The Chloroplast Import Receptor Toc90 Partially Restores the Accumulation of Toc159 Client Proteins in the *Arabidopsis thaliana ppi2* Mutant

Sibylle Infanger[a,2], Sylvain Bischof[b,2], Andreas Hiltbrunner[c], Birgit Agne[d], Sacha Baginsky[d] and **Felix Kessler[a,1]**

a Laboratoire de physiologie végétale, Université de Neuchâtel, Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland
b Department of Biology, ETH Zurich, Universitätstrasse 2, 8092 Zürich, Switzerland
c University of Tübingen, Centre for Plant Molecular Biology (ZMBP), Department of Plant Physiology, auf der Morgenstelle 1, D-72076 Tübingen, Germany
d Institute of Biochemistry and Biotechnology, Martin-Luther-University Halle-Wittenberg, Weinbergweg 22, 06120 Halle (Saale)

ABSTRACT Successful import of hundreds of nucleus-encoded proteins is essential for chloroplast biogenesis. The import of cytosolic precursor proteins relies on the Toc- (translocon at the outer chloroplast membrane) and Tic- (translocon at the inner chloroplast membrane) complexes. In *Arabidopsis thaliana*, precursor recognition is mainly mediated by outer membrane receptors belonging to two gene families: Toc34/33 and Toc159/132/120/90. The role in import and precursor selectivity of these receptors has been intensively studied, but the function of Toc90 still remains unclear. Here, we report the ability of Toc90 to support the import of Toc159 client proteins. We show that the overexpression of Toc90 partially complements the albino knockout of Toc159 and restores photoautotrophic growth. Several lines of evidence including proteome profiling demonstrate the import and accumulation of proteins essential for chloroplast biogenesis and functionality.

Key words: chloroplast; protein import; Toc159; substrate specificity; proteomics.

INTRODUCTION

The chloroplast is a metabolic hub hosting a large number of primary pathways including photosynthesis, starch synthesis and breakdown, amino acid and lipid biosynthesis, as well as secondary metabolism. Chloroplast biogenesis therefore constitutes an essential step in plant growth and development. Of many hundred chloroplast-localized proteins, only about 100 remain encoded by the chloroplast genome. This implicates that nucleus-encoded proteins have to cross the chloroplast envelope membranes to reach their destination inside the organelle. Most nuclear-encoded proteins are synthesized as precursor proteins with cleavable N-terminal transit peptides and use an import route controlled by the Toc-/Tic- (translocon at the outer/inner membrane of the chloroplast) complexes (reviewed in Balsera et al., 2009; Agne and Kessler, 2009). In *Arabidopsis thaliana*, recognition and selectivity of cytosolic precursors are mediated by the members of two small families of GTP-binding receptors, Toc33 and Toc159. The phenotypes of knockouts of the receptor GTPases Toc33 and Toc159 termed *ppi1* (plastid protein import 1) and *ppi2*, respectively, showed their direct involvement in chloroplast biogenesis and the accumulation of photosynthesis-associated proteins (Jarvis et al., 1998; Bauer et al., 2000).

Ppi1 has a pale-green phenotype and can be rescued by the overexpression of Toc34, the homolog of Toc33, indicating functional redundancy (Jarvis et al., 1998; Kubis et al., 2003). The homozygous double mutant of Toc33 and Toc34 (*ppi1*/*ppi3*) was seedling lethal, while a mutant homozygous for *ppi1* and heterozygous for *ppi3* (*ppi1*/*Toc34*–/+) showed a clear deterioration of the pale *ppi1* phenotype (Constan et al., 2004). Toc33 has a much higher expression level than Toc34 during early development and in the different tissues except for roots, suggesting a more pronounced role of Toc34 in the import of non-photosynthetic proteins (Kubis et al., 2003). Immunoblotting, 2D–DIGE proteomics, and a DNA array analysis indicated that the reduction of activity of mainly photosynthesis-associated genes leads to a defect

[1] To whom correspondence should be addressed. E-mail felix.kessler@unine.ch, tel. ++41 32 718 2292.

[2] These authors contributed equally to this work.

at the protein level in *ppi1*. In contrast, genes encoding constitutive chloroplast proteins remained mostly unchanged in their expression (Kubis et al., 2003) and accumulated normally, proving that their import does not depend on Toc33.

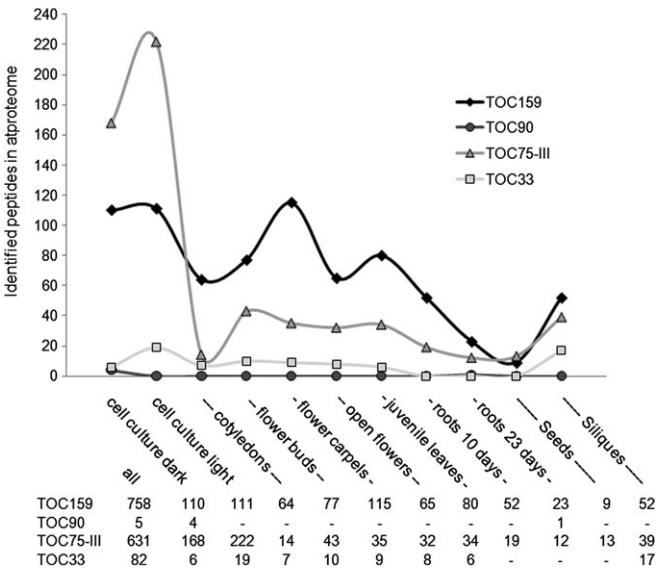
The Toc159 family comprises four different members Toc159/132/120/90. These four members differ mostly in the length of their N-terminal acidic domains, and evidence for the direct involvement of this domain in the functional specialization among these receptors is increasing (Inoue et al., 2010). The knockout of Toc159 results in an albino plant that is not able to grow beyond the cotyledon stage on soil (Bauer et al., 2000), thus underlining its importance for the accumulation of photosynthetic proteins. Transcription and accumulation of a number of abundant photosynthesis-associated proteins are very strongly affected but not completely repressed (Bauer et al., 2000). Interesting hints towards the specialized functions of the Toc159 family members came from three independent reverse genetic studies (Hiltbrunner et al., 2004; Ivanova et al., 2004; Kubis et al., 2004). None of the single knockouts of Toc132, Toc120, and Toc90 had a strong visible phenotype but *toc132*/*toc120* double homozygous plants appeared very pale green (Kubis et al., 2004) or were even embryo lethal (Ivanova et al., 2004), thus suggesting a functional overlap between Toc132 and Toc120. Like Toc34, in comparison to Toc33, Toc132 and Toc120 have a lower expression level than Toc159 in photosynthetic tissues. Major differences were observed when comparing the transcriptome and proteome profiles of the *toc132* knockout with *ppi1*, pointing towards specificity of Toc132 (and Toc120) for non-photosynthetic proteins (Kubis et al., 2004). Moreover, these two receptors exhibited precursor binding properties that were distinct from Toc159 (Ivanova et al., 2004). Last but not least, overexpression of either Toc132 or Toc120 failed to complement *ppi2*.

To date, the role of Toc90 remains elusive. *Toc132*/*toc90* and *toc120*/*toc90* double homozygous mutants showed no differences when compared with the *toc132* and *toc120* single mutants (Kubis et al., 2004). Furthermore, overexpression of Toc90 failed to complement *ppi2* (Kubis et al., 2004). Toc90 was described as a GTP-binding component that is associated with the Arabidopsis chloroplast import machinery (Hiltbrunner et al., 2004). Toc90 has a conserved G- and M-domain but in contrast to Toc159, -132, and -120, lacks the A-domain (Hiltbrunner et al., 2004; Kubis et al., 2004). Like the knockout of Toc120, plants lacking Toc90 termed *ppi4* had no recognizable phenotype (Hiltbrunner et al., 2004). Interestingly, the *ppi2*/*ppi4* double knockout appeared even paler than *ppi2*, and in comparison to *ppi2*, revealed a further reduction in chlorophyll a/b binding protein (CAB), thus suggesting a functional overlap between these two receptors. As for Toc159, the Toc90 transcript was up-regulated by light, suggesting a possible link to the import of photosynthetic proteins (Hiltbrunner et al., 2004). In this study, we show that the overexpression of Toc90 partially complements *ppi2* and restores sufficient protein import for photoautotrophic growth. Immunoblotting, *in vitro* import assays, and shotgun proteomics demonstrate the implication of Toc90 in import and accumulation of Toc159 client proteins.

