A role of Toc33 in the protochlorophyllide-dependent plastid import pathway of NADPH:protochlorophyllide oxidoreductase (POR) A†

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Summary

NADPH:protochlorophyllide oxidoreductase (POR) A is a key enzyme of chlorophyll biosynthesis in angiosperms. It is nucleus-encoded, synthesized as a larger precursor in the cytosol and imported into the plastids in a substrate-dependent manner. Plastid envelope membrane proteins, called protochlorophyllide-dependent translocon proteins, Ptcs, have been identified that interact with pPORA during import. Among them are a 16-kDa ortholog of the previously characterized outer envelope protein Oep16 (named Ptc16) and a 33-kDa protein (Ptc33) related to the GTP-binding proteins Toc33 and Toc34 of Arabidopsis. In the present work, we studied the interactions and roles of Ptc16 and Ptc33 during pPORA import. Radiolabeled Ptc16/Oep16 was synthesized from a corresponding cDNA and imported into isolated Arabidopsis plastids. Crosslinking experiments revealed that import of ^{35}S-Oep16/Ptc16 is stimulated by GTP. ^{35}S-Oep16/Ptc16 forms larger complexes with Toc33 but not Toc34. Plastids of the ppi1 mutant of Arabidopsis lacking Toc33, were unable to import pPORA in darkness but imported the small subunit precursor of ribulose-1, 5-bisphosphate carboxylase/oxygenase (pSSU), precursor ferredoxin (pFd) as well as pPORB which is a close relative of pPORA. In white light, partial suppressions of pSSU, pFd and pPORB import were observed. Our results unveil a hitherto unrecognized role of Toc33 in pPORA import and suggest photooxidative membrane damage, induced by excess Pchlide accumulating in ppi1 chloroplasts because of the lack of pPORA import, to be the cause of the general drop of protein import.

Keywords: Arabidopsis, chloroplast biogenesis, protein import, POR, Oep16, Toc33.

Introduction

Plastids comprise a family of related, partially interconvertible forms, called proplastids, etioplasts, chloroplasts, leucoplasts, and amyloplasts (Kirk and Tilney-Basset, 1978). Despite their structural and functional diversity all of the different plastid types have in common the operation of a sophisticated protein import machinery that is responsible for the import of most of the plastid proteins from the cytosol. Plastids contain only limited coding information in their own DNA and consequently need to import the major part of their protein constituents. In case of chloroplasts, a trimeric protein translocon (Toc) complex in the outer plastid envelope membrane catalyzes the initial recognition, binding and translocation of cytosolic precursors (Ma *et al.*, 1996; Schnell *et al.*, 1994; see also Bauer *et al.*, 2001; Keegstra and Cline, 1999; Schleiff and Soll, 2000 for review). Import requires ATP and is transit sequence-dependent. The transit sequences of cytosolic precursors interact with Toc proteins of 159, 75 and 34 kDa (called Toc159, Toc75 and Toc34) during import (Hiltbrunner *et al.*, 2001; Kouranov and Schnell, 1997; Ma *et al.*, 1996; Perry and Keegstra, 1994).

†This article is dedicated to Professor Dr Diter von Wettstein on the occasion of his 75th birthday.

Toc159 and Toc34 are GTP-binding proteins (Bölter *et al.*, 1998; Chen *et al.*, 2000; Kessler *et al.*, 1994; Seedorf *et al.*, 1995). They share the same basic architecture and consist of a GTP-binding domain (G-domain) and a membrane anchor domain (M-domain; Bauer *et al.*, 2000; Chen *et al.*, 2000). Toc159 additionally contains an NH_2-terminal acidic domain (A-domain) implicated in precursor binding.

Interestingly, two additional atToc159-related genes have been identified by the Arabidopsis genome project (Bayer *et al.*, 2000; Ivanova *et al.*, 2004; Kubis *et al.*, 2004). Moreover, two closely related forms of atToc34 were discovered that exhibited major differences in their expression patterns (Gutensohn *et al.*, 2000; Jarvis *et al.*, 1998). Recent evidence suggests the existence of multiple, regulated protein import pathways involving different combinations of atToc159-related and atToc34-related proteins. These pathways may reflect the need to import an organelle-specific protein complement in order to sustain metabolism (Jackson-Constan and Keegstra, 2001). In line with this hypothesis, Wan *et al.* (1995, 1996) found precursor-specific differences in import of pyruvate kinase isoforms into chloroplasts and leucoplasts. In addition, Dahlin and Cline (1991) observed that protein import is determined by developmental fate and age of the plant, implying a rather flexible nature of the responsible import machineries.

NADPH:protochlorophyllide oxidoreductase (POR) A is the only known example of a nucleus-encoded plastid protein that is imported into plastids in a regulated manner. pPORA import is substrate-dependent (Reinbothe *et al.*, 1995a,b,c, 1996, 1997, 2000). Etioplasts containing high levels of Pchlide imported pPORA *in vitro*, whereas chloroplasts which lack detectable levels of Pchlide did not (Reinbothe *et al.*, 1995a,b, 1996, 1997, 2000). pPORA import into chloroplasts was restored by feeding isolated chloroplasts the Pchlide precursor 5-aminolevulinic acid (5-ALA; Reinbothe *et al.*, 1995a,b, 1996, 1997, 2000). Recent work performed by Kim and Apel (2004) proved that the substrate-dependent import pathway operates *in planta*. Using DNA encoding green jellyfish protein (GFP) fused to either the full-length pPORA or only its transit sequence (transA) transformed into the *fluorescent* (*flu*) mutant of Arabidopsis that overproduces Pchlide in darkness (Meskauskiene *et al.*, 2001), the authors demonstrated co-localization of PORA-GFP and Pchlide fluorescence indicative of substrate-dependent import in cotyledonary plastids. By contrast, no PORA-GFP fluorescence appeared in Pchlide-free plastids. We found that pPORA does not interact with Toc159 and Toc75 in barley, Arabidopsis and other plant species *in vitro* (Reinbothe *et al.*, 2000). Plastid envelope proteins were identified that establish a Pchlide-dependent translocon called the Ptc complex (Reinbothe *et al.*, 2004a,b). Among its identified protein constituents were an ortholog of the previously described outer plastid envelope protein Oep16 (Baldi *et al.*, 1999; Pohlmeyer *et al.*, 1997) and a 33-kDa protein exhibiting amino acid sequence similarity to both Toc33 and Toc34 of Arabidopsis (Jarvis *et al.*, 1998).

Oep16 in barley and pea chloroplasts is a nuclear gene product (Baldi *et al.*, 1999; Pohlmeyer *et al.*, 1997). In contrast to other nucleus-encoded chloroplast proteins, it is synthesized without cleavable chloroplast transit peptide for import. Pohlmeyer *et al.* (1997) suggested that pea Oep16 inserts into the lipid bilayers spontaneously and does not interact with other proteins. By contrast, we revealed an association of pPORA with Oep16 and plastid envelope proteins of 130 kDa (Ptc130), 52 kDa (Ptc52) and 33 kDa (Ptc33) forming the Ptc complex (Reinbothe *et al.*, 2004b).

