

# The Arabidopsis *ppi1* Mutant Is Specifically Defective in the Expression, Chloroplast Import, and Accumulation of Photosynthetic Proteins<sup>W</sup>

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The import of nucleus-encoded proteins into chloroplasts is mediated by translocon complexes in the envelope membranes. A component of the translocon in the outer envelope membrane, Toc34, is encoded in Arabidopsis by two homologous genes, *atTOC33* and *atTOC34*. Whereas *atTOC34* displays relatively uniform expression throughout development, *atTOC33* is strongly upregulated in rapidly growing, photosynthetic tissues. To understand the reason for the existence of these two related genes, we characterized the *atTOC33* knockout mutant *ppi1*. Immunoblotting and proteomics revealed that components of the photosynthetic apparatus are deficient in *ppi1* chloroplasts and that nonphotosynthetic chloroplast proteins are unchanged or enriched slightly. Furthermore, DNA array analysis of 3292 transcripts revealed that photosynthetic genes are moderately, but specifically, downregulated in *ppi1*. Proteome differences in *ppi1* could be correlated with protein import rates: *ppi1* chloroplasts imported the ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit and 33-kD oxygen-evolving complex precursors at significantly reduced rates, but the import of a 50S ribosomal subunit precursor was largely unaffected. The *ppi1* import defect occurred at the level of preprotein binding, which is consistent with a role for *atToc33* during preprotein recognition. The data suggest that *atToc33* is involved preferentially in the import of photosynthetic proteins and, by extension, that *atToc34* is involved in the import of nonphotosynthetic chloroplast proteins.

## INTRODUCTION

Like mitochondria, chloroplasts are descended from a free-living prokaryotic organism that entered the eukaryotic lineage through endosymbiosis. During the course of their evolution, chloroplasts relinquished the majority of their genes so that now, >90% of chloroplast proteins are encoded in the nucleus (Leister, 2003). Chloroplast proteins are synthesized in precursor form on cytosolic ribosomes and targeted post-translationally to chloroplasts (Keegstra and Cline, 1999). Each precursor protein (preprotein) carries an NH<sub>2</sub>-terminal targeting signal called the transit peptide, which guides the protein to the chloroplast (Bruce, 2001). Translocon complexes in the outer and inner envelope membranes of chloroplasts (Toc and Tic, respectively) mediate preprotein import in an ATP- and GTP-dependent process (Chen et al., 2000; Hiltbrunner et al., 2001a; Jarvis and Soll, 2002). Proteins are translocated across both membranes simultaneously, in extended conformation, and then processed to their mature size by a stromal processing peptidase. After translocation, newly imported proteins are folded to their final conformation by molecular chaperones or

targeted to one of a number of internal compartments via separate protein targeting pathways (Keegstra and Cline, 1999).

Several components of the Toc and Tic complexes have been identified in biochemical studies using isolated pea chloroplasts. The core components of the Toc complex are called Toc159, Toc34, and Toc75, according to their molecular weights (Hirsch et al., 1994; Kessler et al., 1994; Perry and Keegstra, 1994; Schnell et al., 1994; Seedorf et al., 1995; Bölter et al., 1998). Toc159 and Toc34 are related GTPases that have been proposed to act as preprotein receptors. Both proteins are anchored in the outer envelope by COOH-terminal domains and project their GTP binding domains into the cytosol. Although their precise mode of action remains unclear (Sveshnikova et al., 2000; Hiltbrunner et al., 2001b), Toc159 and Toc34 interact with incident preproteins (Ma et al., 1996; Kouranov and Schnell, 1997) and mediate their transfer to the translocation channel, of which Toc75 is a major component. Toc75 has a  $\beta$ -barrel structure comprising 16 amphiphilic  $\beta$ -strands and forms a channel with a pore size of  $\sim$ 14 to 26 Å (Hinnah et al., 2002). The Tic complex is less well characterized, and there is even some disagreement in the literature concerning the identity of its components (Jarvis and Soll, 2002). A 100-kD heat-shock protein (Hsp100) homolog, ClpC, held at the stromal face of the Tic complex has been proposed to drive chloroplast protein import, much like Hsp70 proteins drive protein translocation into mitochondria and the endoplasmic reticulum (Nielsen et al., 1997).

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In Arabidopsis, several of these translocon components are encoded by multiple genes. For example, there are two Toc34-related genes in Arabidopsis, *atTOC33* and *atTOC34* (Jarvis et al., 1998; Gutensohn et al., 2000), and four Toc159-related genes, *atTOC159*, *atTOC132*, *atTOC120*, and *atTOC90* (Bauer et al., 2000; Hiltbrunner et al., 2001a). There also are multiple Toc75-related sequences in Arabidopsis, but some other components are encoded by just a single gene (Jackson-Constan and Keegstra, 2001). The reason for this Toc component multiplicity in Arabidopsis is unclear, but it has been proposed that different Toc isoforms have different preprotein recognition specificities (Jarvis et al., 1998). Support for this hypothesis was obtained recently upon characterization of an *atTOC159* knockout mutant called *plastid protein import2 (ppi2)* (Bauer et al., 2000). Homozygous *ppi2* plants have a seedling-lethal, albino phenotype characterized by pronounced defects in chloroplast biogenesis. Interestingly, whereas the expression and accumulation of proteins involved directly in photosynthesis (photosynthetic proteins) are reduced strongly in *ppi2*, proteins involved in chloroplast functions unrelated to photosynthesis (nonphotosynthetic proteins) are expressed and accumulate normally. These observations led to the suggestion that atToc159 has recognition specificity for photosynthetic proteins and that atToc132 and atToc120 are involved preferentially in the import of nonphotosynthetic proteins (Bauer et al., 2000). The existence of such substrate-specific protein import pathways would prevent the bulk flow of highly abundant, photosynthetic proteins from outcompeting the import of less abundant but equally important nonphotosynthetic proteins.

Previously, we described the identification and preliminary characterization of an *atTOC33* knockout mutant called *ppi1* (Jarvis et al., 1998). Homozygous *ppi1* plants are yellow-green in appearance but, unlike *ppi2* plants, they are able to survive to maturity. This difference in phenotype severity may indicate that any specialization among the Toc34 homologs is less pronounced than what exists among the Toc159 homologs and that there is considerable functional redundancy between atToc33 and atToc34. Indeed, overexpression of atToc34 was shown to complement the atToc33 deficiency of *ppi1* plants (Jarvis et al., 1998). Nevertheless, the possibility remains that under normal, physiological conditions, atToc33 and atToc34 operate preferentially in import pathways with different substrate specificities. Some support for this idea was provided recently by the demonstration that, in vitro, atToc33 but not atToc34 is able to inhibit the binding of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit precursor (a photosynthetic preprotein) to chloroplasts in a GTP-dependent manner (Gutensohn et al., 2000). In this study, we took an in vivo approach to further address this question by characterizing the *ppi1* mutant in detail.

## RESULTS

### *atTOC33* Expression Is Induced Strongly in Rapidly Growing Photosynthetic Tissues

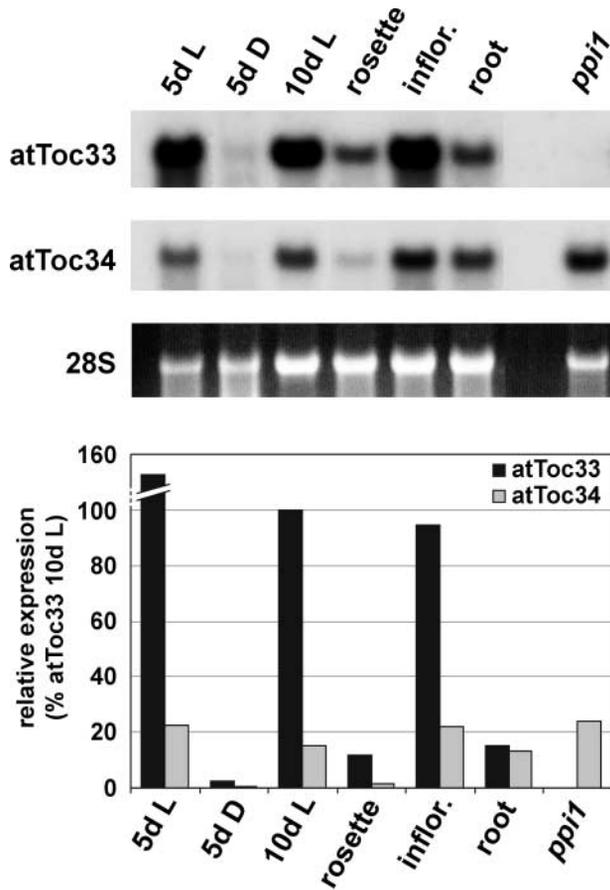
We previously determined the expression levels of *atTOC33* and *atTOC34* over a developmental time course at the whole-

plant level (Jarvis et al., 1998). We found that *atTOC33* is expressed at levels several times greater than *atTOC34* and that both genes are subject to developmental regulation, with the highest levels of expression occurring in young plants. These data demonstrated that both genes are expressed during periods of intense plastid biogenesis, but they provided no clues regarding the possible functional differences between the proteins, because gene expression levels in individual tissues were not investigated. Although subsequent experiments did provide spatial expression profiles for each gene (Gutensohn et al., 2000), these studies did not address the relative levels of expression of the two genes.