RESULTS

Overexpression of Toc90 Partially Complements Toc159 Knockout

Based on the various hints that Toc90 might contribute to the import route for Toc159 client proteins (Kubis et al., 2004; Hiltbrunner et al., 2004), we investigated the function of Toc90 *in vivo*. Complementation of the albino mutant *ppi2* would provide direct evidence for a functional overlap between these two receptors. At the RNA level, the expression of Toc90 in seedlings is only about half that of Toc159 (Kubis et al., 2004; Hiltbrunner et al., 2004). At the protein level, the difference in abundance appears to be much more pronounced. Publicly available data of a large-scale proteome profiling study on Arabidopsis tissues (Baerenfaller et al., 2008) indicate that only five peptides were identified for Toc90 in comparison to a total of 758 for Toc159 in all tissues (Figure 1). Based on these indications, we attempted to strongly overexpress Toc90 in the *ppi2* background. Full-length Toc90 and a hexahistidine-tagged Toc90 (hisToc90; six histidine residues replacing the first 14 amino acids of Toc90) were engineered into the p3300.1 Cambia vector (pCAMBIA3300, CAMBIA, Australia), under the control of the strong constitutive Cauliflower mosaic virus 35S promoter and the nopaline synthase (nos) terminator. These constructs were then used to transform heterozygous *ppi2* plants using the floral dip



	all	cell culture dark	cell culture light	cotyledons	flower buds	flower carpels	open flowers	juvenile leaves	roots 10 days	roots 23 days	Seeds	Siliques
TOC159	758	110	111	64	77	115	65	80	52	23	9	52
TOC90	5	4	-	-	-	-	-	-	-	1	-	-
TOC75-III	631	168	222	14	43	35	32	34	19	12	13	39
TOC33	82	6	19	7	10	9	8	6	-	-	-	17

Figure 1. Protein Abundance of Toc Core Components in Wt Arabidopsis Tissues

The number of peptides identified in various wt Arabidopsis tissues is representative of the protein abundance of the Toc core components. The number of identified peptides is based on a large-scale proteome profiling study of wt Arabidopsis tissues (Baerenfaller et al., 2008; www.atproteome.ethz.ch).

method (Clough and Bent, 1998). Kanamycin resistance allowed selection for the *ppi2* background and BASTA resistance for the presence of the transgene. Tiny and very pale-green F1 plants resistant to both selection markers were obtained for both full-length Toc90 and hisToc90. Many of these plants did not survive. We decided to continue the characterization of a viable line expressing hisToc90 in the *ppi2* background (Figure 2A–2G). The selected line (hisToc90/*ppi2*) showed no segregation in F2 and had a uniformly pale-green phenotype in the following generations, indicating homozygosity of the hisToc90 transgene. Genotyping confirmed the homozygous *ppi2* background as well as the presence of hisToc90 transgene (Figure 2H). Quantitative RT–PCR showed a more than 40 times higher mRNA expression of hisToc90 in the hisToc90/*ppi2* line than in wild-type (wt) (Figure 2J). In contrast to *ppi2*, hisToc90/*ppi2* plants had pale-green cotyledons and were able to grow slowly and set seed on soil (Figure 2G). Chlorophyll measurements confirmed a clear enhancement of the pigment level in hisToc90/*ppi2* over *ppi2* (Figure 2I). But consistent with the pale-green phenotype, the chlorophyll content of hisToc90/*ppi2* leaves was still four times lower than in the wt.

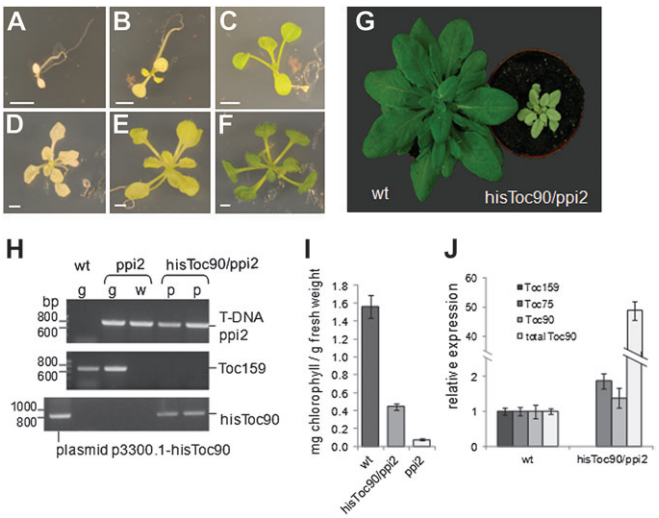


Figure 2. Phenotype, Genotype, Chlorophyll Content, and mRNA Expression Level of hisToc90/*ppi2* line 23.

(A–F) Partial rescue of the near-albino phenotype of 2 and 4-week-old *ppi2* (A, D) by overexpression of hisToc90 (B, E). HisToc90/*ppi2* plants remain paler than wt (C, F). Bar = 2 mm.

(G) Eight-week-old soil-grown hisToc90/*ppi2* plants are delayed in development when compared to wt plants.

(H) PCR validation of the homozygous *ppi2* background and of the presence of the hisToc90 transgene in hisToc90/*ppi2* plants.

(I) Chlorophyll content of 5-week-old *ppi2*, hisToc90/*ppi2*, and wt plants.

(J) RNA expression levels of *Toc159*, *Toc75*, endogenous *Toc90*, and total *Toc90* quantified by RT–PCR in 5-week-old hisToc90/*ppi2* and wt plants.

Recovery of Chloroplast Biogenesis in hisToc90/*ppi2* Plants

Defects in chloroplast biogenesis have been reported in mutants lacking important components of the Toc complex (reviewed in Balsera et al., 2009; Agne and Kessler, 2009). As photoautotrophic growth relies on functional chloroplasts, we wanted to know to what extent the overexpression of hisToc90 could salvage plastid differentiation in *ppi2*. Transverse sections stained by toluidine blue illustrate a severe lack of cellular organization in *ppi2* leaves that is recovered in hisToc90/*ppi2* but not to the level of structuring observed in the wt (Figure 3A–3C). Epidermal margins are regular and the parenchyma clearly contains palisade and spongy tissues. Electron microscopic images of leaf ultrastructure show the lack of abundant thylakoid membranes in *ppi2* plastids as already reported previously (Figure 3D–3E; Bauer et al., 2000; Yu and Li, 2001). In comparison to wt, hisToc90/*ppi2* plastids are not fully developed and lack an extensive thylakoïd system. However, the presence of a rudimentary thylakoïd membrane system suggests that the expression of TOC90 favors the import of photosynthetic proteins and the differentiation of functional chloroplasts.

HisToc90/*ppi2* Plants Import and Accumulate Photosynthetic Proteins

To determine the nature of proteins in hisToc90/*ppi2* plastids, we first analyzed the accumulation of LSU (large subunit of Rubisco), CAB (chlorophyll a/b binding protein), and SSU (small

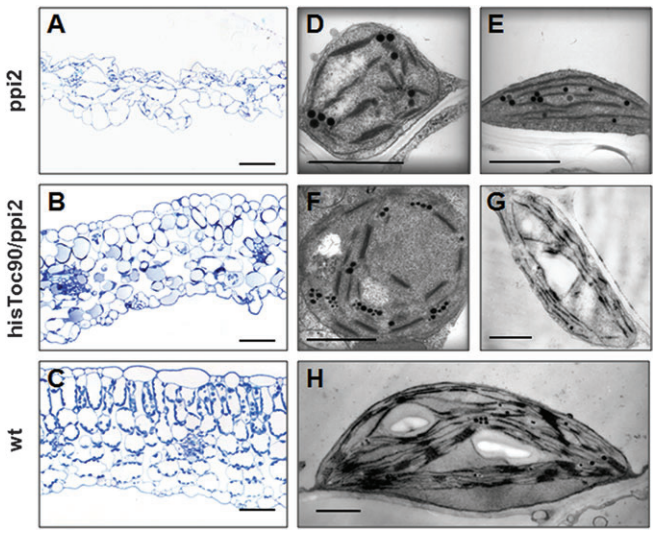


Figure 3. Leaf Structure and Chloroplast Ultra Structure of 5-Week-Old True Leaves.

(A–C) Leaf cross-sections of *ppi2*, hisToc90/*ppi2*, and wt stained with toluidine blue. Bar = 50 μm.