To solve this apparent contradiction and to study the presumed functions and interactions of Oep16 with Toc34 and/or Toc33, the ppi1 mutant of Arabidopsis (Jarvis *et al.*, 1998; Kubis *et al.*, 2003) was used. This knockout mutant is defective in the gene encoding Toc33 (Jarvis *et al.*, 1998). Despite the fact that import of certain precursor proteins was diminished, it showed especially reduced amounts of POR in darkness and formed abnormal prolamellar bodies (Jarvis *et al.*, 1998) where POR is the major protein constituent (Dehesh and Ryberg, 1985). Strikingly, ppi1 etioplasts accumulated massive amounts of a POR-related higher molecular mass protein of 40 kDa (Jarvis *et al.*, 1998), indicative of the PORA precursor (Reinbothe *et al.*, 1996). That etiolated seedlings displayed a delay in greening upon light exposure further suggest a lack of pPORA import. In the present study we demonstrate that Toc33, but not Toc34, is necessary for pPORA import. In white light, a partial suppression of general protein import was observed which is most likely due to photooxidative membrane damage triggered by excess Pchlide accumulating in ppi1 plastids because of the lack of pPORA import. Our results thus unveil a so far unrecognized role of Toc33 in pPORA import and highlight the importance of the substrate-dependent import pathway in keeping Pchlide homoeostasis in plants.

Results

Ppi1 chloroplasts accumulate pPORA at their outer plastid envelope

In a first set of experiments, etioplasts were isolated from dark-grown ppi1 and wild-type plants. As a control, the recently isolated ppi3 mutant (Constan *et al.*, 2004) was used. This mutant (allele 3–2, SALK_059206; http://signal.salkedu; Alonso *et al.*, 2003) does not express detectable levels of atToc34 (Constan *et al.*, 2004). Plastid proteins were then extracted with trichloroacetic acid, separated by SDS-PAGE, blotted onto nitrocellulose membranes, and the filters probed with a POR-specific antiserum by Western blotting. As shown in Figure 1(a), this experiment confirmed accumulation of the 40 kDa protein band in ppi1 etioplasts. In addition,

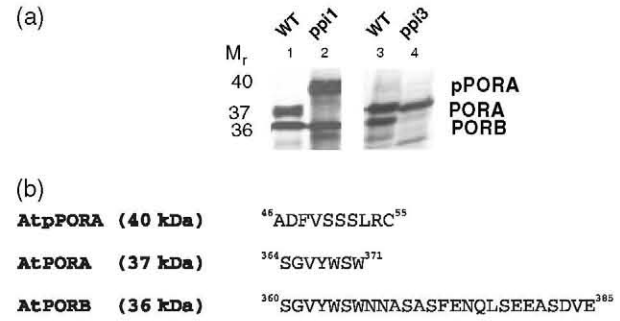

Figure 1. Accumulation of 40 kDa pPORA in ppi1 etioplasts. Etioplasts were isolated from dark-grown wild-type (WT) as well as ppi1 and ppi3 plants and protein subjected to Western blotting (a) and protein sequencing (b).
(a) Detection of POR-related proteins in wild-type, ppi1 and ppi3 etioplasts using a heterologous antiserum against PORA of barley. The size of the different bands is indicated (in kDa).
(b) After two-dimensional separation by isoelectric focusing in the first dimension and SDS-PAGE in the second dimension, individual POR protein spots were treated with endoproteinase LysC and the resulting peptides sequenced. Numbers refer to the published Arabidopsis PORA and PORB protein sequences (Armstrong *et al.*, 1995).

POR-related 37 and 36 kDa protein bands were detected that, based on their differential expression in etiolated plants upon illumination (Armstrong *et al.*, 1995), were tentatively defined as PORA and PORB, respectively. The third, recently identified POR protein of Arabidopsis, PORC (Pattanayak and Tripathy, 2002; Su *et al.*, 2002), is light-induced and was not detected in either etiolated wild-type or ppi1 plants.

When the blots were compared, a specific lack of the 37 kDa band was apparent in ppi1 etioplasts, whereas the 36 kDa band was similarly expressed in ppi1 and wild-type plastids (Figure 1a). In plastid protein extracts of etiolated ppi3 plants, only the 37 kDa band but apparently not the 36 kDa band was detectable. Minor amounts of a larger band as well as several other bands presumably representing POR degradation products were seen. Protein mass spectrometry of peptides mixtures generated with endoproteinase LysC identified the 40 kDa band as pPORA and the 37 and 36 kDa bands as PORA and PORB, respectively (Figure 1a). A third, weak band of approximately 35 kDa was found in ppi1 etioplasts, the identity of which could not be determined (Figure 1a). Because similar PORA protein levels were detected in wild-type and ppi3 etioplasts, we concluded that Toc34 may not play a role in pPORA import and disregarded the ppi3 mutant in most subsequent experiments.

Oep16 and Toc33, but not Toc34, interact with pPORA

In a second set of experiments, energy sources of chloroplasts isolated from light-grown wild-type and ppi1 plants were depleted by keeping the organelles in darkness on ice for 1 h (Theg *et al.*, 1989). Isolated, intact plastids in turn were incubated with ^{35}S-Oep16 synthesized from a corresponding cDNA. Parallel incubations were performed in the