To gain insight into the reasons underlying the existence of two Toc34-related genes in Arabidopsis, we determined the relative expression levels of *atTOC33* and *atTOC34* at different developmental stages and in different tissue types by RNA gel blot analysis (Figure 1). Whereas *atTOC34* was expressed at a relatively uniform, low level at most stages of development, *atTOC33* was induced strongly in photosynthetic tissues undergoing rapid growth (in 5- and 10-day-old light-grown plants and in inflorescence tips). In fully expanded photosynthetic tissues (rosette leaves), the expression of both genes was reduced significantly, although *atTOC33* continued to be expressed at a higher level than *atTOC34*. Most interestingly, in nonphotosynthetic root tissue, *atTOC33* was downregulated specifically such that the two genes were expressed at essentially the same level. Both genes were expressed at low levels in dark-grown plants, which presumably reflects the minimal requirements for plastid biogenesis in these plants. The 10-day-old *ppi1* control indicated that the hybridization probes were specific and confirmed that *atTOC34* expression is not upregulated strongly in the *ppi1* mutant.

### Photosynthetic Proteins Are Specifically Deficient in *ppi1* Chloroplasts

The demonstration that *atTOC33* expression was induced strongly in rapidly expanding photosynthetic tissues suggests that atToc33 may be relatively more important for the assembly of the photosynthetic apparatus than atToc34. To investigate this possibility, we used immunoblot analysis to compare the abundance of a range of different photosynthetic and nonphotosynthetic proteins in chloroplasts isolated from 10-day-old wild-type and *ppi1* plants (Figure 2). In this analysis, antibodies against four photosynthetic proteins (Rubisco small subunit [SSU], light-harvesting chlorophyll *a/b* binding protein [LHCII], photosystem I subunit D [PSI-D], and ferredoxin-NADP<sup>+</sup> oxidoreductase [FNR]) and four nonphotosynthetic chloroplast proteins (Hsp70, the Hsp100 homolog ClpC, triose phosphate/phosphate translocator [TPT], and coproporphyrinogen oxidase [CPO]) were used. To obtain semiquantitative data, a dilution series of each chloroplast extract was examined with each antibody. By comparing the band intensities obtained, we deduced that each of the photosynthetic proteins was reduced in abundance in *ppi1* chloroplasts by ~50% or more. No evidence for preprotein accumulation in *ppi1* was observed, suggesting that most nonimported preproteins are degraded rapidly or, alternatively, that gene expression is correlated closely



**Figure 1.** Expression Levels of *atTOC33* and *atTOC34* during Seedling Development and in Different Tissues of Arabidopsis.

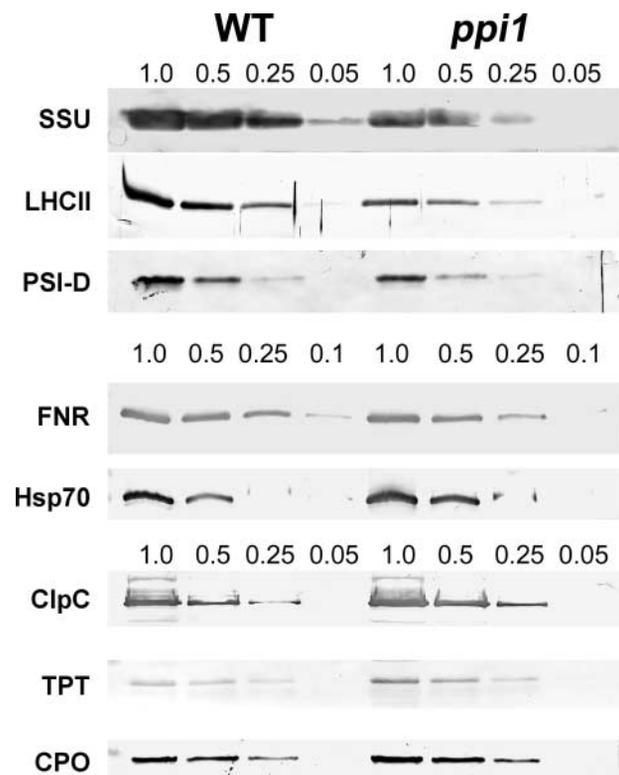
Fifteen-microgram samples of total RNA isolated from Arabidopsis tissues were examined by RNA gel blot analysis. RNA was isolated from wild-type seedlings grown in vitro for 5 days in the light (5d L), 5 days in the dark (5d D), and 10 days in the light (10d L), from three different tissues of 28-day-old wild-type plants grown on soil (rosette leaves, young inflorescence tips, and roots), and from *ppi1* seedlings grown in vitro for 10 days in the light (*ppi1*). Filters were probed using  $^{32}\text{P}$ -labeled *atTOC33* and *atTOC34* cDNA probes with identical specific activities. rRNA (28S) was used as a loading control. The images shown were obtained after a 3-week exposure, but quantification was performed using a shorter exposure. Relative levels of expression of *atTOC33* and *atTOC34* normalized for 28S rRNA are shown in the graph at bottom; y axis values between 100 and 140 have been removed to aid visualization.

with the efficiency of import of the encoded preprotein. By contrast, all four nonphotosynthetic proteins were present at least at wild-type levels in *ppi1* chloroplasts, and some even appeared to be enriched slightly in the mutant.

To corroborate these findings, we next adopted a proteomics approach. The chloroplast proteomes of 10-day-old wild-type and *ppi1* plants were analyzed using difference gel electrophoresis (DIGE). Proteins extracted from isolated chloroplasts were labeled with nonsaturating CyDyeDIGE fluor. In a typical experiment, wild-type proteins were labeled with the Cy5 fluor

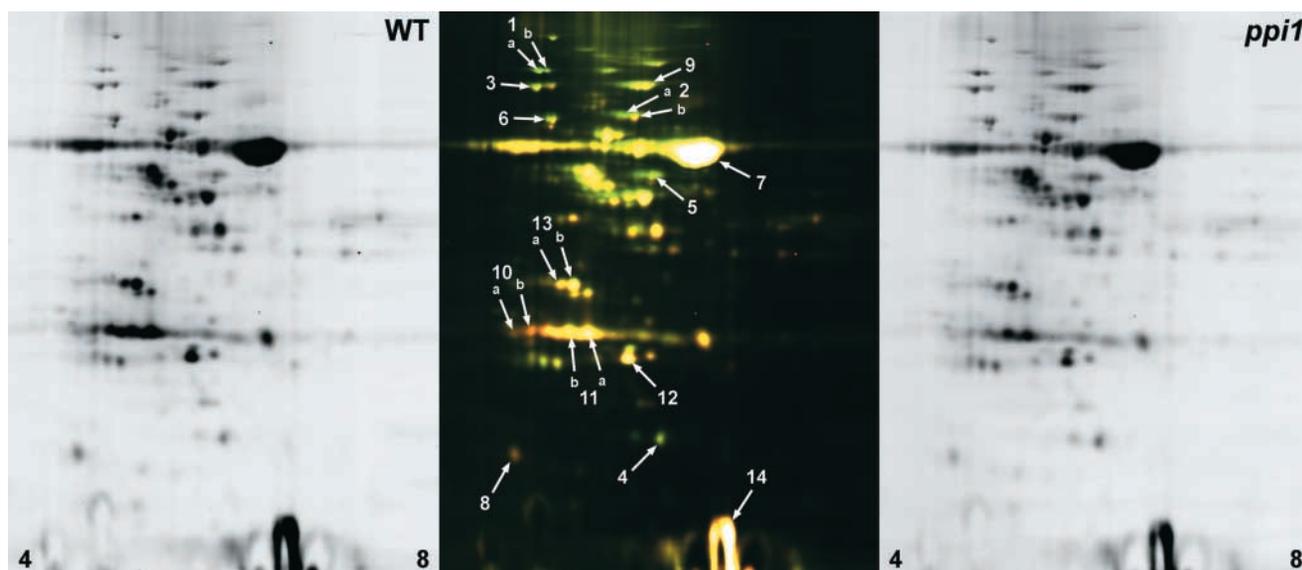
and mutant proteins were labeled with Cy3. The labeled protein samples were mixed and then resolved in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. The Cy5 and Cy3 fluorescent images of a typical gel are shown in Figure 3 (left and right, respectively). Additionally, an overlay of the Cy5 (wild type; false-colored red) and Cy3 (*ppi1*; false-colored green) images has been included to aid the visualization of protein abundance differences (Figure 3, middle); proteins depleted in *ppi1* appear red, whereas those enriched in *ppi1* appear green. The fluorescent signal associated with each protein spot was quantified and used to calculate the degree of enrichment or depletion in *ppi1* chloroplasts.

