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The major protein import receptor of plastids is essential for chloroplast biogenesis

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Light triggers the developmental programme in plants that leads to the production of photosynthetically active chloroplasts from non-photosynthetic proplastids[1]. During this chloroplast biogenesis, the photosynthetic apparatus is rapidly assembled, mostly from nuclear-encoded imported proteins[2–4], which are synthesized in the cytosol as precursors with cleavable amino-terminal targeting sequences called transit sequences. Protein translocon complexes at the outer (Toc complex)[5–7] and inner (Tic complex)[6,8,9] envelope membranes recognize these transit sequences, leading to the precursors being imported. The Toc complex in the pea consists of three major components, Toc75, Toc34 and Toc159 (formerly termed Toc86)[6,7,10,11]. Toc159, which is an integral membrane GTPase[12], functions as a transit-sequence receptor[5–7,13]. Here we show that *Arabidopsis thaliana* Toc159 (atToc159) is essential for the biogenesis of chloroplasts. In an *Arabidopsis* mutant (*ppi2*) that lacks atToc159, photosynthetic proteins that are normally abundant are transcriptionally repressed, and are found in much smaller amounts in the plastids, although *ppi2* does not affect either the expression or the import of less abundant non-photosynthetic plastid proteins. These findings indicate that atToc159 is required for the quantitative import of photosynthetic proteins. Two proteins that are related to atToc159 (atToc120 and atToc132) probably help to maintain basal protein import in *ppi2*, and so constitute components of alternative, atToc159-independent import pathways.

The identification and proposed functions of the Toc components are based on the analysis of *in vitro* import assays using isolated pea chloroplasts and recombinant precursors[5–7], although there is no evidence for the essential roles of the Toc proteins in plastid protein import *in vivo*. The *Arabidopsis thaliana* mutant *ppi1* (for plastid protein import) has a disruption in a gene encoding one of the two homologues of Toc34 (ref. 14), and causes a non-lethal defect in chloroplast development. We investigated Toc159 because its receptor activity indicates that it might be an important component of the import apparatus. To study the function of Toc159 *in vivo*, we used a reverse genetic approach[15] to identify mutants in *Arabidopsis TOC159*. Searches of *Arabidopsis* genomic sequence databases revealed the existence of a family of three putative import receptor proteins related to pea Toc159 (psToc159) (Fig. 1a). These three proteins, which we designate atToc159, atToc132 and atToc120 on the basis of their deduced relative molecular masses, have a tripartite structure (Fig. 1a). The GTP-binding (Fig. 1a, black and white print) and carboxy-terminal membrane anchor domains (Fig. 1a, green) are highly conserved among the three proteins (~65% identity), whereas the N-terminal acidic domains (Fig. 1a, red) vary considerably in sequence (~20% identity) and length. AtToc159 and psToc159 are most closely related (48% overall identity; 74% identity in the GTP-binding and C-terminal domains), indicating that they are functional orthologues[10,16]. The messenger RNAs for all three proteins are present in *Arabidopsis* seedlings, with atToc159 mRNA being five- to tenfold more abundant than those of atToc132 and atToc120 in both etiolated and green seedlings (Fig. 1b). Strikingly, the messages for all three proteins are increased about twofold in light-grown green as opposed to dark-grown etiolated seedlings (Fig. 1b). This finding presumably reflects increased import activity in developing chloroplasts when expression levels of nuclear-encoded photosynthetic proteins are highest[17,18].

Synthetic [^{35}S]atToc159, -132 and -120 (Fig. 2a, lane 1) were inserted into the outer membrane of isolated pea chloroplasts in an *in vitro* import reaction (Fig. 2a, lane 2) to verify that the putative receptor proteins are chloroplast proteins. All three proteins were resistant to alkaline extraction (Fig. 2a, lane 4). Thermolysin treatment of chloroplasts after the import reactions resulted in the quantitative degradation of the imported proteins to membrane anchor fragments of relative molecular mass 52,000 (M_r 52K; Fig. 2a, lane 3), as previously shown for psToc159 (ref. 5). These results confirm that the proteins are found in the outer envelope membrane, and indicate that all three integrate into the membrane with their N-terminal acidic and GTP-binding domains oriented to the cytoplasm. The location and topology of endogenous atToc159 was confirmed by thermolysin treatment of isolated *Arabidopsis* chloroplasts followed by immunoblotting with anti-atToc159$_{1–740}$ antibodies (data not shown). Portions of newly imported [^{35}S]atToc159, -132 or -120 associated with Toc complexes that were immunoaffinity-purified using anti-psToc34 IgG-Sepharose (Fig. 2b, lane 2). No proteins were detected in control experiments using preimmune IgG-Sepharose (Fig. 2b, lane 3). These data lead us to conclude that atToc159, -132 and -120 function as components of the Toc complex.

