroplast development.

The defect in chloroplast development was apparent when the morphology of plastids from the cotyledons and true leaves of the *attoc120-1/atTOC120/attoc132-1/attoc132-1* and wild-type plants were examined by transmission electron microscopy. Chloroplasts from both cotyledons (Figure 9A) and the pale sectors of true leaves (Figure 9B) of the mutant exhibited underdeveloped thylakoid membranes with a pronounced decrease in granal stacks. Although the numbers of plastids per cell did not seem to be significantly different, the overall size of the plastids was uniformly smaller in mutant than in wild-type plants. The defects in chloroplast morphology are consistent with a role for atToc120/132 in plastid protein import and suggest that the defects in plant growth and development are due to disruption of the normal progress of plastid biogenesis.

Interestingly, several heterozygous *ppi2* plants transformed with the 35S-*atTOC132* construct exhibited variegated leaves and retarded growth similar to the *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants (Figure 10A, lines

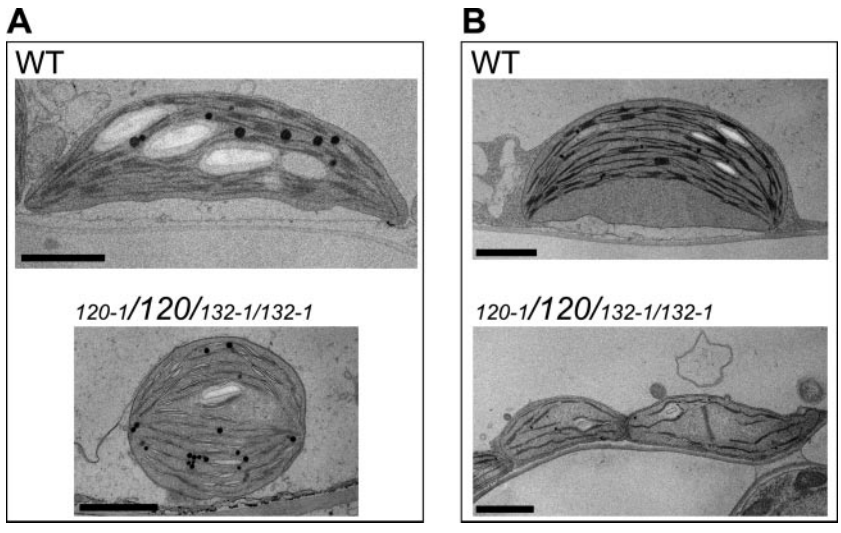


Figure 9. Ultrastructure of plastids from *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants. Transmission electron micrographs of cotyledons (A) and true leaves (B) from wild-type (WT) and *attoc120-1/atTOC120/attoc132-1/attoc132-1* (*120-1/120/132-1/132-1*) plants. The micrographs of *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants were derived from the pale sectors of variegated leaves. Bar, 1 μm.

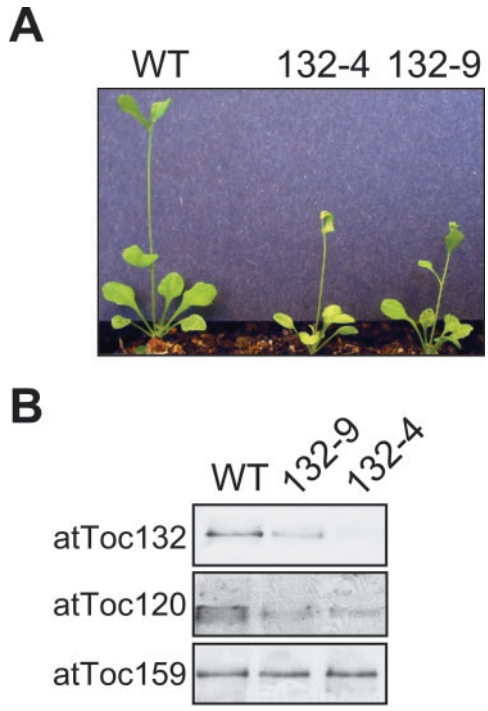


Figure 10. Phenotype of plants underexpressing atToc132 and atToc120. (A) Visual phenotype of 21-d-old wild-type plants (WT) and plants transformed with *atTOC132* that are exhibiting cosuppression of *atTOC132* and *atTOC120* expression (132-4 and 132-9). (B) Immunoblot analysis of the expression of atToc132, atToc120, and atToc159 in the wild-type (WT) and cosuppressed (132-4 and 132-9) plants shown in A.