(D–H) Electron microscopic images of *ppi2*, hisToc90/*ppi2*, and wt leaves. The formation of thylakoïd stacks in hisToc90/*ppi2* (G) in comparison to *ppi2* can be observed (D, E). Nevertheless, chloroplast biogenesis in hisToc90/*ppi2* is not as advanced as in wt (H). Also, some undifferentiated plastids can be observed in hisToc90/*ppi2* (F). Bar = 1 μm.

subunit of Rubisco), which had previously been used as markers in *ppi2* (Bauer et al., 2000) (Figure 4A). Immunoblotting revealed a significantly higher level of all three proteins in hisToc90/*ppi2* leaves when compared to *ppi2*, indicating that the accumulation of these proteins was enhanced. The relative molecular masses of CAB and SSU, identical to those in wt, indicated that these photosynthetic proteins were processed to their mature forms and therefore likely had been imported into the chloroplast. We also looked at the principal components of the Toc import machinery (Figure 4B). Immunoblotting showed no changes in the abundance of the core components of the Toc-complex, Toc75 and Toc33. In contrast, the levels of Toc132 slightly increased in *ppi2* and hisToc90/*ppi2* in comparison to wt.

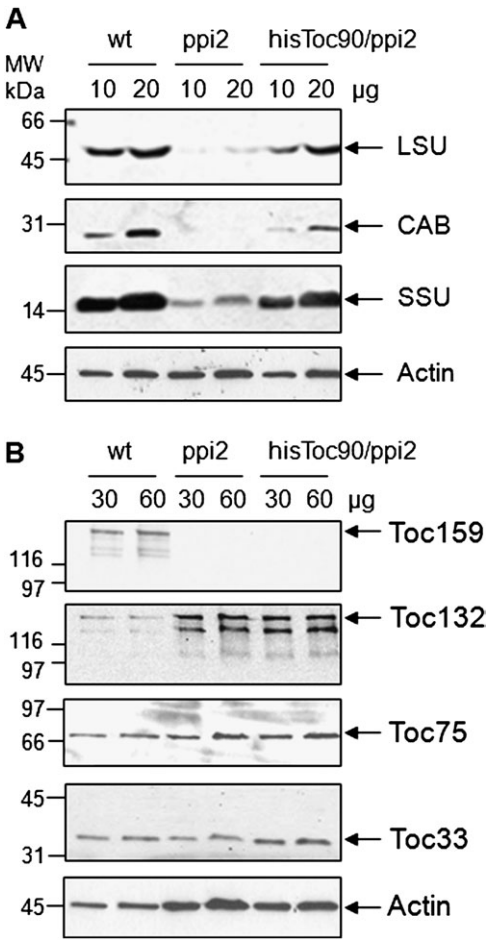


Figure 4. Western Blot Analysis of Selected Photosynthetic Proteins and Toc-Complex Components.

(A) Immunoblotting using antibodies raised against the small and large subunits of Rubisco (SSU and LSU, respectively) and CAB (chlorophyll a/b binding protein). Total proteins were extracted from 4-week-old *ppi2*, hisToc90/*ppi2*, and wt plants. Immunoblotting of 10 and 20 μg total leaf protein was carried out.

(B) Immunoblotting using antibodies raised against the Toc-complex components Toc159, Toc132, Toc75, and Toc33. Immunoblotting of 30 and 60 μg total leaf protein was carried out. Antibodies specific for plant actin were used as an external reference.

In order to test the functionality of precursor import in hisToc90/*ppi2*, we performed protein import assays into isolated plastids using radio-labeled pSSU as a reference import substrate (Figure 5). Isolated hisToc90/*ppi2* chloroplasts imported and processed pSSU but at slightly less than the normal wt rate. Together with the immunoblotting experiments, these import experiments indicated that protein import was partially recovered in hisToc90/*ppi2* and that several photosynthetic proteins accumulated albeit not to wt levels (Figures 4A and 5). These results were consistent with the pale-green phenotype of the hisToc90/*ppi2* plants. Immunoblotting did not allow an unbiased assessment of protein accumulation in hisToc90/*ppi2* because the analysis was restricted to only a few candidate proteins for which antibodies were available. We therefore analyzed the protein accumulation in *ppi2*, hisToc90/*ppi2*, and wt in a small-scale proteomics experiment.

Analysis of Protein Accumulation in *ppi2*, hisToc90/*ppi2*, and wt Cotyledons

To further characterize the role of Toc90 in chloroplast development, we analyzed the most abundant proteins that dominate the proteome of *ppi2*, hisToc90/*ppi2*, and wt cotyledons. Even on a small scale, shotgun proteomics offer the opportunity to identify a large number of proteins (resulting in a 'proteome snapshot') (Bräutigam and Weber, 2009; Joyard et al., 2009). Proteins were extracted from 6-day-old cotyledons grown on soil (Bauer et al., 2000). Equivalent amounts of protein were separated by SDS–PAGE and subjected to tandem mass spectrometry (LC–MS/MS) analysis. In total, 288 non-redundant proteins were identified: 134 in *ppi2*, 165 in hisToc90/*ppi2*, and 164 in wt (Figure 6A). Of these 288 proteins, 52 were exclusively identified in *ppi2*, 46 exclusively in hisToc90/*ppi2*, and 63 were unique to the wt. We assigned an intracellular localization to all identified proteins using the SUBA database. This database integrates different localization criteria from prediction tools, proteomics, and biochemical studies (Heazlewood et al., 2007; http://suba.plantenergy.uwa.edu.au). All identified proteins and their localization annotation are listed in Supplemental Table 1.

Accumulation of chloroplast-localized proteins in the cell does not necessarily indicate that they were successfully imported. *In vitro* import of pSSU into isolated hisToc90/*ppi2* chloroplasts argues in favor of a functional import machinery (Figure 5). In addition, all chloroplast-localized proteins in this study have previously been identified in other proteomics studies of isolated chloroplasts (Kleffmann et al., 2004; Zybailov et al., 2008; Joyard et al., 2009) and recent data suggested that precursor proteins that accumulate in the cytosol are degraded by the proteasome system (Lee et al., 2010). Given that we deliberately analyzed abundant proteins, we can assume that we exclusively identified the mature proteins after import into chloroplasts. Consistent with this assumption, we did not detect any peptides corresponding to the predicted N-terminal

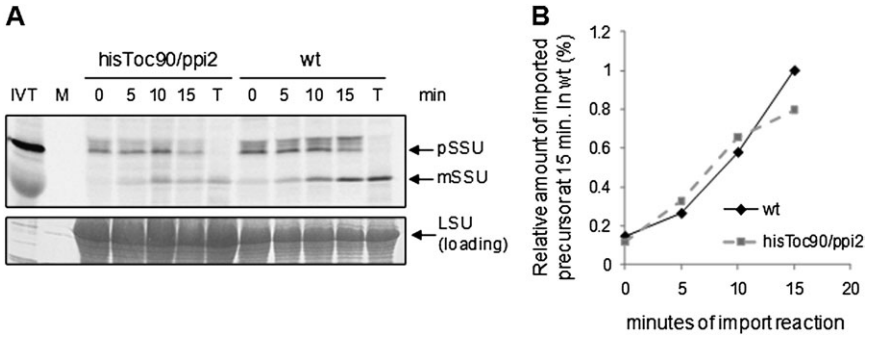


Figure 5. *In vitro* Import of Synthetic [^{35}S]pSSU in Isolated Chloroplasts of hisToc90/*ppi2* and Wt.

(A) *In vitro* import of synthetic [^{35}S]pSSU. Isolated chloroplasts of 4-week-old hisToc90/*ppi2* and wt plants were incubated with *in vitro* translated and [^{35}S]-radiolabeled precursor of the small subunit of Rubisco (pSSU) for 0, 5, 10, and 15 min. Protease resistance after import of the precursor was verified by thermolysin treatment of chloroplasts after 15 min of import (T). Chloroplasts were re-isolated and equal protein amounts were separated by SDS–PAGE followed by Coomassie Blue staining (lower panel). Radiolabeled pSSU and mature SSU (mSSU) were visualized using a PhosphorImager (upper panel).

(B) Quantification of the amount of imported mSSU in three independent import experiments. 100% import was defined as [^{35}S] mSSU accumulated after 15 min of import reaction time. IVT, *in vitro* translate; M, molecular mass standards.

