

Complete maturation of the plastid protein translocation channel requires a type I signal peptidase

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The protein translocation channel at the plastid outer envelope membrane, Toc75, is essential for the viability of plants from the embryonic stage. It is encoded in the nucleus and is synthesized with a bipartite transit peptide that is cleaved during maturation. Despite its important function, the molecular mechanism and the biological significance of the full maturation of Toc75 remain unclear. In this study, we show that a type I signal peptidase (SPase I) is responsible for this process. First, we demonstrate that a bacterial SPase I converted Toc75

precursor to its mature form in vitro. Next, we show that disruption of a gene encoding plastidic SPase I (Plsp1) resulted in the accumulation of immature forms of Toc75, severe reduction of plastid internal membrane development, and a seedling lethal phenotype. These phenotypes were rescued by the overexpression of Plsp1 complementary DNA. Plsp1 appeared to be targeted both to the envelope and to the thylakoidal membranes; thus, it may have multiple functions.

Introduction

The 75-kD component of the translocon at the outer envelope membrane of chloroplasts, Toc75, forms a β -barrel structure and is postulated to be part of the protein translocation channel (Hinnah et al., 1997). In the model plant *Arabidopsis thaliana*, Toc75 is encoded by a single functional gene (*atTOC75-III*) in the nucleus (Jackson-Constan and Keegstra, 2001), and its disruption by a T-DNA insertion resulted in an embryo-lethal phenotype (Baldwin et al., 2005). Toc75 is the only known protein in the outer membrane of plastids or mitochondria that is synthesized as a larger precursor with an NH₂-terminal extension and goes through multiple cleavages during maturation (Tranel et al., 1995; Schleiff and Klösgen, 2001). The Toc75 transit peptide consists of two domains. The first portion targets the protein into the stroma via the general pathway and is removed by a stromal processing peptidase (Tranel and Keegstra, 1996). The second part contains a unique polyglycine stretch that appears to function as a stop transfer domain and is cleaved by an unidentified enzyme (Tranel and Keegstra, 1996; Inoue and

Keegstra, 2003). The intermediate form of Toc75 that lacks the first but still retains the second portion of the transit peptide was found in the plastid outer envelope membrane along with the mature form in young leaves (Tranel et al., 1995). However, whether or not the intermediate form plays any active biological roles remains unknown. Recently, two conserved alanine residues at -3 and -1 to the second cleavage site of the transit peptide were found to be important for complete maturation of Toc75 (Inoue and Keegstra, 2003). This feature is reminiscent of that for signal peptides that direct the export or secretion of proteins and are cleaved by type I signal peptidase (SPase I; Paetzel et al., 2002). Thus, the involvement of SPase I in complete cleavage of the Toc75 transit peptide has been suggested (Inoue and Keegstra, 2003; Inoue et al., 2005).

SPases I exist in a wide range of cell membranes, including plasma membranes of prokaryotes, the endoplasmic reticulum, the mitochondrial inner, and the chloroplast thylakoidal membranes of eukaryotes (Paetzel et al., 2002). A typical SPase I has one transmembrane domain or two domains along with four conserved soluble regions, two of which contain serine and lysine residues, respectively, which together form a catalytic dyad that is essential for enzyme activity (Paetzel et al., 2002). SPase I substrates often contain small, uncharged residues such as alanine at -3 and -1 to the cleavage site (von

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Abbreviations used in this paper: Lep, leader peptidase; OE33, 33-kD component of the oxygen-evolving complex; PLB, prolamellar body; Plsp1, plastidic SPase I; POR, protochlorophyllide oxidoreductase; SPase I, type I signal peptidase; Toc, translocon at the outer envelope membrane of chloroplasts.

Heijne, 1986). The substrate specificity of SPase I *in vitro* is relatively broad; e.g., a thylakoidal processing peptidase was shown to cleave bacterial proteins, whereas a bacterial enzyme processed thylakoidal proteins (Halpin et al., 1989).

In this study, we provide evidence that a plastidic SPase I (Plsp1) is responsible for the full maturation of Toc75. Furthermore, we show that this SPase I is required for the biogenesis of plastid internal membranes.

Results and discussion

As a first step to examine whether SPase I is responsible for the complete maturation of Toc75, we incubated the radiolabeled Toc75 precursor with an SPase I from *Escherichia coli*, leader peptidase (Lep). As shown in Fig. 1, the active but not heat-denatured Lep converted the Toc75 precursor to its mature form regardless of the presence of the first part of the transit peptide (lanes 3, 4, 6, and 7). This finding prompted us to seek SPases I in the model plant *A. thaliana* as candidates for the peptidase that is responsible for full maturation of Toc75. There are three SPases I predicted to be located in the plastid (Inoue et al., 2005). One of them, At2g30440, has already been identified as the thylakoidal processing peptidase (Chaal et al., 1998). The second protein, At1g06870, has not been identified in any proteome libraries, but its gene expression is evidenced by the presence of multiple cDNA clones in the database. Finally, the presence of the third protein, At3g24590, in chloroplasts had been confirmed by multidimensional chromatography (Kleffmann et al., 2004). We named this protein Plsp1 for plastidic type I signal peptidase 1 and decided to focus our research efforts on this protein.