The divergence in the cytoplasmic acidic domains among atToc159, -132 and -120 indicates that they have distinct roles in protein import. To determine the role of atToc159, the most abundant member of the group, we screened T-DNA mutant collections[19] for insertions in the *TOC159* gene. Two independent mutant lines, CS11072 and CS19917, were obtained (Fig. 3a) that contain insertions in *TOC159* at 770 and ~1,600 base pairs (bp), respectively. The mutant lines have identical albino phenotypes (Fig. 3b). CS11072, termed *ppi2*, contains a single T-DNA insertion (data not shown) and so was selected for further characterization. Polymerase chain reaction with reverse transcription (RT-PCR) and immunoblotting analyses confirmed the absence of Toc159 mRNA and protein in extracts of CS11072, indicating that *ppi2* is a null mutant (data not shown). Mutant plants are not viable on soil beyond the cotyledon stage (Fig. 3b), so *ppi2* is seedling lethal.

When viewed by transmission electron microscopy, *ppi2* plastids lack the thylakoid membranes and starch granules of mature chloroplasts (Fig. 3d), and therefore resembled undifferentiated proplastids (Fig. 3c). Furthermore, *ppi2* plastids developed only a rudimentary paracrystalline internal membrane structure (prolamellar body) that is characteristic of etioplasts when grown in the dark on sucrose (data not shown). These results indicate that the ability of *ppi2* proplastids to differentiate into chloroplasts is blocked, but other plastid functions, such as etioplast formation, might also be affected.

Coomassie blue (Fig. 4a) and immunoblot (Fig. 4b) analyses revealed that three major photosynthetic genes, the large (RbcL) and small (RbcS) subunits of Rubisco (a known import substrate of Toc159 in pea[5–7]) and the chlorophyll *a/b* binding protein (Cab) are present in *ppi2* at much lower levels than in wild-type plants. Furthermore, the expression of all three proteins was strongly reduced in *ppi2* plants (Fig. 5a). These data show that *ppi2* results in transcriptional repression of highly expressed photosynthetic genes. The simultaneous repression of RbcS and RbcL, a protein encoded by the chloroplast genome, is expected as their regulation is coupled[20]. In contrast, the expression of the light-induced cytoplasmic enzyme chalcone synthase[21] was not reduced in *ppi2* (Fig. 5a), indicating that the repression of transcription is specific for the genes encoding chloroplast proteins. The selective effect of *ppi2* on the transcription of photosynthetic genes is typical of *Arabidopsis* mutants that are defective in chloroplast biogenesis[22]. Although RbcS and Cab expression was reduced, the proteins appeared to be normally processed in *ppi2* because their electrophoretic mobilities were identical to those in wild-type plants (Fig. 4b). Indeed, RbcS (Fig. 4c) and Cab (data not shown), as in wild-type plants (Fig. 4d), are located exclusively within plastids, rather than accumulated in the cytosol in *ppi2*, as judged by immunoelectron microscopy. Thus, although atToc159 is essential for chloroplast biogenesis, plastids lacking this Toc component are still capable of protein import.