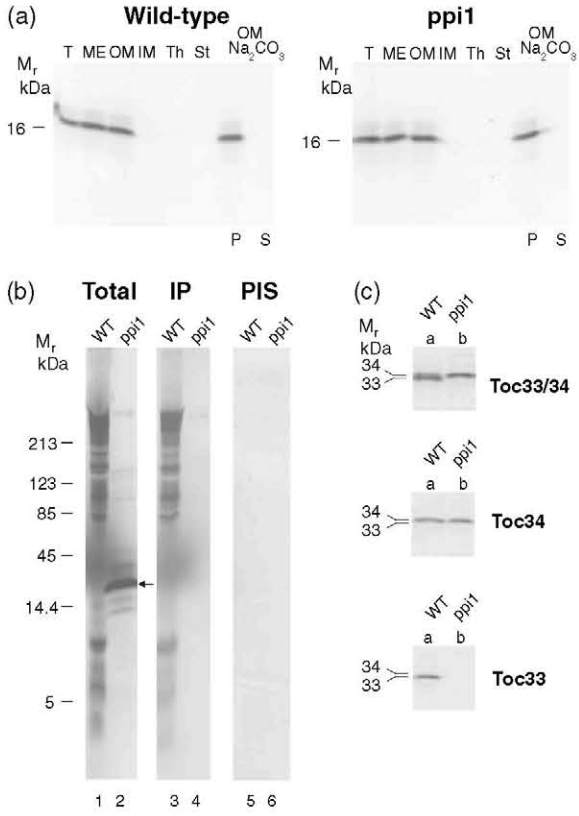

Figure 2. ^{35}S-Oep16 gives rise to a larger protein complex in wild-type but not ppi1 chloroplasts. Chloroplasts were isolated from light-grown wild-type (WT) and ppi1 mutant plants of Arabidopsis, energy-depleted, and incubated with ^{35}S-Oep16 synthesized from a corresponding cDNA clone in the presence of 2 mM Mg-ATP and 0.1 mM Mg-GTP for 15 min in darkness.
(a) Detection of imported ^{35}S-Oep16 by SDS-PAGE and autoradiography in total chloroplasts (T), isolated stroma (St) and thylakoids (Th), as well as mixed envelope membranes (ME), outer (OM) and inner (IM) plastid envelope membranes, and outer envelope membranes extracted with 0.1 M Na_2CO_3, pH 11. P and S denote protein found in the pellet and supernatant fractions, respectively, obtained after centrifugation of the assay mixtures.
(b) Crosslinking of imported ^{35}S-Oep16 to plastid envelope proteins in wild-type and ppi1 chloroplasts of Arabidopsis. Wild-type and ppi1 chloroplasts containing imported ^{35}S-Oep16 were treated with DSP, lysed, and envelopes fractionated on sucrose gradients. After solubilization with LDS, one-tenth of the envelope membrane samples were directly loaded onto an SDS-PAGE gel (total), whereas the remainders were split and subjected to immunoprecipitation with anti-Toc33 antiserum (IP) or respective pre-immune serum (PIS).
(c) Toc33 and Toc34 protein levels in wild-type and ppi1 chloroplasts. Plastid envelopes were recovered from wild-type and ppi1 chloroplasts as described and membrane proteins separated by SDS-PAGE and blotted onto nitrocellulose filters. The filters were then probed with the indicated anti-Toc33 and anti-Toc34 antisera or a mixed anti-Toc33/34 antiserum. Of the two detected bands, only the lower, representing Toc33, was absent from ppi1 chloroplasts.

absence of Mg-ATP or in the presence of 0.1 mM Mg-ATP and 2 mM Mg-ATP. In two batches of samples, the effect of Mg-GTP was analyzed. Replicate samples were incubated with or without 0.1 mM Mg-GTP. Pilot experiments revealed that highest levels of ^{35}S-Oep16 were incorporated into isolated wild-type and ppi1 chloroplasts in the presence of 0.1 mM Mg-GTP plus 2 mM Mg-ATP (see Figure S1). At these nucleoside triphosphate concentrations, wild-type and ppi1

Arabidopsis chloroplasts sequestered similar amounts of ^{35}S-Oep16 in their outer plastid envelopes (Figure 2a).

After prior insertion reactions, the crosslinker dithio*bis*(succinidyl propionate) (DSP) was added to the ^{35}S-Oep16-containing ppi1 and wild-type chloroplasts. We assumed that DSP not only would covalently connect the radiolabeled Oep16 to nearby proteins, but would also connect adjacent proteins to each other, giving rise to larger complexes. DSP-treated chloroplasts in turn were solubilized with LDS and protein-resolved by SDS-PAGE (Lübeck *et al.*, 1997).

Figure 2(b) shows that the envelope-bound ^{35}S-Oep16 was converted into a higher molecular mass complex of approximately 400 kDa when chloroplasts from wild-type Arabidopsis plants were used (lane 1). Immunoprecipitations with an antiserum against a fingerprint motif of Toc33 of Arabidopsis, which is absent from Toc34 (see Experimental procedures), identified Toc33 as part of the recovered complex (Figure 2b, lane 3). With chloroplasts from the Toc33-deficient ppi1 mutant, only trace amounts of this higher molecular mass complex were found (Figure 2b, lanes 2 and 4). Instead, a radioactive band of approximately 32 kDa was the most heavily labeled product (Figure 2b, lane 2). This band did not cross-react with the anti-Toc33 antiserum; it is most likely due to Oep16 dimers formed upon DSP treatment. Control experiments with pre-immune serum confirmed the specificity of the anti-Toc33 antiserum (Figure 2b, lanes 5 and 6). Western blotting highlighted the presence of two closely related bands of which only the lower, 33 kDa band was absent from ppi1 chloroplasts (Figure 2c).

AtToc33 and atToc34 are twin components (Jarvis *et al.*, 1998). Sveshnikova *et al.* (2000) proposed a preprotein receptor role for Toc34 of pea in chloroplasts. Bearing this in mind, we next tested binding of ^{35}S-pPORA and ^{35}S-pPORB to chloroplasts and etioplasts of ppi1 and wild-type Arabidopsis plants. We used 5,5′-dithiobis-2-nitrobenzoic acid) (DTNB)-derivatized ^{35}S-pPORA and ^{35}S-pPORB, because cross-linking would allow us to determine whether the precursors entered a productive import pathway upon binding or not (Tokatlidis *et al.*, 1996). As shown previously for barley chloroplasts (Reinbothe *et al.*, 2004b), DTNB-activated ^{35}S-pPORA crosslinks Oep16, while the