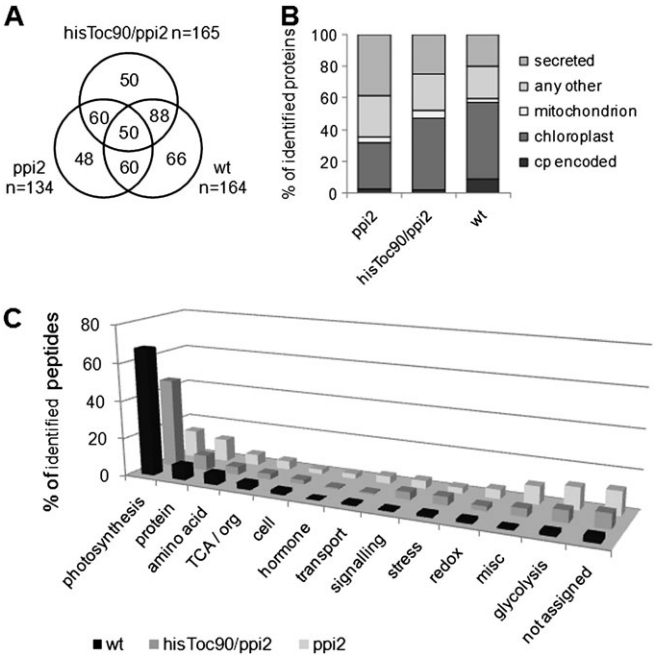


Figure 6. Protein Accumulation in *ppi2*, hisToc90/*ppi2*, and Wt Cotyledons.

(A) Protein accumulation in 6-day-old *ppi2*, hisToc90/*ppi2* and wt cotyledons. Total proteins were extracted, separated by SDS–PAGE, digested by trypsin, and subjected to LC–MS/MS using an LCQ-DECA XP mass spectrometer. Fifty of a total of 288 identified proteins were identified in all three profiled genotypes.

(B) Localization distribution of all proteins identified in *ppi2*, hisToc90/*ppi2*, and wt. Localization was assigned using the SUBA database (Heazlewood et al., 2007; http://suba.plantenergy.uwa.edu.au).

(C) Functional categorization of all peptides identified in *ppi2*, hisToc90/*ppi2*, and wt. Functional categorization is based on Mapman (Thimm et al., 2004; http://mapman.mpimp-golm.mpg.de).

transit peptide regions of the chloroplast-localized proteins (Nielsen et al., 1997; Emanuelsson et al., 2000, 2007; Armbruster et al., 2009).

The differences observed between the proteomes of *ppi2*, hisToc90/*ppi2*, and wt should reflect direct or indirect consequences of the loss of Toc159 in *ppi2* and of the partial re-establishment of protein import by the overexpression of hisToc90 in hisToc90/*ppi2*. Indeed, the number of identified proteins belonging to the chloroplast increased significantly from *ppi2* to hisToc90/*ppi2* to wt (15–38–59%), indicating the recovery of chloroplast biogenesis (Figure 6B). This appears even more striking when comparing the respective number of identified peptides: 161–653–905 (*ppi2*–hisToc90/*ppi2*–wt). These data further support the increased accumulation of chloroplast-localized proteins in hisToc90/*ppi2* compared to *ppi2* but not to wt levels, as already observed in the immunoblotting experiments (Figure 4A). Similarly, we observed that 24 of 27 proteins that were preferentially detected in hisToc90/*ppi2* and wt compared to *ppi2* belong to the chloroplast (Table 1). Twenty of these 24 chloroplast-localized proteins are involved in photosynthesis based on Mapman annotation (Thimm et al., 2004; http://mapman.mpimp-golm.mpg.de/). In contrast, only two of 12 proteins that were preferentially detected in *ppi2* compared to hisToc90/*ppi2* and wt were predicted to localize to the chloroplast (see Supplemental Table 1). The 10 other proteins are predicted to localize to the secretory pathway or to an undefined location and distribute among diverse metabolic functions. Preferentially identified proteins were defined as being identified with at least twice as many peptides in the respective genotype. It has been shown for LC–MS/MS analyses of digested proteomes that the number of matching MS/MS spectra (SPCs) correlates with protein abundance if there are sufficient SPCs per protein

Table 1. Identified Chloroplast-Localized Proteins.

Locus	Description	Total number of peptides			Metabolic bin
		ppi2	hisToc90/*ppi2*	wt	
	Proteins preferentially detected in *ppi2* only (*n* = 2)				
AT2G47730	ATGSTF8 (GLUTATHIONE S-TRANSFERASE 8)	5	2	1	Misc.
AT3G19170	ATPREP1/ATZNMP (PRESEQUENCE PROTEASE 1)	5	0	0	Protein
	Proteins preferentially detected in hisToc90/*ppi2* and wt compared to *ppi2* (*n* = 24)				
AT1G06680	PSBP-1 (OXYGEN-EVOLVING ENHANCER PROTEIN 2)	0	6	8	Photosynthesis
AT1G15820	LHCB6 (LIGHT HARVESTING COMPLEX PSII)	0	6	16	Photosynthesis
AT1G42970	GAPB (GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE B SUBUNIT)	0	9	9	Photosynthesis
AT1G55490	CPN60B (CHAPERONIN 60 BETA)	7	40	23	Photosynthesis
AT1G56190	Phosphoglycerate kinase, putative	7	32	15	Photosynthesis
AT1G61520	LHCA3 (Photosystem I light harvesting complex gene 3)	0	9	18	Photosynthesis
AT1G67090	Ribulose bisphosphate carboxylase small chain 1A	1	5	4	Photosynthesis
AT2G39730	RCA (RUBISCO ACTIVASE)	2	14	9	Photosynthesis
AT3G01500	CA1 (CARBONIC ANHYDRASE 1)	0	4	15	TCA/org
AT3G11630	2-cys peroxiredoxin, chloroplast (BAS1)	4	8	9	Redox
AT3G12780	PGK1 (PHOSPHOGLYCERATE KINASE 1)	0	40	20	Photosynthesis
AT3G26650	GAPA (GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE A SUBUNIT)	0	6	7	Photosynthesis
AT4G04640	ATPC1 (ATP synthase gamma chain 1)	0	8	7	Photosynthesis
AT4G05180	PSBQ/PSBQ-2 (photosystem II subunit Q-2)	0	5	12	Photosynthesis
AT4G10340	LHCB5 (LIGHT HARVESTING COMPLEX OF PHOTOSYSTEM II 5)	2	8	29	Photosynthesis
AT4G20360	AtRABE1b/AtRab8D (Arabidopsis Rab GTPase homolog E1b)	2	4	12	Protein
AT4G21280	PSBQ/PSBQ-1/PSBQA	3	13	21	Photosynthesis
AT4G32260	ATP synthase family	0	4	5	Photosynthesis
AT5G38410	Ribulose bisphosphate carboxylase small chain 3B	0	5	7	Photosynthesis
AT5G50920	CLPC (HEAT SHOCK PROTEIN 93-V)	0	12	12	Protein
AT5G66570	PSBO-1 (OXYGEN-EVOLVING ENHANCER 33)	0	11	17	Photosynthesis
ATCG00120	Encodes the ATPase alpha subunit	2	36	45	Photosynthesis
ATCG00480	Chloroplast-encoded gene for beta subunit of ATP synthase	1	30	55	Photosynthesis
ATCG00490	Large subunit of RUBISCO	30	146	240	Photosynthesis
	Proteins preferentially detected in hisToc90/*ppi2* (*n* = 6)				
AT1G12410	CLPR2 (Clp protease proteolytic subunit 2)	0	5	0	Protein
AT1G29910	CAB3 (CHLOROPHYLL A/B BINDING PROTEIN 3)	6	18	3	Photosynthesis
AT2G28000	CPN60A (chloroplast/60 kDa chaperonin alpha subunit)	8	26	11	Photosynthesis
AT4G24280	CPHSC70-1 (chloroplast heat shock protein 70–1)	8	25	3	Stress
AT5G20720	CPN20 (CHAPERONIN 20)	3	10	5	Protein
AT5G61410	RPE (EMBRYO DEFECTIVE 2728)	2	4	0	Photosynthesis
	Proteins preferentially detected in wt (*n* = 17)				
AT1G03130	PSAD-2 (photosystem I subunit D-2)	0	2	6	Photosynthesis
AT1G31330	PSAF (photosystem I subunit F)	0	2	9	Photosynthesis
AT1G32060	PRK (PHOSPHORIBULOKINASE)	0	1	4	Photosynthesis
AT1G44575	NPQ4 (NONPHOTOCHEMICAL QUENCHING)	0	0	16	Photosynthesis
AT3G04790	Ribose 5-phosphate isomerase-related	0	0	5	Photosynthesis
AT3G47470	LHCA4 (Photosystem I light harvesting complex gene 4)	0	2	5	Photosynthesis
AT3G54890	LHCA1; chlorophyll binding	0	0	5	Photosynthesis
AT3G60750	Transketolase, putative	0	0	22	Photosynthesis
AT5G01530	Chlorophyll A–B binding protein CP29 (LHCB4)	0	0	10	Photosynthesis
AT5G04140	GLU1 (FERREDOXIN-DEPENDENT GLUTAMATE SYNTHASE 1)	2	0	7	N-metabolism
AT5G38430	Ribulose bisphosphate carboxylase small chain 1B	0	0	4	Photosynthesis