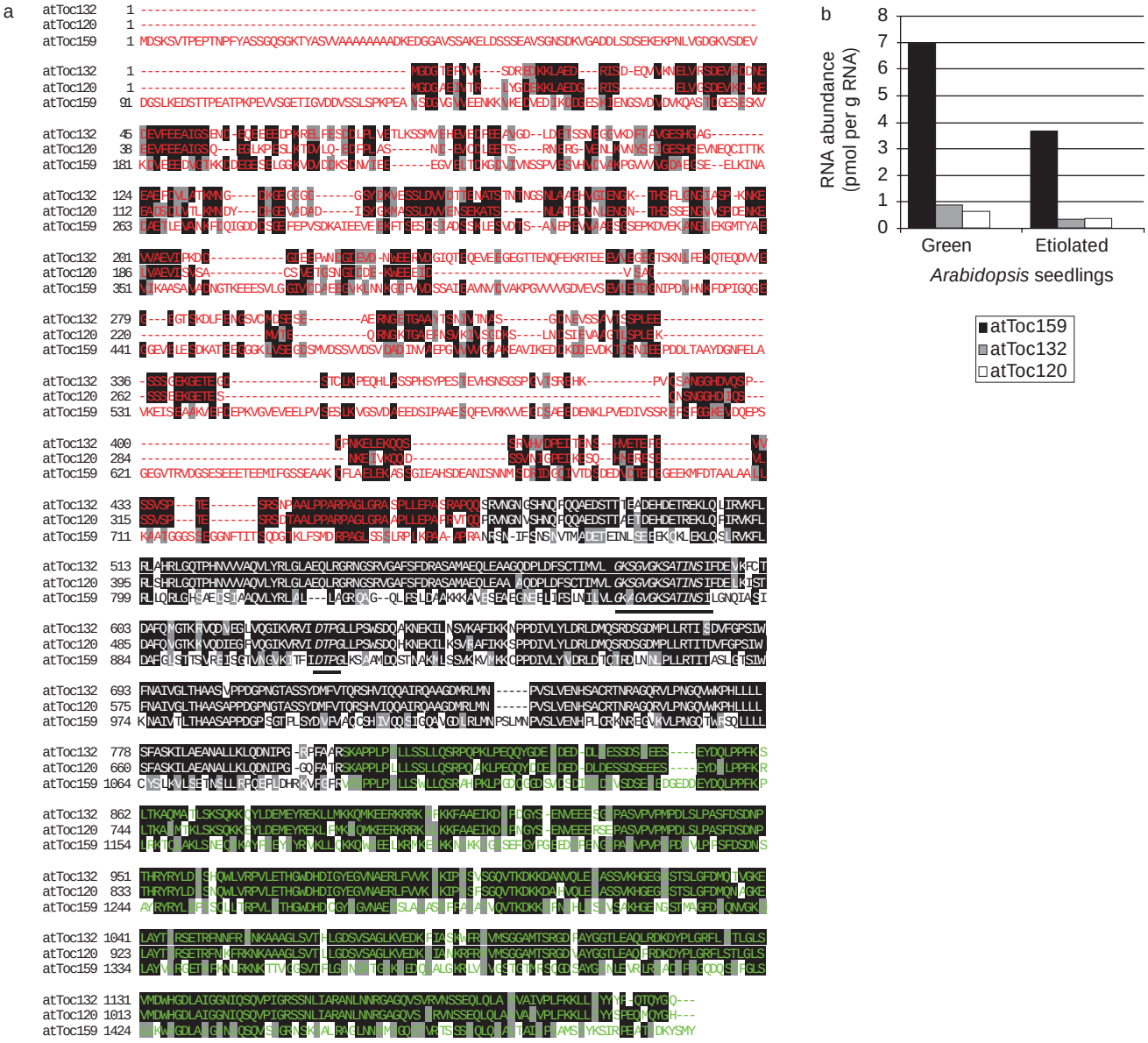

Figure 1 A family of three Toc159-related genes in *Arabidopsis*. **a**, Deduced sequences of atToc159, atToc132 and atToc120 were aligned using the ClustalW 1.7 software. Gaps are introduced to maximize identical sequences. Amino acids identical in at least two of the sequences are shaded in black; conserved substitutions are shaded in grey. Variable acidic domains are in red, the GTPase region is in black and white, and the membrane anchor domain is in green. GTP-binding motives are underlined. **b**, Abundance of atToc159, -132 and -120 RNA in *Arabidopsis* seedlings grown in continuous light (Green) or in the dark (Etiolated) for 6 days.