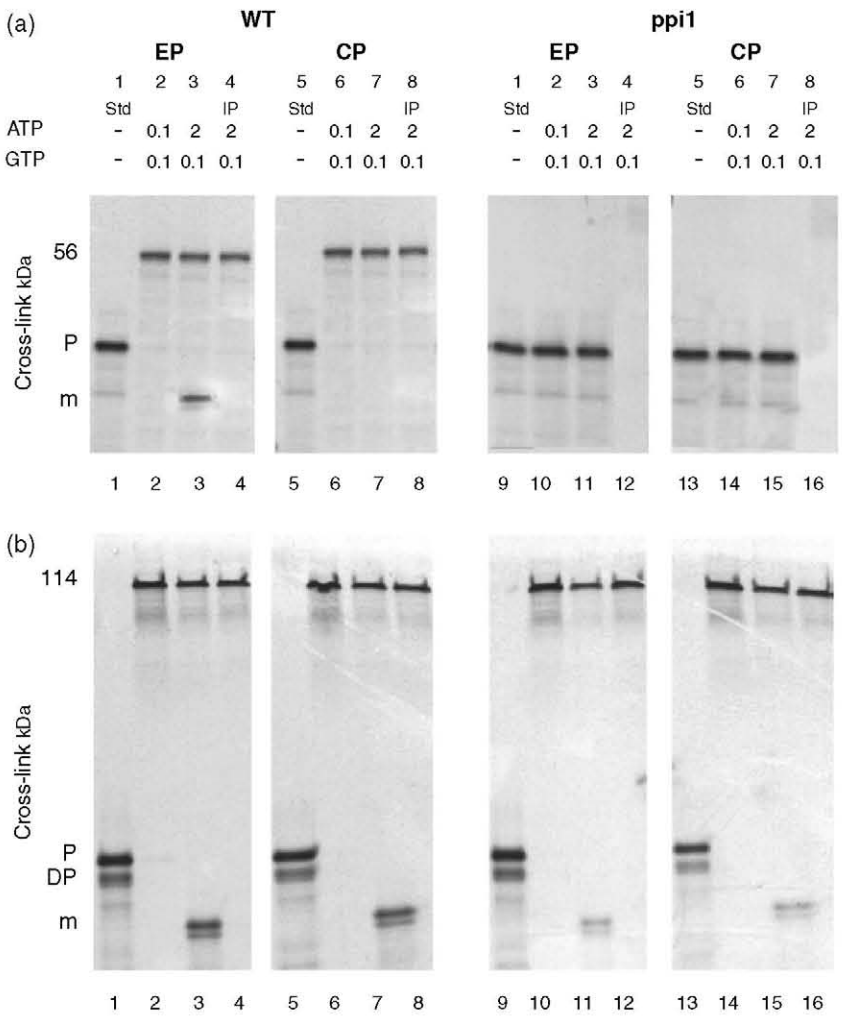

Figure 3. ppi1 plastids bind pPORA, but do not promote the precursor to a productive import pathway. Chloroplasts (CP) and etioplasts (EP) were isolated from light-grown and dark-grown wild-type (WT) and ppi1 plants and incubated with DTNB-activated, urea-denatured ^{35}S-pPORA and ^{35}S-pPORB in the presence of the indicated Mg-ATP and Mg-GTP concentrations for 15 min in darkness. Insertion (lanes 2, 6, 10, and 14) and import (lanes 3, 7, 11, and 15) of the ^{35}S-labeled precursors were determined by non-reducing SDS-PAGE. An aliquot of the assays containing 2 mM Mg-ATP and 0.1 mM Mg-GTP was subjected to co-immunprecipitation (IP), using anti-Oep16 (A) and anti-Toc75 (B) antisera (lanes 4, 8, 12, and 16 each), respectively.
(a) Crosslink product formation and import of ^{35}S-pPORA into wild-type and ppi1 etioplasts (1×10^7) and chloroplasts (5×10^7).
(b) Similar to (a), but showing crosslink product formation and import of ^{35}S-pPORB.
The autoradiograms highlight precursors (P) and respective crosslink products, marked by their M_r, as well as imported, mature proteins (m). Std defines input standards; DP marks a degradation product of pPORB.

DTNB-derivatized ^{35}S-pPORB interacts with Toc75, giving rise to 60 and 118 kDa crosslink products, respectively.

Figure 3 illustrates binding and crosslink product formation for chloroplasts and etioplasts of wild-type Arabidopsis plants. With DTNB-activated pPORA, an approximately 56 kDa product was produced under insertion (0.1 mM Mg-GTP and 0.1 mM Mg-ATP) and import (0.1 mM Mg-GTP and 2 mM Mg-ATP) conditions (Figure 3a, lanes 2 and 6: insertion; lanes 3 and 7: import). This approximately 56 kDa product contained Oep16, as shown by its cross-reactivity toward an antiserum against barley Oep16 (Figure 3a, lanes 4 and 8, respectively). In etioplasts, a fraction of the precursor was imported (Figure 3a, lane 3). By contrast, chloroplasts were unable to import pPORA (Figure 3a, lane 7).

For ^{35}S-pPORB, a DTNB-induced 114 kDa crosslink species was produced (Figure 3b, lanes 2–8). In the presence of 0.1 mM Mg-GTP and 2 mM Mg-ATP, a fraction of the precursor was taken up by both etioplasts and chloroplasts and processed to mature size (Figure 3b, lanes 3 and 7).

When binding, crosslink product formation and import were analyzed for isolated ppi1 plastids, a different picture emerged. While ^{35}S-pPORB behaved essentially in the same way as found for wild-type etioplasts and chloroplasts (Figure 3b), ^{35}S-pPORA could neither be cross-linked to Oep16, to give rise to the 56 kDa crosslink product, nor be imported into ppi1 etioplasts (Figure 3a). Nevertheless, binding of the precursor was well detectable (Figure 3a). Immunoprecipitation showed that this binding did not involve Oep16 (Figure 3a). We assumed that it may be due to other Ptc proteins interacting specifically with the transit sequence of pPORA such as Ptc130 (Reinbothe *et al.*, 2004a). This idea is corroborated by the findings presented in Figure 4. In this experiment, wild-type and ppi1 chloroplasts were incubated with ^{125}I-*N*-[4[(*p*-azidosalicylamido) butyl]-3′(2-pyridyldithio) propionamid (APDP)-derivatized pPORA in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP. ^{125}I-APDP is a hetero-bifunctional, photo-activatable, cleavable crosslinker. In darkness, it forms stable disulfide bridges with protein sulfhydryl groups. Upon UV light illumination it is activated and its reactive azido group then crosslinks to neighboring molecules. Treatment with DTT or β-mercapto-ethanol reduces the disulfide bond such that the precursor is released and the radioactivity transferred onto the crosslinked target protein(s), allowing its (their) detection by SDS-PAGE and autoradiography (Kouranov and Schnell, 1997; Ma *et al.*, 1996).

Figure 4(a) highlights that crosslinking of ^{125}I-APDP-pPORA to ppi1 chloroplasts gave rise to a unique 130 kDa band (lane 1). By contrast, crosslinking of ^{125}I-APDP-pPORA to wild-type plastids produced a pattern of at least four main bands, displaying molecular masses of 90, 52, 33 and 16 kDa, respectively (Figure 4a, lane 2). Protein mass spectrometry and immunoprecipitations (data not shown) indicated that the 52, 33 and 16 kDa bands represent orthologs

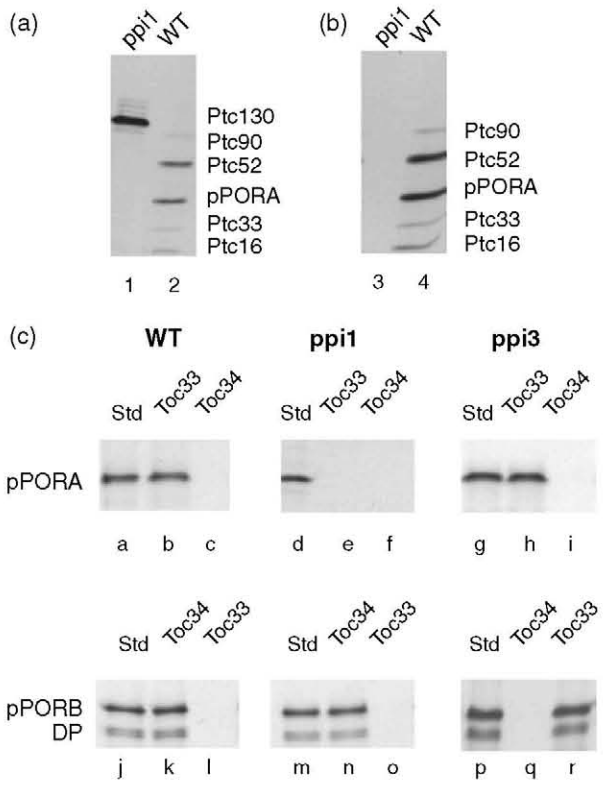

Figure 4. pPORA binds to ppi1 chloroplasts via Ptc130, but no Ptc complex is formed. Chloroplasts were isolated from light-grown wild-type (WT) as well as ppi1 and ppi3 plants and incubated with bacterially expressed ^{125}I-APDP-derivatized (a) or non-derivatized (b, c) pPORA in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP.