132-4 and 132-9). Examination of the expression of atToc132 and atToc120 in these two lines by immunoblotting indicated that the expression of both proteins was significantly reduced (Figure 10B). The reduction presumably resulted from silencing of the *atTOC132* transgene leading to cosuppression of the native *atTOC120* and *atTOC132* genes. Similar cosuppression phenomena have been reported for other genes with high degrees of sequence identity (Ledger *et al*., 2001; Li and Nam, 2002). The level of atToc159 in these plants is unaffected (Figure 10B), indicating that the sequence divergence between the *atTOC159* and *atTOC132* genes is sufficient to avoid cosuppression of this gene. This observation corroborates the phenotype of the *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants and provides additional evidence that atToc120/132 perform functions in plastid biogenesis that are distinct from atToc159.

atToc132 Binds Selectively to Different Plastid Preproteins

Our hypothesis that atToc120/132 and atToc159 define distinct targeting pathways predicts that they exhibit preferences for binding to different preproteins. Consistent with this model, we recently have shown that atToc159 binds selectively to the transit peptides of representative chloroplast-specific preproteins (e.g., presmall subunit of Rubisco [pSSU]) relative to representative constitutively expressed plastid preproteins (e.g., prepyruvate dehydrogenase E1α subunit [pE1α]) (Smith *et al*., 2004). These data predict that pE1α would use an alternate receptor system, such as atToc120/132. To test this possibility, we examined the ability of atToc132 to bind pSSU and pE1α transit peptides in a standard pull-down assay. In vitro-translated, ^{35}S-labeled receptor was incubated with immobilized fusion proteins corresponding to the pSSU and pE1α transit peptides fused to hexahistidine-tagged dihyrofolate reductase ($DHFR_{His}$). As shown in Figure 11A, atToc132 binds in a dose-dependent manner to pE1α-$DHFR_{His}$ with maximum binding (45% of added receptor) observed at 200 pmol of preprotein. AtToc132 binding to the $DHFR_{His}$ control lacking a transit peptide was ~10% of added receptor at the maximum level of fusion protein tested (Figure 11A), indicating that atToc132 binding was specific for the transit peptide. Binding to pSSU-$DHFR_{His}$ exhibited a similar pattern to the $DHFR_{His}$ control, indicating a very low affinity of the receptor for the pSSU transit peptide (Figure 11A).

To confirm the selectivity of atToc132, we examined the ability of soluble fusion proteins to compete with receptor binding to the immobilized pE1α fusion. The competitors were pE1α-$DHFR_{His}$ and pSSU fused to staphylococcal protein A (pSSU-protA). Figure 11B demonstrates that pE1α-$DHFR_{His}$ effectively competed with itself for binding to atToc132, reducing binding to 30% of control levels at the highest concentration tested. In contrast, the pSSU-protA fusion did not compete for binding at comparable concentrations. These data confirm that atToc132 exhibits a preference for binding to the pE1α transit peptide and are consistent with the hypothesis that atToc120/132 mediate the import of essential constitutive plastid proteins.

DISCUSSION

Most nucleus-encoded plastid proteins are targeted to the organelle by their intrinsic transit peptides. Original studies on transit peptide function suggested that they are functionally interchangeable despite the lack of a consensus structure (Mishkind *et al*., 1985; Van den Broeck *et al*., 1985; de Boer *et al*., 1991). This led to the proposal that all preproteins followed a single targeting pathway that converged on a single general import machinery (Soll and Tien, 1998). In this report, we demonstrate that members of the Toc159 family in *Arabidopsis*, atToc120, atToc132, and atToc159, represent structurally and functionally distinct preprotein import receptors. These observations challenge the existence of a "general" import machinery by confirming the existence of separate pathways for protein targeting to plastids. Our conclusions are based on several corroborating observations. First, we detected complexes containing atToc120 and atToc132 as well as atToc120 or atToc132 alone, but neither receptor was found in association with atToc159 (Figure 3). In addition, atToc120/132 and atToc159 assemble differentially with atToc34 and atToc33, respectively (Figure 4). Therefore, atToc120 and atToc132 form structurally distinct Toc translocons from those containing atToc159.