Based on these data, spots were selected for identification by mass spectrometry, and the results obtained are summarized in Table 1. All of the proteins we identified that were depleted in *ppi1* chloroplasts were components of the photosyn-



**Figure 2.** Immunoblot Analysis of Photosynthetic and Nonphotosynthetic Chloroplast Proteins in the *ppi1* Mutant.

Dilution series of chloroplast protein preparations from the wild type (WT) and the *ppi1* mutant (*ppi1*) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed using antibodies against photosynthetic (SSU, LHCII, PSI-D, and FNR) and nonphotosynthetic (Hsp70, ClpC, TPT, and CPO) proteins. In most cases, filters were cut in half and probed for one photosynthetic protein and one nonphotosynthetic protein (the pairings used were SSU/TPT, PSI-D/ClpC, and FNR/Hsp70); the LHCII and CPO data were derived using separate blots. The dilution steps used are given above the gels. Dilution series started with 20  $\mu\text{g}$  for SSU, PSI-D, ClpC, TPT, and CPO, with 10  $\mu\text{g}$  for FNR and Hsp70, and with 2  $\mu\text{g}$  for LHCII. The data shown are representative of three independent experiments.



**Figure 3.** DIGE Analysis of the *ppi1* Chloroplast Proteome.

Total chloroplast protein samples isolated from 10-day-old seedlings were analyzed using CyDyeDIGE technology. Wild-type (WT) and mutant (*ppi1*) protein samples (50  $\mu$ g each) were labeled with Cy5 and Cy3, respectively. The samples were pooled and run on a single 24-cm, pH-3 to -10, two-dimensional gel and then imaged using parameters appropriate for each fluor. The left and right panels show corresponding sections (pH 4 to 8, as indicated) of the Cy5 and Cy3 images, respectively. The middle panel shows an overlay of the two images after inversion and the application of false color: Cy5 (wild type) is shown in red, and Cy3 (*ppi1*) is shown in green. Labels indicate protein spots that were identified by mass spectrometry (see Table 1).

thetic apparatus (such as SSU, OE33, and LHCII), whereas all of those showing enrichment in *ppi1* were nonphotosynthetic proteins, including molecular chaperones and a component of the plastid genetic system (an EF-Tu-like translation elongation factor). Steady state levels of another genetic system compo-

nent (the 50S ribosomal subunit protein, L12-C), an enzyme of carbon metabolism (transketolase), and the plastid-encoded Rubisco large subunit (LSU) were unaffected by the *ppi1* mutation. Because the amounts of LSU and SSU protein normally are closely coordinated (Rodermeil et al., 1996), we conclude

**Table 1.** Proteins Identified by DIGE and Mass Spectrometry

Spot No.	Gene No.	Fold Change in <i>ppi1</i> <sup>a</sup>	Protein
1	At2g04030	a, 2.41 $\pm$ 0.61; b, 1.85 $\pm$ 0.09	Hsp90 homolog
2	At1g55490 At3g13470	a, 1.86 $\pm$ 0.13; b, 1.64 $\pm$ 0.05	Chaperonin 60 $\beta$
3	At4g24280 At5g49910	1.78 $\pm$ 0.07	Hsp70 homolog
4	At3g62030	1.76 $\pm$ 0.29	Peptidyl prolyl isomerase
5	At4g20360	1.72 $\pm$ 0.08	Elongation factor, EF-Tu homolog
6	At2g28000	1.64 $\pm$ 0.19	Chaperonin 60 $\alpha$
7	RbcL	—	Rubisco large subunit, LSU
8	At3g27850	—	Ribosomal subunit, L12-C
9	At3g60750	—	Transketolase-like protein
10	At3g04790	a, 0.63 $\pm$ 0.04; b, 0.56 $\pm$ 0.03	Ribulose-5-phosphate isomerase
11a	At4g10340	0.61 $\pm$ 0.05	Light-harvesting chlorophyll <i>a/b</i> binding protein CP26 ( <i>Lhcb5</i> )
11b	At1g29910 At1g29920 At1g29930 At2g34420	0.61 $\pm$ 0.05	Light-harvesting chlorophyll <i>a/b</i> binding protein LHCII type 1 ( <i>Lhcb1.1</i> , <i>Lhcb1.2</i> , <i>Lhcb1.3</i> , <i>Lhcb1.5</i> )
12	At1g06680	0.58 $\pm$ 0.03	23-kD subunit of oxygen-evolving complex, OE23
13	At3g50820 At5g66570	a, 0.56 $\pm$ 0.09; b, 0.50 $\pm$ 0.06	33-kD subunit of oxygen-evolving complex, OE33
14	At1g67090	0.43 $\pm$ 0.04	Rubisco small subunit, SSU ( <i>Ats1A</i> )

<sup>a</sup> Prefixes "a" and "b" denote protein spots that could not be distinguished upon mass spectrometric analysis. Values are means  $\pm$  SD.

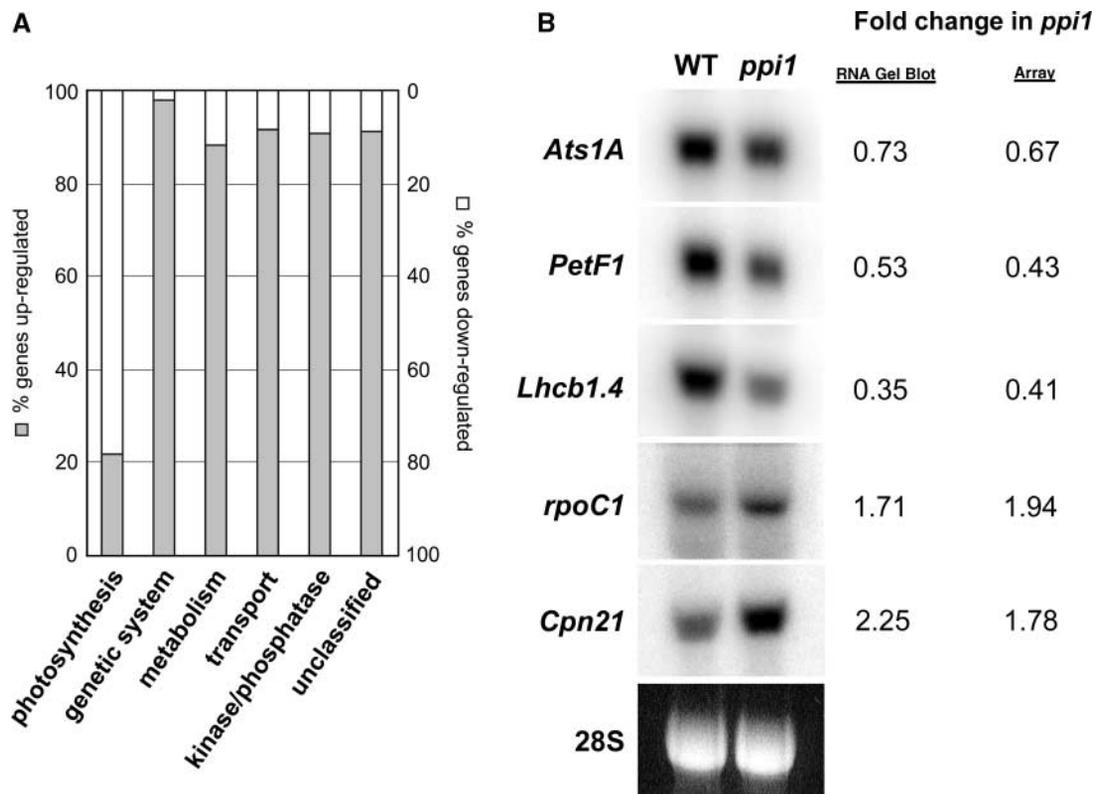
that the moderate SSU deficiency in *ppi1* is insufficient to trigger these coregulatory mechanisms.

### Expression of Photosynthetic Genes Is Downregulated Specifically in *ppi1*

The expression of nuclear genes that encode chloroplast proteins is regulated in response to a variety of signals that emanate from plastids (Surpin et al., 2002; Jarvis, 2003). These retrograde signaling mechanisms ensure that nuclear gene expression is coupled with the needs of the cell's chloroplasts, so that the photosynthetic apparatus and other biosynthetic machineries are assembled in stoichiometric fashion. To gain further insight into the role of the atToc33 protein, we conducted a global analysis of the mRNA expression of genes of plastid-related function in *ppi1* using nylon filter DNA array technology (Kurth et al., 2002; Richly et al., 2003). Of the 3292 genes analyzed, 1461 showed significantly different levels of expression from the wild type (see supplemental data online). The majority of these genes were upregulated moderately in

*ppi1*, with only 161 genes showing reduced levels of expression. Remarkably, most of the genes of known function that were downregulated in *ppi1* encode components of the photosynthetic apparatus. By contrast, genes showing increased expression levels encode components of the chloroplast's endogenous genetic system, factors involved in metabolic processes unrelated to photosynthesis, and a range of other nonphotosynthetic proteins.