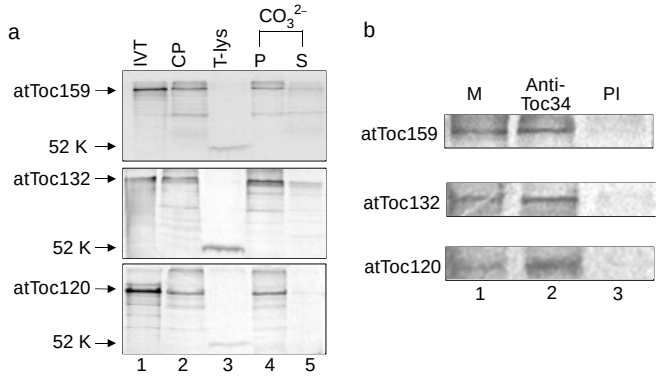

Figure 2 atToc159, atToc132 and atToc120 are components of the Toc complex of the plastid protein import machinery. **a**, Synthetic [^{35}S]atToc159, -132 or -120 (IVT) associate with isolated pea chloroplasts in a standard import reaction (CP). Thermolysin treatment (T-lys) degrades all three imported proteins to membrane-protected fragments of relative molecular mass 52,000 (52K). Imported [^{35}S]atToc159, -132 and -120 remain with the membrane fraction (P) after extraction with alkaline carbonate buffer (CO_3^{2-}). **b**, Imported [^{35}S]atToc159, -132 or -120 associated with the Toc complex. They indirectly bind to anti-Toc34 IgG-Sepharose but not preimmune IgG-Sepharose (PI), indicating that they associate with Toc complexes. Lane 1 contains 10% of the chloroplast membrane fraction (M) used for immunoprecipitations.

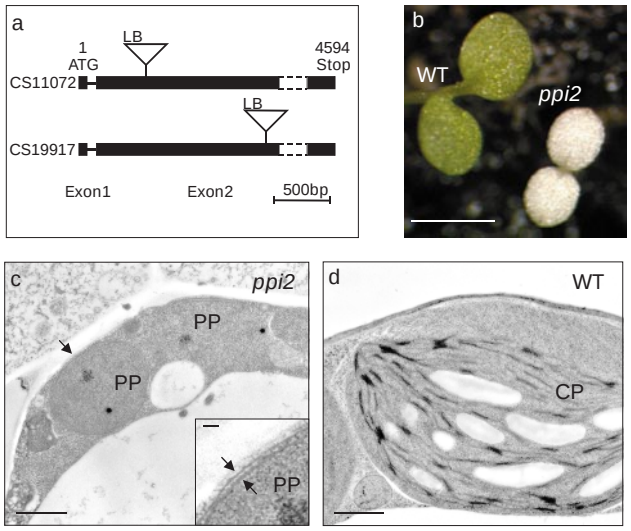

Figure 3 Characteristics of the *ppi2* mutant. **a**, Schematic representation of *TOC159* gene disruptions in the CS11072 and CS19917 *Arabidopsis* lines. Translation initiation (ATG) and termination (Stop) codons are indicated. The T-DNA inserts are represented with the 5′ border sequences of the insert (LB) labelled to indicate the orientation (not to scale). **b**, Visible phenotype of *ppi2*. CS11072 *ppi2* line and wild-type (WT) seedlings were grown in soil for 6 days in long-day conditions (16 hours light: 8 hours dark). Scale bar, 2 mm. **c**, **d**, Ultrastructure of *ppi2* plastids. Transmission electron microscopy of plants grown on soil for 6 days in long-day conditions indicates that *ppi2* plastids (**c**) remain as undifferentiated proplastids (PP) compared to the chloroplasts (CP) present in wild-type (WT) cells (**d**). Scale bar, 0.5 μm. Inset, the double membrane envelope of the *ppi2* proplastids; scale bar, 0.05 μm.

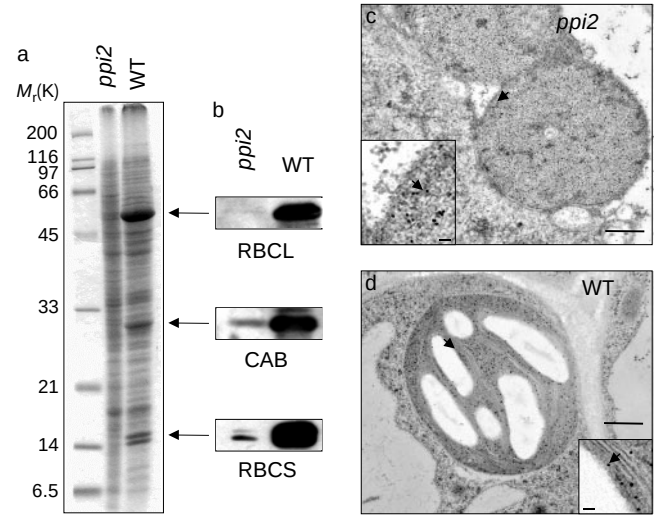

Figure 4 *ppi2* plants contain reduced amounts of photosynthetic proteins. **a**, The reduction in RbcS, RbcL and Cab expression is apparent by SDS–PAGE analysis of wild-type (WT) and *ppi* plants. **b**, Immunoblotting with sera against the large (RBCL) and small (RBCS) subunits of Rubisco and the chlorophyll *a/b* binding protein (CAB) reveals a dramatic reduction of the proteins in *ppi2* plants. RbcS and Cab are processed to their mature forms in the *ppi2* plants. **c**, **d**, Immunogold labelling of RbcS in plastids of *ppi2* (**c**) and wild-type (**d**) cotyledons, indicating that RbcS is imported in the absence of atToc159. Scale bar, 0.5 μm. Arrows indicate the position of the insets. The arrow in the inset points to a gold particle; scale bar, 0.05 μm.