(a) Photo-crosslinking of plastid envelope proteins in ppi1 (lane 1) and wild-type (lane 2) plastids induced by UV light treatment. After solubilization with SDS, plastid envelope proteins were separated by SDS-PAGE on a mini-gel and detected by autoradiography.

(b) Purification of plastid envelope proteins bound to pPORA in ppi1 (lane 3) and wild-type (lane 4) chloroplasts. After the insertion reaction, re-purified plastids were lysed and crude membranes fractionated on sucrose gradients into outer (OM) and inner (IM) plastid envelope membranes, as well as OM–IM junction complexes containing pPORA. From the latter, pPORA and bound proteins were solubilized with Triton X-100 and purified by Ni-NTA chromatography. After elution, protein was separated by SDS-PAGE and detected by Coomassie staining.

(c) Co-immunoprecipitation of plastid envelope proteins interacting with ^{35}S-pPORA and ^{35}S-pPORB. Mixed envelope membranes were prepared from wild-type and ppi1 chloroplasts containing imported ^{35}S-pPORA and ^{35}S-pPORB and solubilized with 1.3% decylmaltoside. Protein in turn was subjected to co-immunoprecipitation, using the indicated anti-Toc33 and anti-Toc34 antisera. Std defines respective import standards.

of barley Ptc52, Ptc33 and Ptc16. In addition, faint amounts of Ptc130 were seen (Figure 4a, lane 2). Basically the same bands were obtained when chloroplasts were allowed to insert a bacterially expressed, hexahistidine [(His)6]-containing, but non-^{125}I-APDP-derivatized pPORA during pre-incubation (Figure 4b, lane 4). After incubation, the plastids were lysed and outer envelope membranes (OM), inner envelope membranes (IM) and OM-IM junction

complexes containing pPORA-$(His)_6$ separated on sucrose gradients (Schnell *et al.*, 1994). From OM-IM junction complexes, pPORA-$(His)_6$ and adhering proteins were solubilized with Triton X-100 and purified on Ni-NTA-agarose. The final protein fraction was subjected to SDS-PAGE and Coomassie staining. The respective banding pattern is shown in Figure 4(b) (lane 4). It basically confirmed that the recovered Ptc complex contained Ptc52, Ptc33, and Ptc16. In addition, faint amounts of the 90 kDa protein detected before and referred to as Ptc90 were detectable (Figure 4b, lane 4). With ppi1 plastids we did not obtain sufficient amounts of OM–IM junction complexes that would have allowed purifying plastid envelope proteins interacting with pPORA (Figure 4b, lane 3). Consistent with previous results (Reinbothe *et al.*, 2004a), OM–IM junction complexes were enriched only in the presence of precursor and functional Ptc complexes.

To obtain further evidence for the presence of Toc33 in the Ptc complex, ^{35}S-pPORA was incubated with chloroplasts from wild-type and ppi1 plants in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP, but in the absence of any cross-linking reagent. For comparison, chloroplasts of the ppi3 mutant lacking Toc34 were used. A different solubilization strategy was employed. The re-isolated chloroplasts were solubilized with 1.3% decylmaltoside according to Lübeck *et al.* (1997). The solubilized proteins and complexes were in turn subjected to immunoprecipitations with the anti-Toc33 and anti-Toc34-antisera described previously.

With wild-type and ppi3 chloroplasts, the anti-Toc33 antiserum, but not the anti-Toc34 antiserum, was able to co-immunoprecipitate the radiolabeled pPORA (Figure 4c, lanes b and c). By contrast, no co-precipitates were obtained for ppi1 chloroplasts (Figure 4c, lanes e and f). Similar, but Toc34-mediated, co-immunoprecipitates were produced for pPORB both with wild-type and ppi1, but not ppi3, chloroplasts (Figure 4c, lanes h and k). In all cases tests with preimmune sera were negative (data not shown).

Protein import into ppi1 etioplasts and chloroplasts

Toc33 was originally regarded as part of the general protein import machinery (Jarvis *et al.*, 1998). Its lack in ppi1 chloroplasts correlated with a drop in general protein import. Precursors to the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU), the light-harvesting chlorophyll *a/b* binding protein of photosystem II (LHCII) and PORB were imported into ppi1 chloroplasts with similar, ca 25–40% lowered efficiencies, compared with wild-type chloroplasts (Jarvis *et al.*, 1998; Kubis *et al.*, 2003).

Heterologous soybean pSSU, *Silene pratensis* precursor ferredoxin (pFd) and barley pPORA and pPORB were used to re-address these previous observations. Import experiments were carried out with wild-type and ppi1 plastids. In a first set of experiments, import of pPORA and pPORB was tested for etioplasts and chloroplasts. All incubations were carried out in darkness. Figure 5(a,b) shows that import of pPORA was quantitatively inhibited in ppi1 etioplasts and chloroplasts. In either case, precursor levels remained constant before and after incubation and no mature PORA protein appeared inside the plastids (Figure 5, lanes 6–10). By contrast, wild-type plastids were able to import pPORA (Figure 5, lanes 1–5). Import was substrate-dependent and occurred only in plastids that contained Pchlide, either produced from endogenous 5-ALA (etioplasts) or exogenously administered 5-ALA (chloroplasts). Consistent with previous results (Reinbothe *et al.*, 1995c), import of pPORB was not dependent on Pchlide. Wild-type and ppi1 etioplasts and chloroplasts readily incorporated and processed the precursor regardless of whether the incubations were performed in the presence or absence of 5-ALA (Figure 5, lanes 11–20).

In a subsequent set of experiments we tested import of soybean pSSU, *S. pratensis* precursor ferredoxin (pFd) as well as barley pPORA and pPORB into wild-type and ppi1 chloroplasts. Parallel incubations were performed in darkness and white light. This experiment not only confirmed that no pPORA uptake occurs into ppi1 chloroplasts but it also highlighted a light-induced destabilization of imported pPORB, leading to its degradation by plastid proteases (Figure 5c,d). As shown previously, PORB imported into chloroplasts is destabilized and degraded as a result of light-driven chlorophyllide formation (Reinbothe *et al.*, 1995c).

The results shown in Figure 5(c) additionally unveiled that no reduction in import of pSSU and pFd was detectable unless the incubations were carried out in white light. The amount of each protein imported into ppi1 chloroplasts, expressed as a percentage of the amount imported into wild-type plastids, dropped to 68% (pFd) and 62% (pSSU) and thus matched previously reported levels (Jarvis *et al.*, 1998). Further, approximately 5–10% reductions in pSSU and pFd import were detected when ppi1 chloroplasts were fed 5-ALA in white light during the import reactions (Figure 5d). Similar, though slightly smaller 5-ALA- plus light-dependent reductions of import occurred for wild-type chloroplasts (Figure 5d).