We assigned all 1461 genes showing differential expression in *ppi1* to six different functional categories as follows: photosynthesis, including Calvin cycle enzymes (55 genes); genetic system (152 genes); metabolism not related directly to photosynthesis (170 genes); transport of substances, including metabolites and proteins (60 genes); kinases and phosphatases (55 genes); and putative, hypothetical, or otherwise unclassifiable proteins (969 genes). The proportion of genes showing increased or decreased expression in *ppi1* in each category is shown in Figure 4A. The fact that photosynthetic genes were downregulated specifically in *ppi1* reinforces the hypothesis that atToc33 operates preferentially in an import pathway with



**Figure 4.** Analysis of the Expression of Nucleus-Encoded Chloroplast Genes in *ppi1*.

**(A)** Total RNA isolated from 10-day-old wild-type and *ppi1* mutant seedlings was used to probe a nylon filter DNA array carrying 3292 gene-specific tags. All 1461 genes showing differential expression in *ppi1* were placed into six different categories, as described in the text. The proportion of genes within each category showing upregulation (gray bars) and downregulation (white bars) in *ppi1* is shown.

**(B)** Ten-microgram samples of the RNA described in **(A)** were analyzed further by RNA gel blot analysis. Five of the differentially regulated genes listed in Table 2 were selected for analysis. Filters were hybridized with  $^{32}\text{P}$ -labeled probes corresponding to the gene-specific tags described in **(A)**. rRNA (28S) was used as a loading control. Radioactivity associated with each band shown was quantified and used to calculate the fold change in expression in *ppi1* (relative to the wild type [WT]) for each gene; the corresponding data derived from the DNA array experiment (**A**); Table 2) are shown for comparison.

specificity for photosynthetic proteins. It is conceivable that the nucleus responds to the perturbation of photosynthetic protein import, communicated via some retrograde signaling pathway, by downregulating the genes that encode the affected proteins.

To further illustrate these gene expression changes, the data for 42 genes representative of four different functional categories (photosynthesis, 12 genes; genetic system, 10 genes; non-photosynthetic metabolism, 10 genes; and protein targeting, 10 genes) as well as 8 genes belonging to other categories are shown in Table 2; the relative levels of expression of these 50 genes are illustrated further in the supplemental data online. Not only did most of the genes that show downregulated expression in *ppi1* encode components of the photosynthetic apparatus, they also tended to be expressed at a higher level than genes that show upregulated expression (Table 2; see also supplemental data online). The plastome-encoded Rubisco large subunit gene, *rbcL*, is one obvious exception. Also of interest is the observation that all of the protein targeting-related genes were upregulated in *ppi1*, except for the gene that encodes Tic20-I, which is a putative channel component of the inner envelope membrane (Jackson-Constan and Keegstra, 2001). Similarly, genes that encode several different molecular chaperones, some of which have been implicated in chloroplast protein import (Schnell et al., 1994; Nielsen et al., 1997; Jackson-Constan and Keegstra, 2001), also were upregulated in *ppi1* (Table 2; see also supplemental data online).

To corroborate the findings of this DNA array experiment, we examined the expression of five genes listed in Table 2 by RNA gel blot analysis. Three photosynthetic genes (*Ats1A*, *PetF1*, and *Lhcb1.4*) and two nonphotosynthetic genes (*rpoC1* and *Cpn21*) were analyzed in this way. As shown in Figure 4B, the gene expression changes observed using RNA gel blot analysis were quantitatively very similar to those observed using DNA array analysis. Therefore, these results confirm the validity of the gene expression data presented in Figure 4A and Table 2.

### ***ppi1* Affects the Import of Different Preproteins to Different Degrees**

Isolated *ppi1* chloroplasts were shown previously to import reduced quantities of in vitro-translated SSU, LHCII, and protochlorophyllide oxidoreductase (POR; a light-sensitive chlorophyll biosynthetic enzyme) over a fixed period of time (Jarvis et al., 1998). However, import rate comparisons for different preproteins were not conducted. To determine if the primary cause of the chloroplast proteome differences observed in *ppi1* (Figures 2 and 3, Table 1) could be a differential effect of the *ppi1* mutation on the import of different preproteins, we compared import rates for three different preproteins using isolated wild-type and mutant chloroplasts. Import rates were determined by measuring the accumulation of imported protein over time. For these experiments, we selected SSU (a component of the photosynthetic dark reactions that occur in the stroma), OE33 (a component of the photosynthetic light reactions that occur in the thylakoids), and L11 (a 50S ribosomal subunit protein and component of the chloroplast's endogenous genetic system) for analysis.

Initial experiments revealed that import rates were linear up to  $\sim 10$  min for SSU preprotein (preSSU) and preOE33 and up to  $\sim 5$  min for preL11 (data not shown); therefore, import rate comparisons were conducted within these periods. Resistance to thermolysin treatment confirmed that the processed forms of all three preproteins had been internalized by intact chloroplasts (see supplemental data online). As shown in Figures 5A and 5B, both preSSU and preOE33 were imported into *ppi1* chloroplasts at significantly reduced rates: on average, preSSU import rates were reduced by  $42.4 \pm 1.9\%$  and preOE33 import rates were reduced by  $79.6 \pm 7.6\%$ . OE33 is targeted to the thylakoid lumen via a stromal intermediate (Keegstra and Cline, 1999) that could be observed between the precursor and mature OE33 bands of Figure 5B. Because this intermediate had already traversed the envelope, it was regarded as imported protein for quantification purposes. By contrast, the effect of *ppi1* on the import of preL11 was slight, and import rates in the different genotypes were almost identical (Figure 5C).

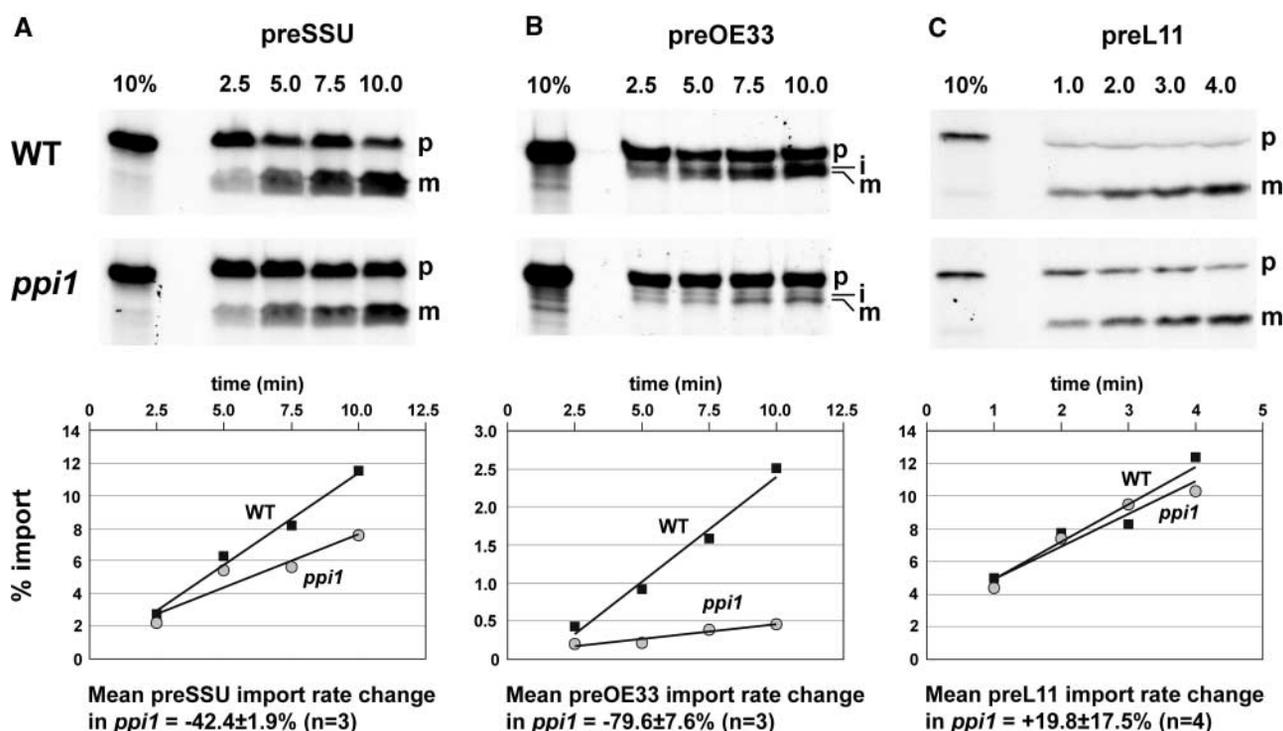
Although OE33 import was affected more strongly than SSU import in vitro (Figure 5), SSU accumulation was affected more strongly than OE33 accumulation in vivo (Figure 3, Table 1). This finding indicates that factors in addition to import efficiency influence steady state protein levels in *ppi1*. Nevertheless, the data clearly demonstrate that *ppi1* affects the import of different preproteins to different degrees; therefore, we conclude that these differential effects are the primary cause of the selective depletion of photosynthetic proteins in *ppi1* chloroplasts. Exactly how the *ppi1* mutation exerts this preprotein specificity is open to interpretation. The most likely explanation is that atToc33 participates preferentially in an import pathway (which perhaps also involves atToc159) that has a degree of substrate specificity. An alternative explanation is that reduced overall levels of a functionally redundant atToc33 + atToc34 pool in *ppi1* affects the import of those preproteins with the lowest affinity for the import apparatus most strongly. However, such a model does not explain why Arabidopsis has retained multiple isoforms of certain components of the import apparatus, such as Toc34 and Toc159, and only single isoforms of others, such as Tic110 and Tic40 (Jackson-Constan and Keegstra, 2001). Furthermore, this model is inconsistent with the fact that *ppi1* significantly affected the import of preSSU (Figure 5A). One of the reasons that preSSU has been used in so many import studies is that it is imported into chloroplasts with high efficiency.