Second, the inability to recover *attoc120-1* and *attoc132-1* double null mutants demonstrates that atToc120 and atToc132 constitute an import receptor subgroup that is required for plant viability (Figure 6). This phenotype is distinct from the conditional lethal phenotype of the atToc159 null mutant, *ppi2*, which can be partially rescued by growth on sucrose (Bauer *et al*., 2000). The essential roles of atToc120/132 are underscored by the observation that severe underexpression of the two receptors in plants with reduced gene dosage (Figure 8) or gene silencing (Figure 10) results in abnormal plastid development and severe growth and developmental defects. These phenotypes are observed even though the expression of atToc159 is normal. Although distinct from atToc159, the functions of atToc132 and atToc120 seem to overlap to the degree that their activities are inter-

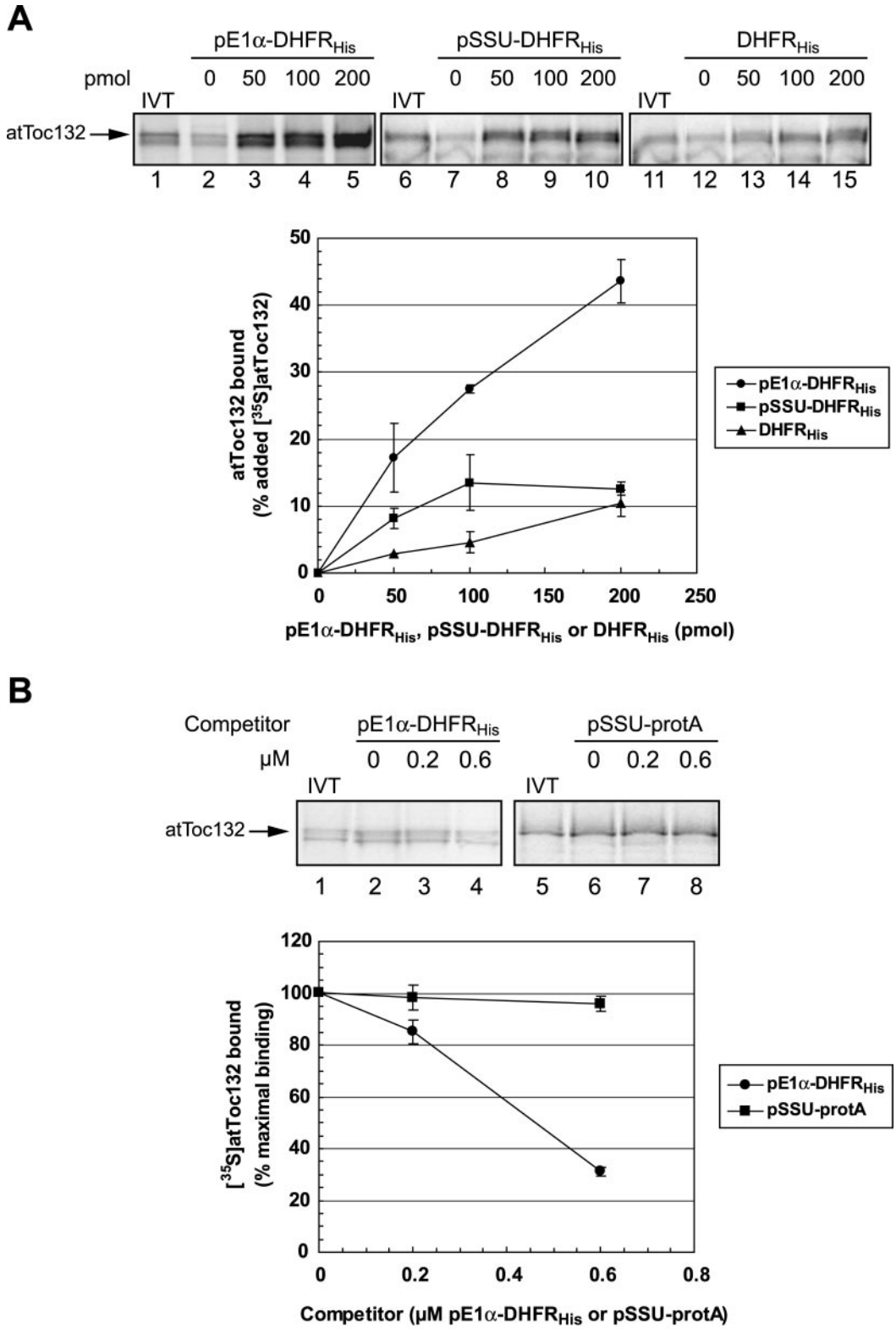


Figure 11. atToc132 selectively binds to the transit peptide of a representative constitutively expressed plastid preprotein. (A) In vitro-translated [^{35}S]atToc132 was incubated with increasing amounts of pE1α-DHFR$_{His}$, pSSU-DHFR$_{His}$, or DHFR$_{His}$ that had been immobilized on Ni^{2+}-NTA resin. Bound proteins were eluted, separated using SDS-PAGE, and analyzed using a PhosphorImager. Lanes 1, 6, and 11 contain 10% of the [^{35}S]atToc132 in vitro translation product (IVT) added to each reaction. Lanes 2, 7, and 12 contain the [^{35}S]atToc132 that bound to the Ni^{2+}-NTA resin in the absence of immobilized protein. (B) [^{35}S]atToc132 was incubated with 50 pmol of IgG-Sepharose–immobilized pE1α-protA$_{His}$ (top left) or 50 pmol of Ni^{2+}-NTA-immobilized pE1α-DHFR$_{His}$ (top right) in the absence or presence of increasing concentrations of pE1α-DHFR$_{His}$ or pSSU-protA, respectively. Bound proteins were eluted, separated by SDS-PAGE, and analyzed using a PhosphorImager. Lanes 1 and 5 contain 10% of the [^{35}S]atToc132 IVT added to each reaction. Binding is presented as the percentage of maximal [^{35}S]atToc132 binding.

changeable in vivo. This conclusion is based on the fact that *attoc120-1* and *attoc132-1* single mutants have no detectable phenotype under normal growth conditions (Figure 6). This is consistent with the high degree of sequence identity between these two receptors relative to atToc159 and atToc90 (Figure 1). The observation that they form mixed or single receptor complexes in the envelope (Figure 3) also is consistent with overlapping functions.