Evidence for photooxidative membrane damage in ppi1 chloroplasts

Plastid envelope membrane proteins were isolated from wild-type and ppi1 chloroplasts and examined by Western blotting. Figure 6(a) shows that both atToc159 and atToc34 were partially degraded in 5-ALA-fed ppi1, but not wild-type, chloroplasts. Consistent with previous results reported for atToc159 (Bauer *et al.*, 2000) and pea Toc159 (Bölter *et al.*, 1998; Chen *et al.*, 2000) treated with protease, major proteolytic products of 86 and 52 kDa were produced

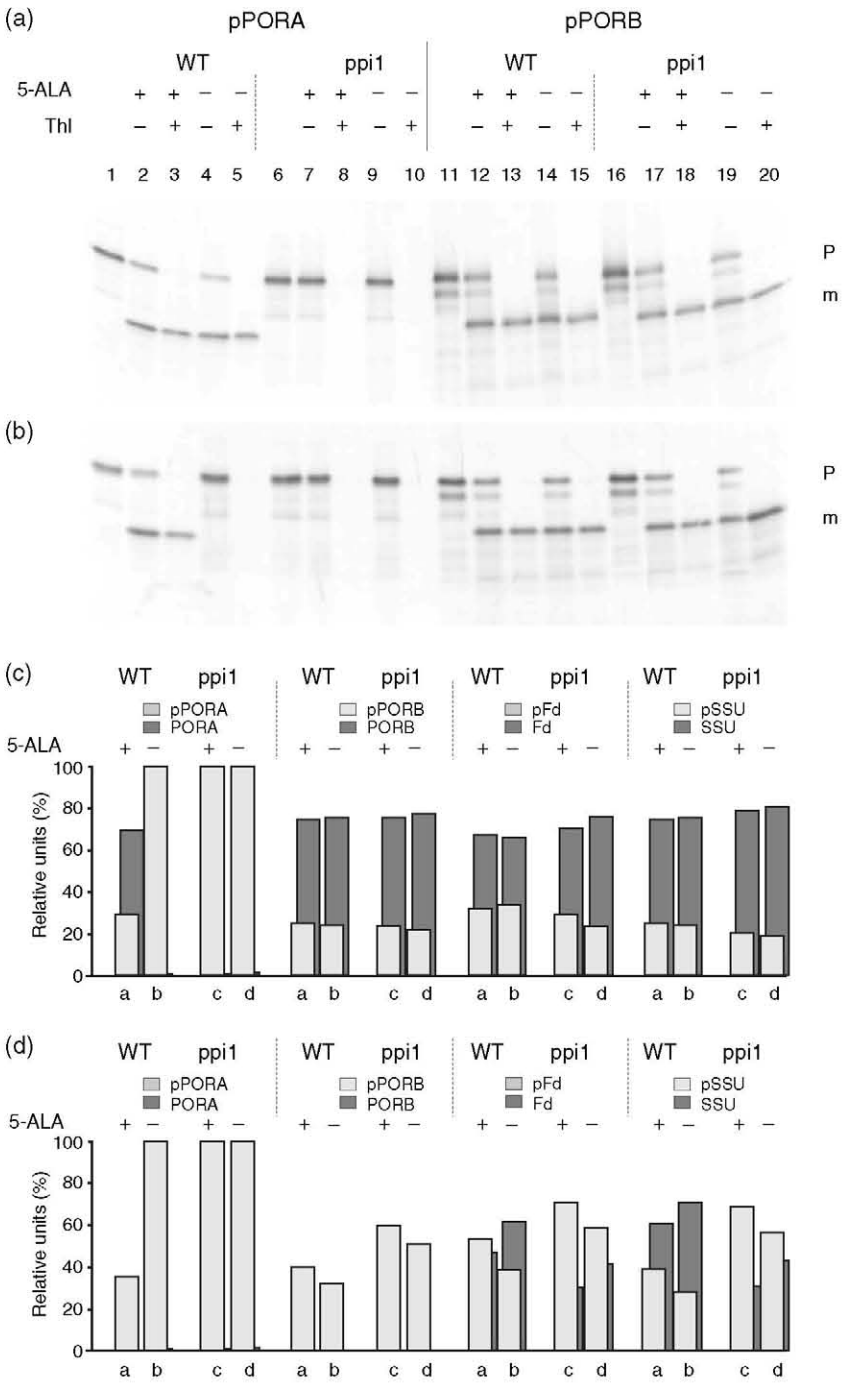

Figure 5. Import of ^{35}S-pPORA and ^{35}S-pPORB tested with ppi1 etioplasts and chloroplasts. ^{35}S-labeled, urea-denatured ^{35}S-pPORA, ^{35}S-pPORB, ^{35}S-pSSU and ^{35}S-pFd were synthesized by coupled *in vitro* transcription/translation. Import reactions in turn were conducted with wild-type (WT) and ppi1 etioplasts and chloroplasts in the presence of 0.1 mM Mg-GTP and 2 mM Mg-ATP at 23°C. As indicated, part of the assay mixtures was supplemented with either phosphate-buffered 5-ALA (+5-ALA), to induce Pchlide synthesis, or phosphate buffer instead of 5-ALA (–5-ALA). After 15 min, import was terminated by the addition of either thermolysin (200 μg ml^{-1} final concentration) (+Thl) or an equal volume of doubly concentrated SDS-sample buffer (–Thl). Protein was resolved by SDS-PAGE and detected by autoradiography. Lanes 1, 6, 11, and 16 each show input standards at time point zero.
(a) ^{35}S-pPORA and ^{35}S-pPORB import into wild-type and ppi1 etioplasts in darkness.
(b) Similar to (a) but showing ^{35}S-pPORA and ^{35}S-pPORB import into chloroplasts in darkness.
(c, d) Quantitative import data for ^{35}S-pPORA, ^{35}S-pPORB, soybean ^{35}S-pSSU and *Silene pratensis* ^{35}S-pFd and 5-ALA-treated (lanes a and c each) and mock-incubated (lanes b and d each) wild-type (WT, lanes a and b each) and ppi1 (lanes c and d each) chloroplasts after dark (c) and light (d) incubations. Light gray and dark gray columns indicate precursor and mature protein levels, relative to the amount of input radioactivity.

(Figure 6a). Moreover, atToc34 was partially degraded in ppi1, but not in wild-type, chloroplasts (Figure 6a), and the resulting banding pattern was reminiscent of that reported previously for pea Toc34 (Chen and Schnell, 1997). By contrast, atToc75 and the inner membrane proteins atTic110 and atTic55 were not proteolytically degraded (Figure 6a). In addition, levels of stromal small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase and thylakoid LHCII were comparable for wild-type and ppi1 plants (Figure 6b). There was no evidence for the accumulation of un-imported preSSU and preLHCII in ppi1 plants (Figure 6b), as would be expected if Toc33 would be involved in import of these precursors.