### ***ppi1* Affects preSSU Import at the Level of Preprotein Binding**

Protein import into chloroplasts can be divided into three distinct stages in vitro based on energetic requirements (Olsen et al., 1989; Olsen and Keegstra, 1992; Ma et al., 1996; Kouranov and Schnell, 1997). These stages are assumed to correspond to sequential steps in the import process that occurs in vivo. The first stage of import is energy-independent binding, and this occurs in the absence of ATP. It involves the reversible interaction of preproteins with receptor components of the Toc complex. The second stage of import is early import intermediate formation, which requires low concentrations of ATP ( $< 100 \mu\text{M}$ ) in the intermembrane space. Preproteins at this stage are

**Table 2.** Selected Genes Showing Differential Expression in *ppi1*

Category/Gene No.	Signal Intensity in the Wild Type	Fold Change in <i>ppi1</i>	Gene Name/Product	Function
<b>Photosynthesis</b>				
<i>rbcL</i>	68.84	1.26	Plastid gene <i>rbcL</i> , Rubisco large subunit	Calvin cycle
At5g38430	46.48	0.73	<i>Ats1B</i> , Rubisco small subunit	Calvin cycle
At4g09650	1.43	0.72	<i>AtpD</i> , ATP synthase $\delta$ -subunit	ATP synthesis
At1g67740	2.55	0.71	<i>PsbY</i> , Y-subunit of PSII	Photosystem II
At5g61410	1.55	0.70	Ribulose-5-phosphate-3-epimerase	Calvin cycle
At1g67090	35.23	0.67	<i>Ats1A</i> , Rubisco small subunit a	Calvin cycle
At1g76100	0.87	0.65	<i>PetE1</i> , plastocyanin	Electron transport
At4g02770	6.58	0.63	<i>PsaD1</i> , D-subunit of PSI	Photosystem I
At3g47470	8.17	0.60	<i>Lhca4</i> , LHCl polypeptide	Light harvesting
At1g56190	1.16	0.43	Phosphoglycerate kinase	Calvin cycle
At1g60950	2.75	0.43	<i>PetF1</i> , ferredoxin	Electron transport
At2g34430	17.01	0.41	<i>Lhcb1.4</i> , LHClI polypeptide	Light harvesting
<b>Genetic system</b>				
At1g34380	0.20	2.34	DNA polymerase type I, putative	DNA replication
<i>rpoC1</i>	1.19	1.94	Plastid gene <i>rpoC1</i> , RNA polymerase	Transcription
At5g26710	0.91	1.45	Glutamyl-tRNA synthetase	Translation
At3g53460	0.78	1.43	RNA binding protein, CP29	RNA stability
At3g62910	0.47	1.38	Translation releasing factor, RF-1-like	Translation
At4g20980	0.49	1.30	Translation initiation factor, eIF3d-like	Translation
At5g24120	0.13	1.30	$\sigma$ -like factor	Transcription
At1g32990	0.91	1.30	50S plastid ribosomal protein, L11	Translation
At3g27850	0.85	1.27	50S plastid ribosomal protein, L12-C	Translation
At1g74980	0.23	1.24	30S plastid ribosomal protein, S9	Translation
<b>Metabolism</b>				
At1g69370	0.38	1.55	Chorismate mutase	Amino acid biosynthesis
At3g48560	0.50	1.44	Acetolactate synthase	Amino acid biosynthesis
At4g18240	0.20	1.40	Starch synthase-like protein	Starch biosynthesis
At1g29900	0.78	1.37	Carbamoyl phosphate synthetase, carb	Pyrimidine biosynthesis
At5g57030	0.53	1.34	Lycopene $\epsilon$ -cyclase	Carotenoid biosynthesis
At3g02610	0.42	1.34	Stearoyl-acyl carrier protein desaturase	Fatty acid biosynthesis
At4g25080	0.67	1.31	Mg-protoporphyrin IX methyltransferase	Tetrapyrrole biosynthesis
At2g26670	0.17	1.31	Heme oxygenase, HO1	Tetrapyrrole biosynthesis
At1g09830	1.46	1.28	Phosphoribosylglycineamide synthetase	Purine biosynthesis
At3g11670	0.85	1.26	Digalactosyldiacylglycerol synthase	Galactolipid biosynthesis
<b>Protein targeting</b>				
At5g28750	0.19	1.85	Tha4	Tat pathway, thylakoids
At4g33350	13.64	1.77	Tic22-IV	Protein import
At5g16620	0.23	1.63	Tic40	Protein import
At2g18710	0.33	1.48	SecY	Sec pathway, thylakoids
At5g03940	0.25	1.46	SRP54	SRP pathway, thylakoids
At4g02510	0.68	1.38	Toc159	Protein import
At4g03320	0.43	1.31	Tic20-IV	Protein import
At1g06950	0.30	1.31	Tic110	Protein import
At3g46740	0.78	1.25	Toc75-III	Protein import
At1g04940	2.96	0.67	Tic20-I	Protein import
<b>Other</b>				
At5g20720	0.49	1.78	Cpn21 chaperonin	Molecular chaperone
At4g39960	0.70	1.54	DnaJ homolog	Molecular chaperone
At3g13470	0.59	1.53	Chaperonin cpn60 $\beta$	Molecular chaperone
At2g28900	0.13	1.48	OEP16 homolog	Outer envelope porin
At4g24280	0.69	1.34	Hsp70 homolog	Molecular chaperone
At5g50920	1.14	1.30	Hsp100 homolog, ClpC-V	Molecular chaperone
At5g24020	0.14	1.28	MinD homolog	Chloroplast division
At5g55280	0.40	1.26	FtsZ homolog	Chloroplast division



**Figure 5.** Comparison of Chloroplast Import Rates in the Wild Type and *ppi1* for Different Preproteins.

In vitro–translated,  $^{35}\text{S}$ -Met–labeled preSSU (A), preOE33 (B), and preL11 (C) were imported into wild-type (WT) and mutant (*ppi1*) chloroplasts for 1.0 to 10.0 min, as indicated. Ten percent of the translation product added to each import reaction was loaded as a control (10%). Precursor (p), intermediate (i; OE33 only), and mature (m) protein forms are indicated at right of the gels. Quantifications for each import experiment are shown in the corresponding graph below each gel. Radioactivity associated with each mature band (intermediate and mature bands together in the case of OE33) was quantified and expressed as a percentage of the total preprotein-associated radioactivity added to each reaction. The data presented in the graphs correspond to the results shown in the gels, which are representative of several independent experiments. Mean import rate changes ( $\pm$ SE) derived from repeated experiments are given at bottom below the graphs.

inserted across the outer envelope membrane and in contact with components of the Tic complex; therefore, progression to this stage of import can be viewed as outer envelope translocation. The final stage of import is complete translocation, which requires high concentrations of ATP ( $>100 \mu\text{M}$ ) in the stroma. Progression to this stage requires both binding and outer envelope translocation as well as inner envelope translocation and processing by the stromal processing peptidase.