Third, ectopic overexpression of atToc132 does not rescue the *ppi2* phenotype (Figure 7), providing additional evidence that atToc159 and atToc120/132 represent functionally distinct receptor subgroups. Although the inability to complement *ppi2* with atToc132 does not completely eliminate the possibility that the different receptor subgroups might have limited overlapping specificities for some preproteins, it does demonstrate that each receptor class is required for the targeting of certain sets of essential plastid preproteins.

Finally, we demonstrate that atToc132 selectively binds the transit peptide of a constitutively expressed plastid preprotein, pE1α, relative to a chloroplast-specific preprotein, pSSU (Figure 11). This is opposite of the results obtained when the binding of atToc159 to preproteins was examined (Smith *et al.*, 2004). The previous study demonstrated a distinct binding preference of atToc159 for the transit peptides of several photosynthetic proteins. These data are consistent with our hypothesis that the specificities of the different receptors define distinct targeting pathways.

The possibility that different preproteins might engage distinct import components was first raised by the observation that preproteins were differentially imported into chloroplasts and leucoplasts (Wan *et al.*, 1996). This led Jarvis *et al.* (1998) to propose that atToc33 and atToc34 might represent distinct targeting pathways for plastid preproteins. Our genetic and biochemical data provide direct in vivo and in vitro evidence for the existence of structurally and functionally distinct preprotein translocons at the outer envelope membrane. It remains to be determined whether the targeting pathways represent distinct pathways for each step in the import process. Although the Toc complexes seem to be distinct, it is possible that these translocons will associate with the same Tic components at the inner envelope membrane. The convergence of the import pathways at this point would account for the observation that a variety of different preproteins can effectively compete with one another for import (Row and Gray, 2001).