Table 1. Continued

Locus	Description	Total number of peptides			Metabolic bin
		ppi2	hisToc90/*ppi2*	wt	
AT5G54270	LHCB3 (LIGHT-HARVESTING CHLOROPHYLL BINDING PROTEIN 3)	0	0	4	Photosynthesis
ATCG00020	Encodes chlorophyll binding protein D1	0	0	9	Photosynthesis
ATCG00270	PSII D2 protein	0	0	14	Photosynthesis
ATCG00280	Chloroplast gene encoding a CP43 subunit of the photosystem II reaction center	0	0	4	Photosynthesis
ATCG00540	Encodes cytochrome f apoprotein	0	0	9	Photosynthesis
ATCG00680	Encodes for CP47, subunit of the photosystem II reaction center	0	0	24	Photosynthesis
	Proteins not matching above categories (*n* = 7)				
AT4G31990	ASP5 (ASPARTATE AMINOTRANSFERASE 5)	4	4	0	Metabolism
AT1G12900	GAPA-2; glyceraldehyde-3-phosphate dehydrogenase	5	2	6	Photosynthesis
AT2G21330	Fructose-bisphosphate aldolase, putative	1	4	3	Photosynthesis
AT2G37220	29 kDa ribonucleoprotein, chloroplast, putative	3	6	5	RNA
AT4G38970	Fructose-bisphosphate aldolase, putative	7	7	4	Photosynthesis
AT5G14740	CA2 (BETA CARBONIC ANHYDRASE 2)	6	10	16	TCA/org
AT5G35630	GS2 (GLUTAMINE SYNTHETASE 2)	3	0	4	N-metabolism

Chloroplast-localized proteins identified with at least four unique peptides in one genotype. Localization annotation was based on the database SUBA (Heazlewood et al., 2007; http://suba.plantenergy.uwa.edu.au). Preferentially identified proteins were defined as being identified with at least twice as many peptides in the respective genotype.

(Lu et al., 2007; Zybailov et al., 2009). Therefore, only proteins detected with at least four peptides were taken into account.

Despite the deficiency in chloroplast development, 32 out of 76 proteins identified in both hisToc90/*ppi2* and *ppi2* were chloroplast-localized and half of these are involved in photosynthesis (Figure 6A; see Supplemental Table 1). This indicates that some photosynthetic and non-photosynthetic proteins already accumulate in *ppi2* despite the absence of Toc159 (Figure 6C). The presence of other import receptors such as Toc132, Toc120, and endogenous Toc90 are probably sufficient to allow import in *ppi2* but at a limited rate (Figure 4B). Furthermore, 50 proteins of these 76 were also identified in the wt (Figure 6A). Twenty-three of these 50 proteins are chloroplast-localized and 14 of these 23 are involved in photosynthesis. An overview of the distribution of the metabolic functions for all identified peptides is given in Figure 6C.

DISCUSSION

The aim of this work was to assess the role of the plastid import receptor Toc90 in chloroplast biogenesis. Proteomics studies indicate that Toc90 is a far less abundant than the prominent import receptor Toc159 in chloroplasts (Baerenfaller et al., 2008; Joyard et al., 2009). Here, were demonstrate that overexpression of Toc90 results in partial complementation of the albino *ppi2* mutant lacking Toc159, thus revealing functional redundancies between Toc90 and Toc159. Partially complemented hisToc90/*ppi2* plants have a pale-green phenotype and are able to grow on soil. In contrast, *ppi2* plants are heterotrophic and must rely on external sucrose supply from the growth medium. Compared to *ppi2*, hisToc90/*ppi2* plants have higher chlorophyll content and contain more developed thylakoid membranes. These data are somewhat surprising, considering that an earlier attempt to complement *ppi2* with Toc90 failed (Kubis et al., 2004). In that study, Toc90 expression was 10 times lower than reported here. This may have been insufficient for *ppi2* complementation and suggests a strong dosage dependence. Also, those complementation experiments were performed in a different genetic background. Kubis and colleagues used an introgressed Columbia-0 (Col-0) ecotype *toc159* line. In this ecotype, they did not observe any obvious effects on *toc159* phenotypic severity in the homozygous double knockout of Toc90 and Toc159 line. In contrast in the Wassilewskija ecotype (Ws), the absence of Toc90 led to a clear deterioration of the *ppi2* phenotype (Hiltbrunner et al., 2004). Nonetheless, in both Col-0 and Ws ecotypes, functional redundancy between Toc132 and Toc120 was shown, and the overexpression of neither one could complement *ppi2* (Ivanova et al., 2004; Kubis et al., 2004).

Partial complementation of *ppi2* strongly supports a functional overlap between Toc159 and Toc90. High mRNA expression levels of Toc90 seem important but only result in partial complementation, suggesting that the functional overlap is limited. Despite the 40-fold mRNA overexpression of hisToc90 in partially complemented *ppi2* plants, we were not able to detect the his-tagged protein by immunoblotting or by LC–MS/MS, but in view of the extremely low levels of Toc90 protein in wt tissues (Figure 1), this is not entirely surprising.

The molecular features that determine the partial functional overlap are not obvious. Toc159 and Toc90 share only 30.5% identity over their entire length and 44.3% identity in their respective G-domain (Kubis et al., 2004). In contrast,

Toc132 and Toc120, which are highly functionally redundant, share 68.9 and 93.4% sequence identity over their entire length and in their G-domain, respectively, and are most different in their A-domains. It therefore appears likely that currently unidentified features in the G- and M-domains determine the extent of the functional overlap between Toc159 and Toc90. GTP-binding and -hydrolysis activities are important features regulating protein import. But, recently, a Toc159 mutant affected in GTP-binding and -hydrolysis was described to target and assemble into Toc complexes, and to still support protein import *in vivo* (Agne et al., 2009).

Differences in precursor binding abilities might influence the import efficiency of Toc159 and Toc90. Removal of the A- and G-domains has been shown to abolish detectable binding of precursors at the chloroplast surface, although precursor translocation could still occur (Chen et al., 2000). Precise docking sites for cytosolic precursors are not known yet but it has recently been demonstrated that the A-domains determines specificity of Toc159 towards 'photosynthetis-associated' preproteins and that of Toc120/-132 towards 'housekeeping' preproteins (Inoue et al., 2010).

The M-domains of Toc159 and Toc90 are only 30.9% similar, which might potentially be a reason for a less efficient association of Toc90 with the outer membrane and the Toc core components Toc33 and Toc75. Although it has clearly been shown that Toc90 is targeted to the chloroplast outer membrane as a GFP-fusion protein, that Toc90 associates with the Toc core complex *in vitro*, and that its G-domain binds to the G-domain of Toc33 (Hiltbrunner et al., 2004), it is conceivable that Toc90 import efficiency may be reduced at that level.

Photosynthesis-associated proteins were preferentially accumulating in hisToc90/*ppi2* over *ppi2* (Figure 6C). The ability of Toc90, which lacks an A-domain to support protein import in the absence of Toc159, confirms that the A-domain is not absolutely required for translocation. But it may still contribute towards client specificity. A potential regulatory or specificity-enhancing role of the A-domain that improves import efficiency appears all the more plausible given that the overexpression of Toc90 does not restore wt import rates. The absence of the Toc159-typical A-domain may render Toc90 promiscuous towards either 'house-keeping' or 'photosynthesis-associated' preprotein classes (Kessler and Schnell, 2009) and may thereby allow it to partially complement *ppi2*. It remains to be seen whether Toc90 also complements Toc132 mutant plants. It has recently been shown that the A-domain of Toc159 is intrinsically disordered and may therefore extend far into the cytosol (Richardson et al., 2009) but how the A-domain of the Toc159 receptor family would influence import specificity still remains to be elucidated. Hyperphosphorylation of the A-domain may well contribute to the regulation of preprotein specificity (Agne et al., 2010).