To determine the stage of import affected by *ppi1*, we quantified the amount of preSSU bound to ATP-depleted chloroplasts in import reactions containing different, limiting concentrations of ATP (Figure 6A). All binding reactions were performed in the dark to prevent ATP synthesis, but control import reactions were performed in the light to confirm that the import competence of the chloroplasts was high (data not shown). In the absence of ATP, binding of preSSU to chloroplasts was reduced by  $\sim 20\%$  in *ppi1* compared with the wild type (Figure 6A). This finding indicates that *ppi1* affects import at the level of energy-independent binding. Increased preprotein binding as ATP is increased to micromolar concentrations is indicative of early import intermediate formation. Because the reduction in binding observed in *ppi1* remained constant as ATP concentrations were increased up to  $100 \mu\text{M}$  (on average,

preSSU binding under ATP-limiting conditions was  $22.0 \pm 2.3\%$  lower for *ppi1* than for the wild type), it seems unlikely that *ppi1* also affects outer envelope translocation (Figure 6A). The slight decline in the amount of bound preSSU at  $100 \mu\text{M}$  ATP (seen in both genotypes) has been observed previously (Olsen et al., 1989) and presumably reflects the fact that a proportion of the preprotein has been translocated.

The efficiency of inner envelope translocation cannot be measured simply by quantifying the amount of protein imported in reactions containing high ATP concentrations ( $>100 \mu\text{M}$ ), because this depends not only on the efficiency of inner envelope translocation but also on the efficiency of energy-independent binding and outer envelope translocation. To separate binding and outer envelope translocation from translocation across the inner envelope membrane, we first bound preSSU to wild-type and *ppi1* chloroplasts under ATP-limiting conditions ( $50 \mu\text{M}$ ). After the formation of import intermediates, we divided each import reaction in two. To half of each reaction, we added 5 mM ATP, whereas to the other half, no additional ATP was added. The amount of translocated protein then was expressed as a percentage of that bound initially, taking into account any differences in binding efficiency (Figure 6B). Once again, an  $\sim 20\%$  deficiency in binding/outer envelope translocation was

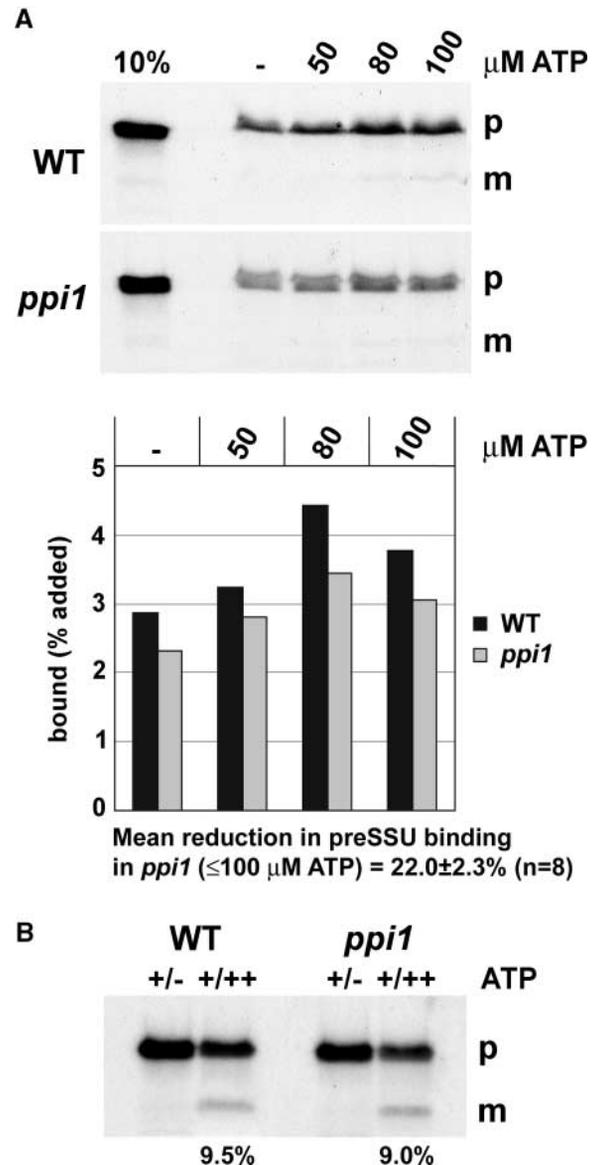
observed. Although the amount of protein translocated into *ppi1* chloroplasts was less in absolute terms, when normalized according to the amount of preSSU bound in each case, it was found to be essentially the same as that in the wild type (Figure 6B): 9.5% of bound protein was translocated in the wild type, and 9.0% of bound protein was translocated in *ppi1*. These data indicate that the effect of *ppi1* is restricted to preprotein binding, which is consistent with a role for atToc33 in preprotein recognition.

## DISCUSSION

We determined the relative levels of expression of *atTOC33* and *atTOC34* at different developmental stages and in different tissues to gain insight into the reason for the existence of these two homologous genes in Arabidopsis. The results showed that whereas *atTOC34* expression is relatively uniform and low level throughout development, *atTOC33* expression is upregulated strongly in new and expanding photosynthetic tissues (Figure 1). Our previous studies revealed that *atTOC33* and *atTOC34* both are expressed at higher levels in young plants and leaves than in older plants and leaves, but individual photosynthetic and nonphotosynthetic tissues were not investigated (Jarvis et al., 1998). The spatial expression patterns observed here are broadly in agreement with those described previously (Gutensohn et al., 2000), but this earlier study failed to address the relative expression levels of the two genes.

Our data correlate nicely with observations made using promoter- $\beta$ -glucuronidase (GUS) fusions and by in situ hybridization (Gutensohn et al., 2000). The strongest expression of *atTOC34-GUS* was observed in roots and floral organs, whereas *atTOC33-GUS* expression was strongest in leaves and, in flowers, occurred only in the external, photosynthetic tissues of the sepals. In situ hybridization experiments confirmed these floral expression patterns and, furthermore, revealed that in stems, *atTOC33* expression is restricted largely to the photosynthetic mesophyll tissues (Gutensohn et al., 2000). Together, these data suggest that *atTOC33* is particularly important for the biogenesis of photosynthetic proteins. The observation that *ppi1* affects the development of chloroplasts but not root plastids is consistent with this hypothesis (Jarvis et al., 1998; Yu and Li, 2001).

Examination of the *ppi1* chloroplast proteome by immunoblot analysis and DIGE revealed that proteins involved directly in photosynthesis are specifically deficient (Figures 2 and 3, Table 1). Interestingly, we also observed a slight enrichment of several nonphotosynthetic proteins in *ppi1* chloroplasts. One possible explanation for the apparent enrichment of nonphotosynthetic proteins in *ppi1* is a simple concentration effect caused by the selective depletion of photosynthetic proteins. Another possibility is that these proteins are upregulated specifically in *ppi1* in an attempt to compensate for perturbations in chloroplast metabolism. The upregulation of molecular chaperones could be an attempt to maximize the activity and/or half-life of those photosynthetic components that are imported successfully. Whatever the reason, the fact that *ppi1* chloroplasts are able to accumulate increased quantities of these proteins sug-



**Figure 6.** Effect of *ppi1* at Different Stages in the Import of preSSU.

**(A)** ATP-depleted wild-type (WT) or mutant (*ppi1*) chloroplasts and  $^{35}\text{S}$ -Met-labeled preSSU were incubated together in the absence or presence of ATP at the indicated concentrations for 10 min in darkness. Quantification of each chloroplast-bound preSSU band in the assay shown is given in the corresponding graph. The data shown are representative of four independent experiments, from which the mean reduction in preSSU binding ( $\pm\text{SE}$ ) was derived. m, mature; p, precursor.

**(B)** Import-chase results. Chloroplasts were incubated with preSSU under ATP-limiting conditions ( $50 \mu\text{M}$ ). The chloroplasts then were reisolated and divided into two aliquots. Half were incubated in the absence of additional ATP (left lanes; indicated +/-), and the other half were incubated in the presence of 5 mM ATP (right lanes; indicated +/+). Values shown below the fluorograph indicate the amount of imported SSU in the lanes labeled +/+, expressed as a percentage of the amount of bound preSSU in the lanes labeled +/- . The data shown are representative of repeated experiments.

gests that the effect of *ppi1* on protein import is, at least to some degree, preprotein specific.

To gain a more global view of the effects of the *ppi1* mutation, we used DNA array technology to survey the expression of 3292 different genes: 2661 genes with predicted or actual chloroplast transit peptides and 631 others. Remarkably, the *ppi1* mutation resulted in the specific downregulation of highly expressed, photosynthetic genes. By contrast, genes related to the plastid genetic system, nonphotosynthetic metabolism, and various transport processes were upregulated moderately in *ppi1* (Figure 4, Table 2; see also supplemental data online). Comparison of these data with those obtained previously (Kurth et al., 2002; Strand et al., 2003) indicates that the gene expression changes observed in *ppi1* are not a general response to the perturbation of chloroplast biogenesis. An Arabidopsis knockout for the L11 protein of the 50S ribosomal subunit (Pesaresi et al., 2001)—a mutant that has a visible phenotype similar to that of *ppi1*—was found to exhibit upregulated expression of both photosynthetic and plastid genetic system genes (Kurth et al., 2002). On the other hand, more severe defects in chloroplast biogenesis caused by the growth of plants on the herbicide norflurazon resulted in the downregulation of both photosynthetic and plastid genetic system genes (Strand et al., 2003). Therefore, it seems plausible that the specific downregulation of photosynthetic genes observed in *ppi1* is an adaptive response to the reduction in import efficiency of the corresponding proteins.