Discussion

Our analysis of the interactions and roles of Oep16 and Toc33 in pPORA import reveals that both proteins interact

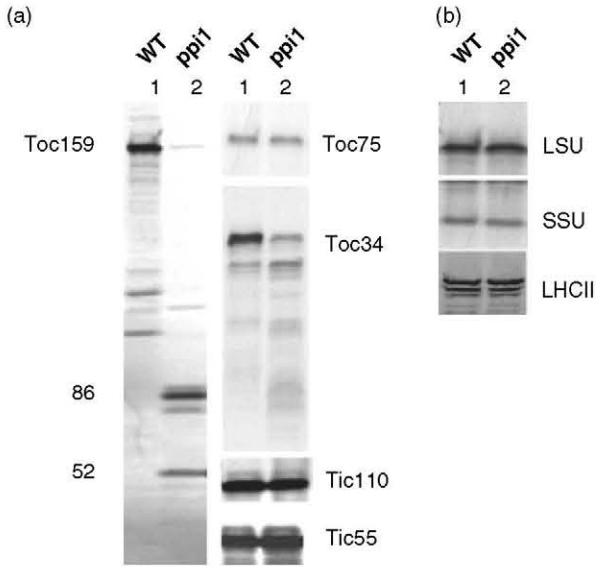

Figure 6. Photooxidative membrane damage in ppi1 chloroplasts. Chloroplasts were isolated from light-grown wild-type (WT) and ppi1 plants, fed 5-ALA, re-purified, lysed and fractionated into mixed outer and inner envelope membranes, stroma, and thylakoids. Protein was extracted from the various fractions, separated by SDS-PAGE and subjected to Western blotting, using the indicated antisera.
(a) Levels of atToc159, atToc34, atTic110, and atTic55 in wild-type and ppi1 chloroplasts.
(b) Levels of large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU and LSU, respectively) and the light-harvesting chlorophyll *a*/*b* binding protein of photosystem II (LHCII) in wild-type and ppi1 chloroplasts.

with each other and also with pPORA. This result supports previous evidence (Reinbothe *et al.*, 2000, 2004a,b) that pPORA does not interact with Toc159 and Toc75 which are presumed to cooperate during the recognition, binding and translocation of precursor proteins related to photosynthesis in the chloroplast (Ma *et al.*, 1996; Perry and Keegstra, 1994; Schnell *et al.*, 1994; Wu *et al.*, 1994). Employing the ppi1 mutant of Arabidopsis we demonstrate that Toc33, but not Toc34, constitutes the Ptc complex. Ppi1 chloroplasts lacking Toc33 were unable to import pPORA but imported pSSU, pFd and pPORB in darkness. In white light, partial suppressions of pSSU, pFd and pPORB uptake were measured. We observed that Arabidopsis chloroplasts are prone to light-dependent modifications in their plastid envelope membrane constituents, such as atToc159 and atToc34, which were partially proteolytically degraded in 5-ALA-fed ppi1 chloroplasts. Findings from Pineau *et al.* (1986) and Joyard *et al.* (1990) point to plastid envelope tetrapyrroles as potential photosensitizers. Protochlorophyllide is one such tetrapyrrole that because of its documented photodynamic properties (Chakraborty and Tripathy, 1992; Tripathy and Chakraborty, 1991) could trigger light-dependent modifications in membrane lipids and protein import complexes, including their disassembly and subsequent protein degradation. Overproduction of free photoexcitable Pchlide in ppi1 chloroplasts led to the observation of a general drop of protein import. In support of this view, op den Camp *et al.* (2003) demonstrated the generation of hydroxyoctadecatrienoic acid that could be attributed to oxidation of the unsaturated membrane fatty acid α-linolenic acid in the *flu* mutant of Arabidopsis. *Flu* is impaired in the negative feedback loop normally limiting excess Pchlide accumulation (Meskauskiene *et al.*, 2001).

AtToc33 and atToc34 are highly related in their amino acid sequences (Jarvis *et al.*, 1998). However, Northern analyses have highlighted their overlapping expression patterns (Gutensohn *et al.*, 2000; Jarvis *et al.*, 1998). Functional tests have mainly made use of pull-down assays employing atToc33 and atToc34 mutant proteins lacking their M-domains, called atToc33ΔM and atToc34ΔM, respectively. Gutensohn *et al.* (2000) reported that atToc33ΔM bound higher levels of pSSU than atToc34ΔM when both proteins were pre-incubated in the presence of 1 mM GTP. However, atToc33ΔM and atToc34ΔM pre-incubated without GTP showed no difference in the binding capacity for pSSU. The experiments employed used a competition assay in which the amount of non-bound precursor was determined by its sequestration by isolated spinach chloroplasts. The use of high GTP concentrations (1 mM instead of 0 or 0.1 mM) for analyzing the initial steps of protein translocation into isolated plastids makes comparisons difficult. Biochemically, cross-linking of pea Toc34 to preproteins occurs only in the absence of additional GTP or ATP (Kouranov and Schnell, 1997). Interestingly, the phenotype (especially the chlorophyll deficiency) of the ppi1 mutant was complemented by overexpression of either Toc33 or Toc34 (Jarvis *et al.*, 1998) implying that both proteins are able to bind the same set of precursors, at least when plants were grown in continuous white light. In addition, in two recently isolated knockout lines that were deficient in atToc34, called ppi3-1 and ppi3-2, respectively, no changes were observed with respect to chlorophyll content, chloroplast ultrastructure, endogenous levels of chloroplast proteins, and chloroplast protein import (Constan *et al.*, 2004). At first glance, these results implicate redundant functions of atToc33 and atToc34 *in planta*. However, we noted that ppi3 chloroplasts considerably import pPORA, whereas ppi1 chloroplasts do not. In addition, both components are expressed in varying degrees during the early stages of plant development following seed germination and atToc33 levels largely exceed those of atToc34 (Gutensohn *et al.*, 2000; Jarvis *et al.*, 1998). These findings suggest that atToc33 and atToc34 could interact with different receptors and translocation channel components such as atToc159, atToc130, atToc125 and atToc90, as well as atToc75 and atOep16 and be involved in different import pathways (see Jackson-Constan and Keegstra, 2001; Reinbothe *et al.*, 2004a,b). Indeed, pull-down assays have identified only a fraction of atToc33M interacting with

atToc75 (Bauer *et al.*, 2002; Hiltbrunner *et al.*, 2001), whereas another fraction of atToc33, as shown in this work, is associated with Oep16 and establishes the Ptc complex.