In vitro import assays of pSSU confirmed the ability of isolated hisToc90/*ppi2* plastids to import proteins but at a slightly lower rate than wt. The relatively moderate difference in import efficiency might appear surprising regarding the pale phenotype of partially rescued hisToc90/*ppi2* in comparison to wt. For the *in vitro* import assays, the number of chloroplasts used was normalized according to the respective chlorophyll content. Since hisToc90/*ppi2* has a reduced chlorophyll content, the number of hisToc90/*ppi2* plastids used for the import assays may have been slightly higher than for the wt. However, the import assays demonstrated that isolated hisToc90/*ppi2* plastids were able to import proteins quite efficiently, essential for the functional rescue of *ppi2*. It seems that the high expression of Toc90 in hisToc90/*ppi2* plants reestablishes a protein import rate sufficiently rapid for limited chloroplast biogenesis and the onset of photoautotrophic growth. Supplying growth medium with sucrose allows *ppi2* to grow beyond the cotyledon stage and even to flower. Remarkably, LSU and mature SSU accumulated to low levels even in *ppi2* (Figure 4A). Proteome profiling revealed an additional 32 chloroplast-localized proteins that were identified in hisToc90/*ppi2* and in *ppi2* (see Supplemental Table 1). This indicates that protein import is probably already functional at low rates in almost undifferentiated *ppi2* plastids. The presence of the other Toc receptors Toc132, Toc120, and the weakly expressed Toc90 probably accounts for the limited protein accumulation observed in *ppi2* plastids. Immunoblotting showed a stable protein level of the core components of the Toc-complex, Toc75 and Toc33. A slight up-regulation of Toc132 in *ppi2* and hisToc90/*ppi2* was observed when compared to the wt. This might potentially be explained by a compensatory mechanism to stabilize the import complex deprived of Toc159, one of its key components. A specific functional rescue of Toc159 by Toc132 seems unlikely based on the failure to complement *ppi2* by overexpressing Toc132 in two independent studies (Kubis et al., 2004; Ivanova et al., 2004).

The Western blotting experiments were extended by a small-scale proteomics experiment ('proteome snapshot'). The proteomics data obtained here give useful information on the accumulation of highly abundant proteins in the chloroplast upon partial restoration of protein import in hisToc90/*ppi2* plants. Of the 24 chloroplast-localized proteins preferentially detected in hisToc90/*ppi2* and wt compared to *ppi2*, 20 are involved in photosynthesis and four are non-photosynthetic (Table 1). The first of these four is a protein that is similar to the ATP-dependent Clp protease ATP-binding subunit and is therefore probably involved in protein homeostasis. The second is the translation elongation factor RABE1b probably involved in protein synthesis. The third is a carbonic anhydrase, a homolog of which has been shown to be shuttled to the chloroplast through the ER (Villarejo et al., 2005; Kitajima et al., 2009; Armbruster et al., 2009). The fourth is a two-cys peroxiredoxin involved in redox regulation of plastid metabolism. This may suggest that similar to Toc159, Toc90 is mostly involved in the import of photosynthetic proteins, but not exclusively. However, care must be taken when discussing client proteins for the import receptors because it has been shown that many nuclear-encoded chloroplast proteins destined to the chloroplast are down-regulated at the gene

expression level upon plastid perturbation (Biehl et al., 2005). In this light, it appears reasonable to assume that the import rate obtained upon hisToc90 overexpression in *ppi2* was sufficient to at least partially rescue and trigger the genetic program required for the onset of photoautotrophic growth. Other factors such as the abundance of the different precursors might also play a role in client specificity. In this view, future work appears necessary to understand the client specificities as well as the client promiscuity of the Toc159/90 and Toc132/120 import receptors.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ppi2 mutant (line CS11072, ecotype Wassilewskija; Bauer et al., 2000) was used in this study. Plants were grown either on soil ('Rasenerde Top Dressing' from Ricoter AG) under long-day conditions (16 h light (160 μmol m^{-2} s^{-1}), 8 h dark, 21°C, 70% relative humidity) or on medium containing 0.5x Murashige and Skoog medium (MS, purchased at Duchefa Biochemie) and 0.8% w/v sucrose under short-day conditions (8 h light (160 μmol m^{-2} s^{-1}), 16 h dark, 21°C, 70% relative humidity).

DNA Constructs Used for Complementation

p3300.1 is a derivative of pCAMBIA3300 (CAMBIA, Australia) containing the terminator of the nopalin synthase gene. A pa terminator fragment was amplified from pCAMBIA3300 using the primers pa 3′ *Mun*I (5′-GAT TAC CAA TTG CTG AAT TAA CGC CGA ATT AA-3′) and pa 3′ *Sac*I (5′-CAT GGA GCT CGA AGA TCT TCG ACG TCG GAA TTC GTT TCT CCA TAA TAA TGT G). The PCR fragment was digested with *Mun*I and *Sac*I and ligated into the *Eco*RI/*Sac*I site of pCAMBIA3300. A 35S promoter fragment was PCR amplified from pCAMBIA3300 using the primer pair 35 5′ *Pst*I (5′-AAA ACT GCA GAA CAT GCC ATG GAG AGA TAG ATT TGT AGA GAG-3′)/35 3′ *Hind*III (5′-CCC AAG CTT TTG GCT AGA GCA GCT TGC CA-3′) and cut with *Pst*I/*Hind*III. This fragment was ligated into the *Pst*I/*Hind*III site of pCAMBIA3300 already containing the pa terminator fragment, which resulted in p3300.1.

To generate p3300.1-H6-Toc90, a Toc90 fragment was PCR amplified from EST-clone H4C12T7 using the primers H6-atToc90 5′ *Nco*I (5′-CAT GCC ATG GGG CAT CAC CAT CAC CAT CAC GTC ATG AAA GGC TTC AAA GAC TG-3′) and atToc90 3′ *Eco*RI (5′-CCG GAA TTC TTA GGA AAC GAG AAA ATT CAC AA-3′). The PCR fragment was digested with *Nco*I and *Eco*RI and ligated into the *Nco*I/*Eco*RI site of p3300.1 to obtain p3300.1-H6-Toc90.

p3300.1-MAH_6Toc90: a fragment of *toc90* was amplified from the EST-clone H4C12T7 using the following primer pair: 90MAH6 *Nco*I (5′-CAT GCC ATG GCT CAT CAC CAT CAC CAT CAC TCA TCT AGA CCA CTT TTG GG-3′) and H4C12T7 T7.4 (5′-GTA TCA GAC ACA GCC TGA TG-3′). p3300.1-MAH_6Toc90 was obtained by ligation into the *Nco*I/*Pst*I sites of p3300.1-H_6-Toc90 described above. This construct is referred as HisToc90 later on. p3300.1-MAH_6Toc90 and p3300.1-Toc90 were transformed into *Agrobacterium tumefaciens* C58.

Plant Transformation and Selection for Complementing Plants

Stable transformation of heterozygous *ppi2* plants with the constructs p3300.1-MAH_6Toc90 and p3300.1-Toc90 were carried out as described in Bauer et al. (2002) using the floral dip method (Clough and Bent, 1998). Seeds of transformants (T0 generation) were grown on soil and sprayed with phosphinotricine (150 mg l^{-1}, commercial solution) for selection. Seeds (T1 generation) of resistant plants were further selected on medium supplemented with 50 mg l^{-1} kanamycine and 30 mg l^{-1} phosphinotricine. Resistant plants were transferred to soil. Seeds of these plants (T2 generation) were grown on selection medium and subsequently used for PCR and Western analysis.

DNA Extraction and Diagnostic PCR

Genomic DNA for PCR was isolated from 4-week-old plants as described in Bauer et al. (2002). The following specific primer pairs were used to identify the T-DNA insertion in *ppi2* plants (*ppi2*: 5′-GAA TAG GGT TTT AAT CGG AAG-3′ and 5′- GAT GCA ATC GAT ATC AGC CAA TTT TAG AC-3′) and the wild-type atToc159 gene (Toc159: 5′-GAA TAG GGT TTT AAT CGG AAG-3′ and 5′-CAT CCC CAA TTT GAT CAA AC-3′) or the transgenes of Toc90 (tToc90: 5′-ATG TTG TGT GGA ATT GTG AG-3′ on the vector and 5′-CGA GTA CAC CAG ATC TTC TC-3′ reverse on TOC90).

RNA Isolation and q/RT–PCR

RNA used for reverse transcription was extracted from 4-week-old plants grown on medium using the RNeasy Plant Mini Kit (Qiagen). 1 μg of total RNA was treated with DNase RQ1 (Promega) according to the supplier's recommendations. After DNase heat inactivation, reverse transcription was carried out using 200 U of moloney murine Leukemia virus Reverse Transcriptase in presence of oligo(dT)15-mers, 0.6 mM dNTPs, and RNasin RNase inhibitors (all from Promega) according to the supplier's recommendations. cDNAs were diluted four times and aliquoted and used in RT–PCR and real-time qPCR. Real-time qPCR was performed using the Absolute SYBR Green Fluorescein Mix (Absolute™QPCR from ABgene) with 0.5-μM primers each and 1-μl cDNA and run on an iCycler from BioRad. Data were analyzed using the Gene Expression Analysis for iCycler iQ® Real-Time PCR Detection System program by BioRad with algorithms outlined by Vandesompele et al. (2002).