Although the complete substrate specificities of the pathways remain to be established, the analyses of *ppi2* suggest that atToc159 is required for the import of light-induced proteins that are expressed predominantly in chloroplasts (Bauer *et al.*, 2000; Smith *et al.*, 2004). The fact that *ppi2* is partially rescued by supplementation with sucrose (Bauer *et al.*, 2000) and the observation that atToc159 expression is highest in green tissues (Figure 2) are consistent with a particular role for this receptor in photomorphogenesis and chloroplast biogenesis. We attribute this unique role to a selectivity of the receptor for binding a set of photosynthetic preproteins. In contrast, the lethality of *attoc120-1/attoc132-1* double mutants under all growth conditions tested (Figure 6) and the constitutive expression of atToc120 and atToc132 in green and nongreen tissues (Figure 2) suggest that they are required for the import of proteins that provide essential functions in multiple plastid types. This interpretation is consistent with the preference of atToc132 for binding pE1α versus pSSU. The pale sectors in variegated plants deficient in atToc120 and atToc132 likely result from a defect in the import of factors required for plastid function/development in general. This possibility provides an explanation for the variegated phenotype of the *attoc120-1/atTOC120/attoc132-1/attoc132-1* plants. The reduced capacity of the atToc120/132 targeting pathway(s) in these plants could limit the ability of key plastid proteins to reach the necessary threshold levels required for proper development. If the import of a limiting factor(s) happens to exceed the threshold, plastid biogenesis will not be disrupted and chloroplasts will develop normally as indicated by the green sectors. Import within cells represented by the pale sectors presumably did not reach the threshold, thereby derailing the developmental process.

On the basis of our results and those of previous studies, we propose that the combined activities of the Toc159 and Toc34 family members contribute to the formation of distinct Toc complexes with selectivity for classes of preproteins. This proposal is consistent with the observation that atToc120/132 and atToc159 associate differentially with atToc34 and atToc33 (Figures 3 and 4). The ability of the Toc159 and Toc34 GTPases to assemble in different combinations would provide a mechanism by which plant cells could generate multiple receptor complexes with distinct affinities for different preproteins. The distinct pathways generated by these combinations of receptors would allow for the simultaneous import of preproteins with very different expression levels, thereby preventing competition for import between different preprotein classes. The individual Toc complexes are unlikely to represent entirely exclusive targeting pathways, but rather translocons with limited overlap in preprotein specificity. This model is consistent with the fact that minor amounts of atToc33 and atToc34 are associated with atToc120/132 and atToc159 complexes, respectively. It also accounts for the fact that low levels of photosynthetic proteins are imported into *ppi2* plastids in the absence of atToc159 (Bauer *et al.*, 2000; Yu and Li, 2001).

Recent binding studies with recombinant atToc33 and atToc34 suggest that they bind preproteins with unequal affinities (Gutensohn *et al.*, 2000; Jelic *et al.*, 2003; Kubis *et al.*, 2003), consistent with the proposal that these two GTPases contribute to the formation of distinct targeting pathways. However, they are unlikely to provide the single element of substrate specificity for Toc complexes because they are functionally interchangeable in vivo (Jarvis *et al.*, 1998; Gutensohn *et al.*, 2000). In addition to the observation that atToc33 and atToc34 bind preproteins, atToc33 has been shown to serve as the docking site for the cytosolic form of atToc159 at the chloroplast surface (Smith *et al.*, 2002b; Wallas *et al.*, 2003). Soluble atToc159 has been proposed to function as a soluble preprotein receptor. This suggests that atToc33 and atToc34 might contribute to targeting pathway specificity not only by selective preprotein binding but also selective binding of atToc159 and atToc120/132 receptors during cycles of preprotein targeting.

The diversity of plastid morphology and function is dictated by the composition of nucleus-encoded plastid preproteins that are expressed over the course of plant development. Key developmental events trigger dramatic changes in the profiles of plastid protein expression, requiring remarkable adaptability in the protein import apparatus. One striking example is the dramatic increase in the expression of light-induced photosynthetic proteins during photomorphogenesis. The data presented here, in conjunction with previous studies, suggest that distinct targeting pathways operate in parallel to maintain the balance of cargo and prevent competition for import between different classes of essential preproteins (e.g., constitutive vs. photosynthetic proteins). Defining the range of specific substrates for these pathways and the structural elements of transit

peptides that dictate pathway specificity will be major challenges for future studies.

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