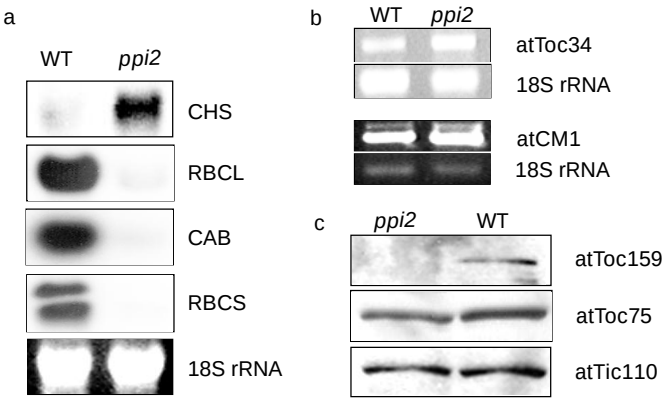

Figure 5 Characterization of the chloroplast biogenetic defect in *ppi2* plants. **a**, Comparative northern-blot analysis of light-induced mRNAs from wild-type (WT) and *ppi2* 6-day-old seedlings. RbcL, RbcS and Cab transcription is repressed in the *ppi2* mutant, whereas the light-regulated gene of cytoplasmic chalcone synthase (CHS) is not repressed. 18S ribosomal RNA was used to normalize loading. **b**, Comparative RT-PCR analysis of non-photosynthetic genes from wild-type and *ppi2* plants. The amount of mRNA encoding chorismate mutase 1 (atCM1) is unchanged in *ppi2* plants. atToc34 mRNA levels are upregulated in the *ppi2* mutant. **c**, Immunoblot analysis of atTic110 and atToc75 in protein extracts from wild-type and *ppi2* plants. The expression and processing of atTic110 and atToc75 are normal in mutant plants, indicating that these proteins are imported in the absence of atToc159.

In contrast to RbcS and Cab, the expression of chorismate mutase (atCM1), a plastid protein that is not specific to chloroplasts[23], was unaffected by *ppi2* (Fig. 5b). The expression of the gene encoding atToc34 (ref. 14) was increased in *ppi2*, possibly to compensate for the loss of atToc159. Furthermore, immunoblots of *ppi2* and wild-type extracts show that expression and processing of atToc75 and atTic110 (refs 8, 9), a component of the Tic-complex, were comparable (Fig. 5c). Toc75 and Tic110 are targeted to chloroplasts by N-terminal transit sequences through the Toc complex[18,24]. The expression and import of the non-photosynthetic proteins tested is therefore not affected in *ppi2*. The presence of atToc34, atToc75 and atTic110 in *ppi2* plants provides additional evidence that the import apparatus is expressed and functional in the absence of atToc159. A likely explanation for this observation is that atToc132 and/or atToc120, which are expressed in *ppi2* (data not shown), partly compensate for the absence of atToc159.

The characteristics of the *ppi2* mutant demonstrate that protein import is a limiting process in chloroplast biogenesis and reveal alternative pathways for targeting proteins to plastids. The atToc159 defect limits the capacity of plastids to import a set of highly expressed photosynthetic proteins that are essential for chloroplast biogenesis. atToc132 and atToc120 might represent additional import complexes with distinct but partly overlapping substrate specificities, which might account for the ability of *ppi2* plastids to import other proteins. The functional differences among the import receptors are likely to involve the acidic cytoplasmic domains where atToc159, -132 and -120 are most divergent. □