The following primer pairs were used: for Toc159: (5′-CAC AGT CTT GCT CTA GCT AG-3′ and 5′-CTC CTC TGA CCA CAT ATG CC-3′), for Toc75: (5′-CCG AGA TAC AGA GAC TTC C-3′ and 5′-CAT TTC CAT CTC CAC CAC CAC-3′), for endogenous Toc90: (5′-GAA AGG CTT CAA AGA CTG GG-3′ and 5′-GAG AGA GAG GAG GCA GAA TTT-3′), for HisToc90: (5′-ATG GCT CAT CAC CAT CAC CAT CAC-3′ and 5′-AAC TTT AAC TCC ACT GAC AG-3′), for total Toc90: (5′-CCG TGA CCC TCA TCA AGA

AC-3′ and 5′-GAG AGA GAG GAG GCA GAA TTT-3′) and for S16 as control: QuantiTect Primer Assay, Qiagen, QT00833819 (F20L16.100/At5g18380).

Chlorophyll Content

Chlorophyll content of 5-week-old plants grown on medium was measured according to Sundberg et al. (1997).

Protein Extraction and Immunoblot Analysis

Total proteins from leaves were isolated from 4-week-old plants grown on medium according to Bauer et al. (2000). The extraction buffer was supplemented with 0.5% v/v protease inhibitor cocktail for plant cell extracts (Sigma P9599) to prevent proteolytic degradation. Proteins were concentrated by chloroform-methanol precipitation and after separation by SDS–PAGE blotted onto nitrocellulose membranes. Antibodies specific to atToc159, atToc75, and atToc33 were used (Bauer et al., 2000; Hiltbrunner et al., 2001; Agne et al., 2009). Anti-atToc132 was a gift from Dr D.J. Schnell (University of Massachusetts, Amherst, MA, USA), anti-chlorophyll a/b binding protein (CAB) from Dr K. Apel (ETH Zürich, Zürich, Switzerland), anti-Rubisco from Dr Stieger (University of Neuchâtel, Neuchâtel, Switzerland), and monoclonal anti-actin was purchased at Sigma (A0480).

Leaf Sections and Ultrastructure Analysis of Chloroplasts

Five-week-old plants grown on medium were fixed overnight at 4°C in 50 mM sodium cacodylate containing 2.5% w/v glutaraldehyde, post-fixed with 1% w/v osmium tetroxide for 2 h at 20°C, dehydrated in ethanol and acetone series, and incubated in Spurr epoxy resin (Polyscience). Semi-thin sections were stained with 1% w/v toluidine blue O in 1% w/v sodium borate and observed and photographed using a light microscope. Ultrathin sections of 90–60 nm were prepared using an Ultracut-E microtome (Reichert-Jung), mounted on copper grids, and contrasted with uranyl acetate and Reynolds lead solution (Reynolds, 1963). Sections were observed with a Philips CM100 electron microscope operating at 60 kV.

Chloroplast Isolation and Import Assay

Intact chloroplasts from 4-week-old plants grown on medium were isolated according to Fitzpatrick and Keegstra (2001) with the following modifications. Cellulase Onozuka R-10 and macerozyme R-10 (Serva 16419, 28302) were reduced to 1 and 0.25% w/v, respectively. To the breakage buffer, 0.1% w/v L-ascorbat and 0.05% v/v protease inhibitor cocktail for plant cell extracts (Sigma P9599) were added. Intact chloroplasts were harvested from the interphase of a Percoll step gradient (40% v/v, 85% v/v) rather than a linear gradient. For each import reaction, chloroplast corresponding to 15 μg of chlorophyll and 5 μl of *in vitro* translated (TNT® T7 Quick Coupled Transcription/Translation System, Promega) [^{35}S] labeled preprotein of the small subunit of Arabidopsis' Rubisco (pSSU) were used (Agne et al., 2009). Import reactions were performed on ice in import buffer (5 mM ATP, 10 mM L-methionine, 0.33 M Sorbitol, 50 mM Hepes-KOH pH 7.5, 40 mM KOAc, 2 mM $Mg(OAc)_2$, 25 μM DTT) for 0, 5, 10, and 15 min. The reactions were stopped by adding ice-cold HS buffer (330 mM Sorbitol and 50 mM Hepes-KOH pH 8). Thermolysin digestion (Sigma) was carried out after 15 min of import in a total volume of 100 μL HS containing 1 μg μl^{-1} thermolysin and 30 μM $CaCl_2$ for 20 min on ice and stopped by adding ice-cold 5 mM EDTA in HS. Chloroplasts were re-isolated and resolved by SDS–PAGE. [^{35}S] signals in Coomassie Blue stained and dried SDS–PAGE gels were detected and analyzed using a PhosporImager (Personal Molecular Imager Fx, BioRad Laboratories) and the Quantity One® software version 4.6 (BioRad Laboratories).

Sample Preparation for Shotgun Proteomics, Mass Spectrometry, and Data Mining

Cotyledons from 6-day-old *ppi2*, hisToc90/*ppi2*, and wt plants grown on soil were collected, directly snap-frozen in liquid nitrogen, and afterwards stored at –80°C until use. Plant material was homogenized using a small pestle in an Eppendorf tube. Proteins were solubilized in extraction buffer (40 mM Tris pH 6.8, 40 mM DTT, 4% SDS, 2x protease inhibitor cocktail (Roche)). Homogenates were centrifuged twice at 20′000 rcf for 20 min at RT. Protein concentration was determined using a BCA Protein Assay Kit (Thermo scientific) before adding DTT and 300 μg of each protein extract were subjected to SDS-PAGE on 12% gels. After electrophoresis, the gels were cut into eight pieces per fraction. Each gel slice was diced into small pieces. In gel trypsin, digestion was performed according to Shevchenko et al. (1996). After digestion and drying, the peptides were re-suspended in 3% acetonitrile 0.2% trifluoretic acid and cleaned up using a C18 ZipTip (Millipore Corporation). Clean samples were dried and re-suspended in 12 μL 3% acetonitrile 0.2% formic acid for mass spectrometry.

Mass spectrometry measurements were performed on an LCQ-DECA XP (Thermo Finnigan), coupled with a HTC PAL (Analytics) autosampler system and a CPS-LC Rheos 2000 (Flux Instruments). Peptide mixtures were loaded onto laboratory-made capillary columns (75-μm inner diameter, 8-cm length, packed with Magic C18 AQ beads, 5 μm, 100 Å (Microm)). Peptides were eluted from the column by an increased acetonitrile concentration in the mobile phase from 3% acetonitrile, 0.2% formic acid to 80% acetonitrile, 0.2% formic acid over 75 min, followed by a 10-min wash step at 3% acetonitrile, 0.2% formic acid. Peptide ions were detected in a survey scan from 450 to 2000 amu followed by three data-dependent MS/MS scans (isolation width 1 amu, relative collision energy 35%, dynamic exclusion enabled, repeat count 2, followed by peak exclusion for 1 min).

Spectra obtained from each individual gel slice were searched individually. Identified peptides of all eight gel slices of each genotype were then pooled for further analysis. MS/MS spectra were searched with Sequest and PeptideProphet using the Trans-Proteomic Pipeline (TPP v2.9) against the *Arabidopsis thaliana* TAIR9 protein database with concatenated decoy database supplemented with contaminants. The search

parameters were: requirement for tryptic ends, one missed cleavage allowed, mass tolerance = ±5 Da, variable modification of methionine (M, PSI-MOD name: oxidation, ModAccession: MOD:00412, mono Δ = 15.9994) and static modification of cysteine (C, PSI-MOD name: iodoacetamide derivative, ModAccession: MOD:00397, mono Δ = 57.0513). For PeptideProphet, the cutoff was set to a minimum probability of 0.9. The false positive rate at peptide level was of 0% for both *ppi2* and hisToc90/*ppi2* and of 0.17% for wt. The false positive rate corresponds to the ratio between the number of peptides identified from the TAIR9 protein decoy database and the number of peptides identified from the TAIR9 protein database. Proteins extracts from pools of 50 6-day-old cotyledons were analyzed in one single experiment by mass spectrometry resulting in a simple 'proteome snapshot'.