When we analyzed the import kinetics of three different preproteins in *ppi1*, we found that the effect of the mutation varied according to the preprotein in question (Figure 5). Interestingly, the two preproteins that were affected most strongly (preSSU and preOE33) are components of the photosynthetic apparatus, whereas the preprotein that was affected least (preL11) is a component of the chloroplast's endogenous genetic system. These data indicate that the selective depletion of photosynthetic proteins in *ppi1* most likely is caused by a differential effect of the mutation on the import of different preproteins. Consequent deficiencies in photosynthetic proteins would, in turn, be predicted to cause downregulated expression of the corresponding nuclear genes through retrograde signaling (Surpin et al., 2002; Jarvis, 2003). If we extend this argument to its logical conclusion, one might suppose that all of the genes that are downregulated in *ppi1* encode preproteins that require atToc33 for their import into chloroplasts. However, it also is possible that *ppi1* affects the import of just a few proteins and that it is the consequent deficiencies in these proteins that perturb, indirectly, the expression and/or accumulation of a range of other (functionally related) proteins. Thus, although it seems likely that many of the proteins listed in Tables 1 and 2 use atToc33 preferentially during their import into chloroplasts, it is not necessarily the case that they all do.

Although studies on protein import kinetics in *ppi1* have not been conducted previously, *ppi1* chloroplasts were shown to import reduced quantities of SSU, LHCII, and two different isoforms of POR over a fixed period of time (Jarvis et al., 1998). Although POR is not involved directly in photosynthesis, it is a light-dependent enzyme that catalyzes the committed step in chlorophyll biosynthesis. These import data are therefore con-

sistent with the results presented here and with the idea that atToc33 is involved preferentially in the import of abundant proteins that play roles closely related to photosynthesis.

By manipulating the ATP concentration in import assays, we found that the effect of *ppi1* on preSSU import is restricted to preprotein binding (Figure 6). This finding is consistent with a role for atToc33 in preprotein recognition. The precise role played by atToc33 (and other Toc34-related proteins) during preprotein binding remains uncertain, however, because current data suggest two different models for Toc receptor function. In the first model, Toc34 and Toc159 remain stably associated with the outer envelope membrane and interact with incident preproteins directly, much like the mitochondrial protein import receptors Tom20 and Tom22 (Pfanner and Geissler, 2001). In support of this model, preproteins can be cross-linked to pea Toc34 (psToc34) and/or psToc159 very early during import into pea chloroplasts (Perry and Keegstra, 1994; Ma et al., 1996; Kouranov and Schnell, 1997), and a direct and specific interaction between preproteins and psToc34 has been observed in vitro (Sveshnikova et al., 2000; Schleiff et al., 2002). However, not all cross-linking studies resulted in the identification of psToc34 (Perry and Keegstra, 1994; Ma et al., 1996), and in those that did, psToc34 appeared to interact with the mature region of the preprotein rather than with the transit peptide, as would be expected of a receptor (Kouranov and Schnell, 1997).

In the second model, soluble Toc159 binds to preproteins in the cytosol and then mediates their targeting to the chloroplast surface by docking at membrane-bound Toc34. In support of this model, an abundant cytosolic form of atToc159 has been observed (Hiltbrunner et al., 2001b), and the crystal structure of psToc34 suggests that heterodimerization between Toc34 and Toc159 may be possible (Sun et al., 2002). This model is reminiscent of signal recognition particle (SRP)-dependent protein targeting, in which the GTP binding protein SRP54 initiates preprotein translocation by docking at receptors that are themselves GTP binding proteins (Keenan et al., 2001).

Thus, preprotein recognition specificity by atToc33 may be mediated by its preferential interaction with a particular subset of preproteins, or alternatively by its preferential interaction with one or more of the different Toc159 isoforms. Bearing these possibilities in mind, it is interesting that atToc33 and atToc34 have been shown to exhibit differential competitor properties in an in vitro preSSU chloroplast binding assay: heterologously expressed atToc33 inhibited the binding of preSSU (translated using a rabbit reticulocyte system) to chloroplasts in a GTP-dependent manner, whereas atToc34 did not (Gutensohn et al., 2000). These data support the former possibility and suggest that the specific effect of *ppi1* on photosynthetic protein import is a reflection of differential preprotein binding properties of atToc33 and atToc34. Interestingly, during our analyses of protein import in the *ppi1* mutant, we consistently observed increased binding of unprocessed preL11 to mutant chloroplasts (Figure 5C). Increased abundance of a receptor with specificity for nonphotosynthetic preproteins (not necessarily associated with Toc complexes) might account for this observation. Although *atTOC34* transcript levels were not increased strongly in *ppi1* (Figure 1), it is possible that atToc34 accumulates to a

higher level in *ppi1* as a result of post-transcriptional processes.

Whatever the mode of action of the Toc GTPases, it seems likely that the proposed substrate specificity of the import apparatus is dependent on differences between the transit peptides of different preproteins. Transit peptides share a few general characteristics (e.g., they are rich in hydroxylated residues and deficient in acidic residues), but they differ widely in size and primary sequence and, unlike mitochondrial presequences, they do not share a common secondary structure (Bruce, 2001). This variability, together with the absence of precise information regarding the recognition process, makes it very difficult to predict which preproteins might be imported through a particular Toc complex or import pathway. Nevertheless, examination of the transit peptide sequences of the three preproteins used in this study revealed one property that correlated with import efficiency in the *ppi1* mutant: at pH 7.0, the preSSU, preOE33, and preL11 transit peptides are predicted to have net charges of 2.877, 3.046, and 6.206, respectively. It is conceivable that the higher charge of the preL11 transit peptide favored interaction with atToc34 (or atToc132- and atToc120-related proteins in the wheat germ translation system) and that the less highly charged preSSU and preOE33 transit peptides interact more favorably with atToc33 (or atToc159-related wheat germ proteins).

We have demonstrated that the *ppi1* mutation affects, with remarkable specificity, the expression, chloroplast import, and accumulation of photosynthetic proteins. Furthermore, the *ppi1* import defect appears to be restricted to the preprotein binding step, which is consistent with a role for atToc33 in preprotein recognition. Together, these data suggest that atToc33 is involved preferentially in the recognition of highly abundant, photosynthetic preproteins and that atToc34 may have preference for less abundant, nonphotosynthetic preproteins.

## METHODS

### Isolation of Arabidopsis Chloroplasts

Chloroplasts were isolated from 10-day-old wild-type and *ppi1* *Arabidopsis thaliana* plants (both Columbia-0 ecotype) grown in vitro as described previously (Aronsson and Jarvis, 2002). Plant material was homogenized for 3 to 4 s (wild type) or 2 to 3 s (*ppi1*) using a Polytron. After the first centrifugation, the homogenate (in 0.5 to 2 mL of isolation buffer) was loaded onto a linear Percoll gradient and centrifuged in a swing-out rotor at 7800g for 10 min. Intact chloroplasts were recovered and washed once with HS buffer (50 mM Hepes and 0.3 M sorbitol). The yield and intactness of the chloroplasts were determined as described previously (Aronsson and Jarvis, 2002).

### Immunoblot Analysis

Standard methods were used for SDS-PAGE, protein gel blot analysis, and detection. Wild-type and *ppi1* chloroplast preparations were quantified using Bradford reagent (Bio-Rad) and solubilized in 2× SDS-PAGE sample buffer (20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 4% [w/v] SDS, 0.125 M Tris/HCl, pH 6.8, and 0.04% [w/v] bromophenol blue). Amounts of protein loaded per lane ranged from 20 to 0.1 μg. To confirm equal loading of the samples, control gels were stained with Coomassie

Brilliant Blue R 250 (Fisher), and five different bands, selected at random, were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). On average, differences between the samples were ~6%. Where possible, filters were cut across the lanes so that one half could be probed with a “photosynthetic” antibody and the other half could be probed with a “nonphotosynthetic” antibody. Primary antibodies were raised in rabbits and were kindly provided by Kenneth Cline (University of Florida, Gainesville) (Hsp70; raised against pea stromal Hsp70), Ulf-Ingo Flügge (University of Cologne, Germany) (TPT; raised against the spinach protein), Bernhard Grimm (Humboldt University, Germany) (CPO; raised against the tobacco protein), Neil Hoffman (National Science Foundation, Arlington, VA) (LHCII; raised against the pea protein), Kenneth Keegstra (Michigan State University, East Lansing) (ClpC; raised against pea stromal ClpC), Kenton Ko (Queen’s University, Kingston, Ontario, Canada) (SSU; raised against the pea protein), and Henrik Scheller (The Royal Veterinary and Agricultural University, Copenhagen, Denmark) (PSI-D and FNR; both raised against barley proteins). The secondary antibody was an anti-rabbit IgG alkaline phosphatase conjugate (Sigma), and the detection reagent was 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate (Sigma).