AtToc159 is present to a large extent in a soluble form in Arabidopsis cells. This finding suggests that atToc159 may assemble in the outer plastid envelope membrane in a precursor-complexed form (Bauer *et al.*, 2002; Hiltbrunner *et al.*, 2001; Smith *et al.*, 2002). How the specificity toward one or the other translocon complex containing closely related Toc33 and Toc34 proteins may be regulated is currently unknown. Previous studies using truncated atToc33ΔM and atToc34ΔM thus have provided interesting, but only limited, insights into the function and assembly of the protein import machineries in chloroplasts. In three recent papers, the existence of different atToc159-related proteins correlated with different precursor specificities *in vitro* (Ivanova *et al.*, 2004; Kubis *et al.*, 2004; Smith *et al.*, 2004). Nevertheless, novel approaches, making use of the established ppi1, ppi2 and ppi3 mutants of Arabidopsis and, for example, the tandem affinity purification method (Puig *et al.*, 2001) or GFP technology (Kim and Apel, 2004), are need to confirm these data and to further dissect the actual functions and assemblies of the different protein import machineries of plastids.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana used in this study was of the ecotype Col-0. The ppi1 mutant has been described (Jarvis *et al.*, 1998). The ppi3-2 mutant was created by the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) and provided by the NASC Nottingham Arabidopsis Stock Centre (http://arabidopsis.info). Surface-sterilized seeds were imbibed for 24 h at 4°C and plated on 1x Murashige–Skoog medium (Gibco-BRL, Grans Island, NY, USA) containing 0.5–1% (w/v) sucrose and 0.6% agar, or were sown on soil.

DNA constructs

Construction and expression of cDNA clones encoding the pPORA and pPORB (Holtorf *et al.*, 1995; Schulz *et al.*, 1989) have been described (Reinbothe *et al.*, 2004a,b). cDNAs for Arabidopsis pPORA and pPORB were a kind gift of Dr R.B. Klösgen, Halle, Germany. Barley Oep16 DNA was a gift from Dr P. Baldi, Italy. AtOep16 DNA was produced by the polymerase chain reaction and subcloned into the pBlueScript SK vector.

Plastid isolation, manipulation, and protein import

Seeds of *A. thaliana* were germinated at 25°C under standard conditions and grown either in complete darkness or under continuous white light illumination provided by fluorescent bulbs (30 W m^{-2}) for appropriate periods. Plastids were isolated from leaf homogenates by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech Europe, Saclay, France) (Reinbothe *et al.*, 1995a). Energy depletion was achieved as described by Theg *et al.* (1989). Treatment of isolated chloroplasts with phosphate-buffered 5-aminolevulinic acid (5-ALA) was performed as described previously (Reinbothe *et al.*, 1995a). Controls were mock-incubated with phosphate buffer instead of 5-ALA.

Re-isolated, intact plastids were resuspended in import buffer lacking ATP (Reinbothe *et al.*, 1995a) and added to the radiolabeled precursors given in the text. Either bacterially expressed precursors (see below) or *in vitro*-synthesized precursors were used. *In vitro* synthesis of ^{35}S-containing precursors was made in a wheat germ system by coupled transcription/translation of the cDNA clones described previously. If necessary, the precursors were concentrated by ammonium sulfate precipitation. All precursors were denatured in 8 M urea prior to use (Reinbothe *et al.*, 2000). To study import, aliquots were withdrawn and diluted such that the final urea concentration in the assays did not exceed 0.2 M. Final 50-μl import mixtures usually consisted of 25 μl of doubly concentrated import buffer (Reinbothe *et al.*, 1995a), 10 μl of the plastid suspension containing, if not stated otherwise, 5×10^7 plastids, 5 μl of the different urea-denatured, radiolabeled precursors, and Mg-ATP and Mg-GTP as indicated in the text. If needed, double-distilled water was added to adjust the final reaction volume. All assay mixtures were assembled on ice under a dim green safe light; the actual import reactions were performed at 23°C for 15 min in darkness. Plastids were re-isolated on Percoll after import (Reinbothe *et al.*, 1995a). Post-import protease treatment of plastids with thermolysin and extraction of membranes with sodium carbonate, pH 11, or 1 M NaCl were carried out as described (Cline *et al.*, 1984). Plastid subfractionation into envelopes, stroma and thylakoids was according to Li *et al.* (1991). Protein was precipitated with trichloroacetic acid (5 % w/v final concentration), resolved by SDS-PAGE on 10–20 % (w/v) polyacrylamide gradients (Laemmli, 1970) either under reducing or non-reducing conditions (Tokatlidis *et al.*, 1996) and detected by autoradiography. The gels shown in Figure 4(a,b) are 15% (w/v) mini-gels.

Crosslinking

^{35}S-pPORA-$(His)_6$ and ^{35}S-PORB-$(His)_6$ precursors were expressed in *Escherichia coli* strain SG13009 (Qiagen, Hilden, Germany) in the presence of ^{35}S-methionine and recovered from inclusion bodies by dissolution in 8 M urea containing 20 mM imidazole-HCl, pH 8.0. Protein was passed over a G-25 column which yielded approximately 85% pure precursor fractions. Derivatization of precursors was achieved with DTNB (Habeeb, 1972; Tokatlidis *et al.*, 1996). For the experiment described in Figure 4, non-^{35}S-labeled, bacterially expressed, purified pPORA-$(His)_6$ and pPORB-$(His)_6$ were used. Derivatization of precursors with ^{125}I-*N*-[4[(*p*-azidosalicyl-amido)butyl]-3′(2-pyridyldithio) propionamid (APDP) was made according to Ma *et al.* (1996). In all cases, precursors were denatured with urea and diluted prior to use as indicated above. Incubations were carried out with isolated, energy-depleted plastids in the presence of 0.1 mM Mg-ATP and 0.1 mM Mg-GTP (insertion) or 2.0 mM Mg-ATP and 0.1 mM Mg-GTP (import) for 15 min in darkness. Plastid work-up after crosslinking has been described elsewhere (Reinbothe *et al.*, 2004a).

Treatment of chloroplasts with DSP, processing of the plastids, and subsequent membrane solubilization were essentially as described by Lübeck *et al.* (1997).

Immunologic techniques

Antisera were raised against Toc33 and Toc34 by using the following synthetic peptides (Jarvis *et al.*, 1998) as antigens:

^{283}QGAIRNDIKTSGKPL^{297} (Toc33) and ^{286}VRAIKSDVSRESKLA^{300} (Toc34). Antisera against pea Toc86, Toc75, Toc34, Tic55, Tic110 and Oep16 as well as barley Oep16 have been described and characterized elsewhere (Baldi *et al.*, 1999; Lübeck *et al.*, 1997; Ma *et al.*, 1996; Pohlmeyer *et al.*, 1997; Schnell *et al.*, 1994). Antisera against atToc159, atToc75 and atTic110 were a gift of Dr F. Kessler, Université Neuchatel, Neuchatel, Switzerland. Immunoprecipitation and co-precipitation were performed according to Wiedmann *et al.* (1987). Western blotting was carried out as described by Towbin *et al.* (1979), using either an ECL system (Amersham) or the anti-rabbit, anti-goat, alkaline phosphatase system.

Miscellaneous

Protein sequencing was performed according to Chang (1983) and Chang *et al.* (1978).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2353/TPJ2353sm.htm

Figure S1. ^{35}S-Oep16 import into isolated wild-type and ppi1 chloroplasts of Arabidopsis.

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