Protein localization assignment was performed using the database SUBA, which integrates different localization criteria from prediction tools, annotation, proteomics, and biochemical studies (Heazlewood et al., 2007; http://suba.plantenergy.uwa.edu.au/). Functional categorization of identified proteins is based on Mapman (Thimm et al., 2004; http://mapman.mpimp-golm.mpg.de).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by SNF grant 3100A0-112565 to S.B. and SNF grant 31003A_127380/1 to F.K. The work was in part supported by the University of Neuchâtel, the NCCR Plant Survival and SystemsX PGCE.

ACKNOWLEDGMENTS

S. Infanger is grateful to J. Smutny, V. Douet, and M. Vlimant for excellent technical work. S. Bischof is very grateful to the members of the Functional Genomics Center Zürich, especially Dr Bernd Roschitzki, for all their support in mass spectrometry and bioinformatics. No conflict of interest declared.

REFERENCES

Agne, B., and Kessler, F. (2009). Protein transport in organelles: the Toc complex way of preprotein import. FEBS J. **276,** 1156–1165.

Agne, B., Andrès, C., Montandon, C., Christ, B., Ertan, A., Jung, F., Infanger, S., Bischof, S., Baginsky, S., and Kessler, F. (2010). The acidic A-domain of Arabidopsis TOC159 occurs as a hyperphosphorylated protein. Plant Physiol. **153,** 1016–1030.

Agne, B., Infanger, S., Wang, F., Hofstetter, V., Rahim, G., Martin, M., Lee, D.W., Hwang, I., Schnell, D., and Kessler, F. (2009). A toc159 import receptor mutant, defective in hydrolysis of GTP, supports preprotein import into chloroplasts. J. Biol. Chem. **284,** 8670–8679.

Armbruster, U., et al. (2009). Chloroplast proteins without cleavable transit peptides: rare exceptions or a major constituent of the chloroplast proteome? Mol. Plant. **2,** 1325–1335.

Baerenfaller, K., Grossmann, J., Grobei, M.A., Hull, R., Hirsch-Hoffmann, M., Yalovsky, S., Zimmermann, P., Grossniklaus, U., Gruissem, W., and Baginsky, S. (2008). Genome-scale proteomics reveals *Arabidopsis thaliana* gene models and proteome dynamics. Science. **320,** 938–941.

Balsera, M., Soll, J., and Bölter, B. (2009). Protein import machineries in endosymbiotic organelles. Cell Mol. Life Sci. **66,** 1903–1923.

Bauer, J., Chen, K., Hiltbunner, A., Wehrli, E., Eugster, M., Schnell, D., and Kessler, F. (2000). The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature. **13,** 203–207.

Bauer, J., Hiltbrunner, A., Weibel, P., Vidi, P.A., Alvarez-Huerta, M., Smith, M.D., Schnell, D.J., and Kessler, F. (2002). Essential role of the G-domain in targeting of the protein import receptor atToc159 to the chloroplast outer membrane. J. Cell Biol. **159,** 845–854.

Biehl, A., Richly, E., Noutsos, C., Salamini, F., and Leister, D. (2005). Analysis of 101 nuclear transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. Gene. **344,** 33–41.

Bräutigam, A., and Weber, A.P. (2009). Proteomic analysis of the proplastid envelope membrane provides novel insights into small molecule and protein transport across proplastid membranes. Mol. Plant. **2,** 1247–1261.

Chen, K., Chen, X., and Schnell, D.J. (2000). Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. Plant Physiol. **122,** 813–822.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

Constan, D., Patel, R., Keegstra, K., and Jarvis, P. (2004). An outer envelope membrane component of the plastid protein import apparatus plays an essential role in Arabidopsis. Plant J. **38,** 93–106.

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. **2,** 953–971.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. **300,** 1005–1016.

Fitzpatrick, L.M., and Keegstra, K. (2001). A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. Plant J. **27,** 59–65.

Heazlewood, J.L., Verboom, R.E., Tonti-Filippini, J., Small, I., and Millar, A.H. (2007). SUBA: the Arabidopsis Subcellular Database. Nucleic Acids Res. **35,** 213–218.

Hiltbrunner, A., Bauer, J., Vidi, P.A., Infanger, S., Weibel, P., Hohwy, M., and Kessler, F. (2001). Targeting of an abundant cytosolic form of the protein import receptor at Toc159 to the outer chloroplast membrane. J. Cell Biol. **154,** 309–316.

Hiltbrunner, A., Grünig, K., Alvarez-Huerta, M., Infanger, S., Bauer, J., and Kessler, F. (2004). AtToc90, a new GTP-binding component of the Arabidopsis chloroplast protein import machinery. Plant Mol. Biol. **54,** 427–440.

Inoue, H., Rounds, C., and Schnell, D.J. (2010). The molecular basis for distinct pathways for protein import into Arabidopsis chloroplasts. Plant Cell. **22,** 1947–1960.

Ivanova, Y., Smith, M.D., Chen, K., and Schnell, D.J. (2004). Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. Mol. Biol. Cell. **15,** 3379–3392.

Jarvis, P., Chen, L.J., Li, H., Peto, C.A., Fankhauser, C., and Chory, J. (1998). An Arabidopsis mutant defective in the plastid general protein import apparatus. Science. **282,** 100–103.

Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., and Rolland, N. (2009). Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways. Mol. Plant. **2,** 1154–1180.

Kessler, F., and Schnell, D.J. (2009). Chloroplast biogenesis: diversity and regulation of the protein import apparatus. Curr. Opin. Cell Biol. **21,** 494–500.

Kitajima, A., et al. (2009). The rice alpha-amylase glycoprotein is targeted from the Golgi apparatus through the secretory pathway to the plastids. Plant Cell. **21,** 2844–2858.

Kleffmann, T., Russenberger, D., von Zychlinski, A., Christopher, W., Sjölander, K., Gruissem, W., and Baginsky, S. (2004). The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. Curr. Biol. **9,** 354–362.

Kubis, S., Baldwin, A., Patel, R., Razzaq, A., Dupree, P., Lilley, K., Kurth, J., Leister, D., and Jarvis, P. (2003). The Arabidopsis ppi1 mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. Plant Cell. **15,** 1859–1871.

Kubis, S., Patel, R., Combe, J., Bédard, J., Kovacheva, S., Lilley, K., Biehl, A., Leister, D., Ríos, G., Koncz, C., and Jarvis, P. (2004). Functional specialization amongst the Arabidopsis Toc159 family of chloroplast protein import receptors. Plant Cell. **16,** 2059–2077.

Lee, S., Lee, D.W., Lee, Y., Mayer, U., Stierhof, Y.D., Lee, S., Jürgens, G., and Hwang, I. (2010). Heat shock protein cognate 70–4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in Arabidopsis. Plant Cell. **21,** 3984–4001.

Lu, P., Vogel, C., Wang, R., Yao, X., and Marcotte, E.M. (2007). Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. Nat. Biotechnol. **25,** 117–124.

Nielsen, H., Engelbrecht, J., Brunak, S., and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10,** 1–6.

Reynolds, E.S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17,** 208–212.

Richardson, L.G., Jelokhani-Niaraki, M., and Smith, M.D. (2009). The acidic domains of the Toc159 chloroplast preprotein receptor family are intrinsically disordered protein domains. BMC Biochem. **30,** 35.

Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68,** 850–858.

Sundberg, E., Slagter, J.G., Fridborg, I., Cleary, S.P., Robinson, C., and Coupland, G. (1997). ALBINO3, an Arabidopsis nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria. Plant Cell. **9,** 717–730.

Thimm, O., Blaesing, O., Gibon, Y., Nagel, A., Meyer, S., Krüger, P., Selbig, J., Müller, L.A., Rhee, S.Y., and Stitt, M. (2004). MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. **37,** 914–939.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT–PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3,** RESEARCH0034.

Villarejo, A., et al. (2005). Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. Nat. Cell Biol. **7,** 1224–1231.

Yu, T.S., and Li, H.M. (2001). Chloroplast protein translocon components atToc159 and atToc33 are not essential for chloroplast biogenesis in guard cells and root cells. Plant Physiol. **127,** 90–96.

Zybailov, B., Friso, G., Kim, J., Rudella, A., Rodríguez, V.R., Asakura, Y., Sun, Q., and van Wijk, K.J. (2009). Large scale comparative proteomics of a chloroplast Clp protease mutant reveals folding stress, altered protein homeostasis, and feedback regulation of metabolism. Mol. Cell Proteomics. **8,** 1789–1810.

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., and van Wijk, K.J. (2008). Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS One. **3,** e1994.