### Chloroplast Proteomics

Isolated chloroplasts were precipitated using 0.1 M ammonium acetate in 100% methanol and resuspended in 10 mM Tris/HCl, pH 8.5, 8 M urea, and 2% (w/v) amidosulfobetaine-14 (Calbiochem). Protein concentrations were determined using a detergent-compatible protein quantification kit (Bio-Rad). Protein samples from wild-type and mutant chloroplasts (50 μg each) were labeled with complementary CyDyeDIGE fluors (Cy3 and Cy5; Amersham Biosciences) (Unlu et al., 1997), pooled, and run on either 13- or 24-cm, pH-3 to -10 or pH-4 to -7 immobilized pH gradient strips (Amersham Biosciences). Resolution in the second dimension was performed using Hoeffer SE 600 or Ettan DALT SDS-PAGE systems (Amersham Biosciences). Gels were scanned using a 2920-2D MasterImager (Amersham Biosciences), and images were exported as 16-bit TIFF files. Image analysis was performed using DeCyder software (Amersham Biosciences). Protein spots showing >1.5-fold change in volume ratio were excised manually after colloidal Coomassie blue staining and digested to peptides using trypsin on a MassPrepStation (Micro-mass, Manchester, UK). The resulting peptides were examined by liquid chromatography/tandem mass spectrometry (Q-Tof, CapLC, Micro-mass/Waters, www.waters.com). Fragmentation data were used to search the National Centre for Biotechnology Information database using the MASCOT search engine. Multiple chloroplast preparations from each genotype were analyzed. Each combination of samples was run in triplicate for each pH range, including one experiment in which the Cy-DyeDIGE fluor labeling was reversed. Spots excised from each gel were treated individually so that multiple identifications were performed for each protein.

### Protein Import into Arabidopsis Chloroplasts

Template DNA for the in vitro transcription/translation of preproteins was amplified by PCR from cDNA clones using M13 primers. The preSSU and preL11 cDNA clones were described previously (Aronsson and Jarvis, 2002), and the preOE33 cDNA clone was obtained from the ABRC (Columbus, OH) as clone 119E10T7. Transcription/translation was performed using a wheat germ system (Promega) containing <sup>35</sup>S-Met and T7 RNA polymerase according to the manufacturer’s instructions.

Import reactions were performed in HMS buffer (50 mM HEPES, 3 mM MgSO<sub>4</sub>, and 0.3 M sorbitol) containing 20 mM gluconic acid (potassium salt), 10 mM NaHCO<sub>3</sub>, and 0.2% (w/v) BSA (Aronsson and Jarvis, 2002).

Each 150- $\mu$ L import assay contained  $10^7$  chloroplasts, 5 mM MgATP, 10 mM Met, and translation product not exceeding 10% of the total volume. Import, thermolysin treatment, and quantification were performed according to Aronsson and Jarvis (2002).

To deplete endogenous ATP, chloroplasts were kept in the dark at room temperature for 20 min. Small molecules, including ATP, were removed from the translation products by Sephadex G 25 filtration (Pharmacia). MgATP was added to import reactions containing dark-adapted chloroplasts and ATP-depleted preprotein at different concentrations (50 to 5000  $\mu$ M) or was omitted completely. Reactions were incubated in the dark at 25°C for 10 min and then analyzed as usual.

Import-chase experiments used similarly prepared, ATP-depleted chloroplasts and translation products and were performed in the dark. First,  $2 \times 10^7$  chloroplasts were incubated with translation product in the presence of 50  $\mu$ M ATP, in a total volume of 300  $\mu$ L, for 10 min at 25°C. The chloroplasts then were reisolated by spinning through a 35% Percoll cushion in HS buffer at 2000g for 6 min and resuspended (with a cut 0.2-mL pipette tip) in 300  $\mu$ L of buffer containing all of the components of an import reaction except chloroplasts, ATP, and translation product. The reaction then was divided into two equal aliquots of 150  $\mu$ L. To one aliquot, ATP was added to a final concentration of 5 mM, and to the other aliquot, an equal volume of water was added. The import reactions were allowed to proceed for 10 min at 25°C and then analyzed as described previously.

### RNA Isolation

RNA was isolated from plant material grown in vitro (5 and 10 days old) or on soil (28 days old). Plant material (up to 4 g) was ground to a powder in liquid nitrogen, and nucleic acids were extracted with equal volumes (~5 mL each) of 100 mM Tris/HCl, pH 9.0, and phenol in 15-mL tubes. Samples were centrifuged at 3000g for 30 min at 4°C and then purified by phenol:chloroform:isoamyl alcohol (25:24:1) extraction. Nucleic acid was precipitated by adding 1.5 mL of 3.0 M NaOAc, pH 5.2, and 2 volumes of 100% ethanol and incubating overnight at -20°C. After centrifugation at 3000g for 45 min at 4°C, samples were dried, resuspended in 500  $\mu$ L of water, and transferred to 1.5-mL tubes. RNA was precipitated by adding 200  $\mu$ L of 10 M LiCl and incubating on ice overnight. Samples were centrifuged at 20,000g for 15 min at 4°C, and the pellets were washed with 70% (v/v) ethanol, dried, and resuspended in sterile water. Heating to 65°C was necessary to dissolve the RNA.

### RNA Gel Blot Analysis

Total RNA was fractionated by electrophoresis on 1.3% (w/v) agarose gels containing 1.85% (v/v) formaldehyde and Mops buffer [20 mM 3-(*N*-morpholino)-propanesulfonic acid, 1 mM EDTA, pH 8.0, and 50 mM NaOAc, pH 7.0] and transferred to Hybond NX membranes (Amersham Pharmacia Biotech) in  $10 \times$  SSC ( $1 \times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate). Membranes were probed with  $\alpha$ - $^{32}$ P-dCTP-labeled DNA fragments (Feinberg and Vogelstein, 1983) in 0.3 M sodium phosphate buffer, pH 7.2, containing 7% (w/v) SDS, 1 mM EDTA, pH 8.0, and 2% (w/v) BSA at 65°C overnight. Filters were washed at 65°C twice with  $2 \times$  SSC and 0.1% (w/v) SDS for 30 min, once with  $1 \times$  SSC and 0.1% (w/v) SDS for 20 min, and once with  $0.5 \times$  SSC and 0.1% (w/v) SDS for 10 min. Band quantification was performed using ImageQuant software (Molecular Dynamics).

Identical filters were probed simultaneously with *atTOC33* and *atTOC34* probes. The *atTOC33* and *atTOC34* probes corresponded to full-length cDNAs and were almost identical in length (1132 and 1158 bp, respectively) and GC content (40.99 and 41.28%, respectively). The probes were labeled simultaneously under identical conditions using the same isotope and were shown to have identical specific activities by scintillation counting. Hybridization, washing, and exposure steps were performed simultaneously under identical conditions. Cross-hybridiza-

tion of the probes between *atTOC33* and *atTOC34* (sequence homology of 50.2%) can be excluded, because the wash stringency used allowed only probe/target combinations with >83% identity to remain hybridized.

### DNA Array Analysis

The 1827 gene-specific tags (GSTs) described previously (Kurth et al., 2002) were combined with an additional 1465 GSTs from genes that encode proteins featuring a chloroplast transit peptide predicted by TargetP (Emanuelsson et al., 2000), resulting in a 3292-GST array (Richly et al., 2003). Amplification, quantification, verification, and spotting (in duplicate) of PCR products on nylon filters were performed as described (Varotto et al., 2001; Kurth et al., 2002). At least three experiments with different filters and independent cDNA probes from plant pools were performed for each condition, thus minimizing variation between individual plants, filters, or probes. cDNA probe synthesis was primed by a mixture of oligonucleotides matching the 3292 genes in the antisense orientation and hybridized to the GST array as described (Kurth et al., 2002). Images were read using a Storm PhosphorImager (Molecular Dynamics) imported into the ArrayVision program (version 6.0; Imaging Research, St. Catharines, Ontario, Canada), in which data were normalized with reference to all spots on the array (Kurth et al., 2002). After import of expression data into the ArrayStat program (version 1.0; Imaging Research), a *z* test (nominal  $\alpha$  set to 0.05) was performed to identify statistically significant differential expression values. The data obtained for all significantly differentially regulated genes are given in the supplemental data online.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Paul Jarvis, rpj3@le.ac.uk.

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