Methods

Isolation of complementary DNAs and production of antisera

A cDNA encompassing the coding region of the atToc159 gene was amplified directly from *Arabidopsis* genomic DNA by PCR. The 5′ PCR primer (CAT GCC ATG GAC TCA AAG TCG GTT ACT CCA GAA CCA ACC AAC CCC TTC TAC GCT TCT TCG GGG CAA TCA GGA AAA ACC TAT GCT TCT GTT GTC) incorporated a 5′ *Nco*1 site and encoded the entire first exon and 26 bases of the 5′ end of the second exon of the atToc159 gene (Genbank accession no. AC002330). The 3′ primer corresponded to the 3′ end of the gene and incorporated a 3′ *Sac*I site. The resulting atToc159 cDNA was ligated into pET21d (Novagen). The atToc132 (Genbank accession no. AC005825) and atToc120 (Genbank accession no. AB02217) cDNAs were amplified from total RNA of 10-day-old *Arabidopsis* seedlings using primers corresponding exactly to the 5′ and 3′ ends of the coding regions. atToc132 and atToc120 cDNAs were ligated into pCR-XL-TOPO (Invitrogen). Anti-atToc159 and anti-atTic110 antibodies were raised against *Escherichia coli*-expressed N-terminal portions of atToc159 (amino acids 1 to 740) and atTic110 (amino acids 1 to 498), respectively. The antibodies were affinity purified against the respective antigen before use.

Isolation of T-DNA insertion mutants

PCR-based identification of T-DNA insertions in *TOC159* was performed according to published protocols[15]. DNA pools from the *Arabidopsis* libraries of T-DNA insertion mutants were obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus, Ohio. PCR reactions required 29-mer primers corresponding to the 5′ end (forward primer, ATG GAC TCA AAG TCG GTT ACT CCA GAA CC) and 3′ end (reverse primer, TTA GTA CAT GCT GTA CTT GTC GTT CGT CG) of atToc159 and the left and right border of the T-DNA[15], respectively. Line CS11072 carried a single insertion consisting of two concatameric right-border to right-border T-DNA molecules, as indicated by Southern blot and PCR analysis and the 3:1 segregation of the T-DNA kanamycin-resistance marker.

Chloroplast import experiments

[^{35}S]atToc159, -132 and -120 were synthesized in a coupled *in vitro* transcription and translation system in the presence of [^{35}S]methionine (Promega). Protein import into isolated pea chloroplasts, thermolysin treatment, chloroplast-membrane isolation and carbonate extraction were performed as described[25]. Immunoaffinity chromatography using anti-Toc34 IgG-Sepharose was performed as described[26].

Characterization of the *ppi2* mutation

Wild-type and mutant plants were grown for 6 days either on soil under long-day conditions (16 hours light: 8 hours dark) or on agarose plates containing 0.5× Murashige-Skoog medium and 1% sucrose (MS-medium) in continuous light. Protein extracts for SDS–PAGE and immunoblot analyses were prepared by direct extraction of seedling tissue in boiling SDS–PAGE sample buffer. Samples corresponding to equivalent amounts of fresh mass were loaded onto SDS–PAGE gels. Antisera were raised against *Arabidopsis* chlorophyll *a/b* binding protein, pea large subunit of Rubisco, *Chlamydomanas reinhardtii* small subunit of Rubisco, and pea Toc75. Northern blots containing 5 μg of total RNA from 6-day-old *Arabidopsis* plants grown on MS medium were hybridized to random-primed [^{32}P]cDNA or genomic DNA probes. The probes were generated by PCR amplification of chalcone synthase (Swiss-Prot accession no. P13114, base pairs 346–1082), large and small subunits of Rubisco (Genbank accession no. U91966 (base pairs 1–1481) and g16195 (base pairs 1–552)), and the chlorophyll *a/b* binding protein (Genbank accession no. X03908 (base pairs 507–999) of *Arabidopsis*. Relative competitive RT-PCR analysis was performed according to the manufacturer's protocols (Ambion). Specific primer pairs to the following genes were used: atToc34 (expressed sequence tag clone 190I17T7: forward, ATG GCC ATG GGG TCT CTC GTG CGT GAA TGG; reverse, TGC GGA TCC TTA AAG TGG CTT TCC ACT TGT); atCM1 (Genbank accession no. Z26519: forward, ATG AGA TCG TCT TGT TGC TCC; reverse, TCA GTC CAG TCT TCT GAG CAA G); atToc159 (forward, CAC AGT CTT GCT CTA GCT AGC CGG TTC; reverse, GCT GTA CTT GTC GTT CGT CGC TTC); atToc132 (forward, GAT TCG GTT TCT GCG GGG TTG; reverse, TCA TTG TCC ATA TTG CGT TTG CGG); atToc120 (forward, AAT GCT GGG AAG GAA TTA GCG TAC ACT A; reverse, TCA GTG TCC ATA TTG CAT TTG CTC AGG). Electron microscopy and immunoelectron microscopy were performed according to previously published protocols[27].

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