We cloned a coding sequence of Plsp1 by RT-PCR from *A. thaliana* seedlings. Our initial attempts to express it and assay the activity of Plsp1 protein in *E. coli* were, however, unsuccessful. Thus, we took a genetic approach to test whether Plsp1 is involved in the maturation of Toc75. We identified a T-DNA mutagenized line of *A. thaliana* with an insertion in the fourth intron of *PLSP1* and named it *plsp1-1* (Fig. 2 A). Plants that were heterozygous for the insertion were apparently indistinguishable from wild type. All of their seeds were germinated on plates containing Murashige-Skoog (MS) media with 1% sucrose. About 25% of resultant seedlings were albino (Fig. 2 B) and died before they developed complete true leaves. We confirmed that they were homozygous for the insertion by genomic PCR (Fig. 2 C, lanes 4 and 5). Furthermore, *PLSP1* transcript was not detectable in mutant plants (Fig. 2 D, lane 2). The seedling lethal phenotype of homozygous *plsp1-1* plants was rescued by expressing a Plsp1 coding sequence with Cauliflower mosaic virus 35S promoter (Fig. 2, B–D). These data confirm that the phenotype of the mutant plants was caused by the disruption of *PLSP1*.

We also examined the morphology of plastids. In cotyledons of homozygous *plsp1-1* mutants, plastids were surrounded by a double-membrane envelope and were similar to wild-type chloroplasts in their sizes; however, the development of thylakoidal membranes was severely retarded (Fig. 2, E and F). We also obtained etiolated seedlings of both wild-type and *plsp1-1* plants. They did not show any apparent visible differ-

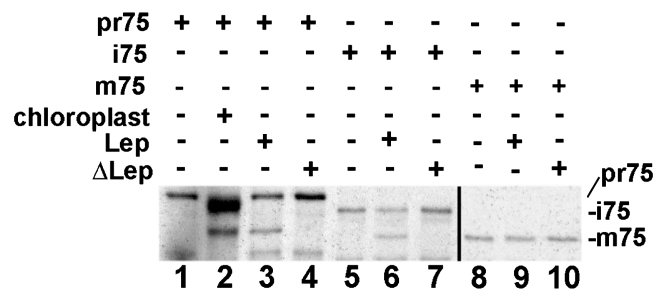


Figure 1. A bacterial SPase I can convert Toc75 precursor to its mature form. Radiolabeled precursor (pr75; lane 1; 10% input) was converted to the intermediate (i75) and mature (m75) forms by chloroplast protein import assay *in vitro* (lane 2). When pr75 or i75 was incubated with Lep, the production of m75 was detected, whereas m75 was not processed (lanes 3, 6, and 9, respectively). Heat-denatured Lep was used as a negative control (lanes 4, 7, and 10). The black line indicates grouping of images of different exposures.

ences. By EM analysis, however, we found a distinctive phenotype of *plsp1-1* plastids: they did not develop the proper internal compartments, such as prolamellar bodies (PLBs), that were present in wild-type plastids (Fig. 2, G and H). Altogether, these data indicate that Plsp1 plays a role in the biogenesis of plastid internal membranes.

To examine the effect of *PLSP1* disruption on the processing and/or accumulation of Toc75, we analyzed proteins from etiolated seedlings by immunoblotting. As shown in Fig. 3, mutant plants mainly accumulated an 84-kD protein, which corresponds to the intermediate form of Toc75 as well as to several minor proteins of sizes larger than 75 kD (lane 2). In contrast, the complemented plants accumulated mature-sized Toc75 (Fig. 3, lane 3). The presence of the intermediate form in the complemented plants (Fig. 3, lane 3) may be caused by the ectopic expression of *PLSP1* by the constitutive promoter. These data indicate that Plsp1 is required for proper maturation of Toc75. Previously, Toc75 was shown to be essential for embryogenesis (Baldwin et al., 2005). Together with the current data, we suggest that the immature forms of Toc75 can form protein translocation channels to support at least minimal development of plastids and, thus, embryogenesis.

We also tested whether accumulation and sizes of other nuclear-encoded plastid proteins were affected by the disruption of *PLSP1* (Fig. 3). An inner envelope protein, Tic40 (Chou et al., 2003), and a stromal chaperone, Hsp93 (Constan et al., 2004), appeared to be processed and accumulated properly in mutant plants (Fig. 3, lanes 4–9). Interestingly, although *plsp1-1* plastids did not develop PLBs correctly, they contained protochlorophyllide oxidoreductase (POR), the major constituent of PLBs (Ryberg et al., 1983), in an amount similar to wild type (Fig. 3, lanes 10–12). This is reminiscent of the case in *crisis* plants, in which the accumulation of POR was not affected even though the development of PLBs was disturbed because of the lack of carotenoid isomerization (Park et al., 2002).

The 33-kD component of the oxygen-evolving complex (OE33) is located in the thylakoidal lumen and has been found both in chloroplasts and in etioplasts (Ryrie et al., 1984). It is synthesized with a bipartite transit peptide, which consists of a

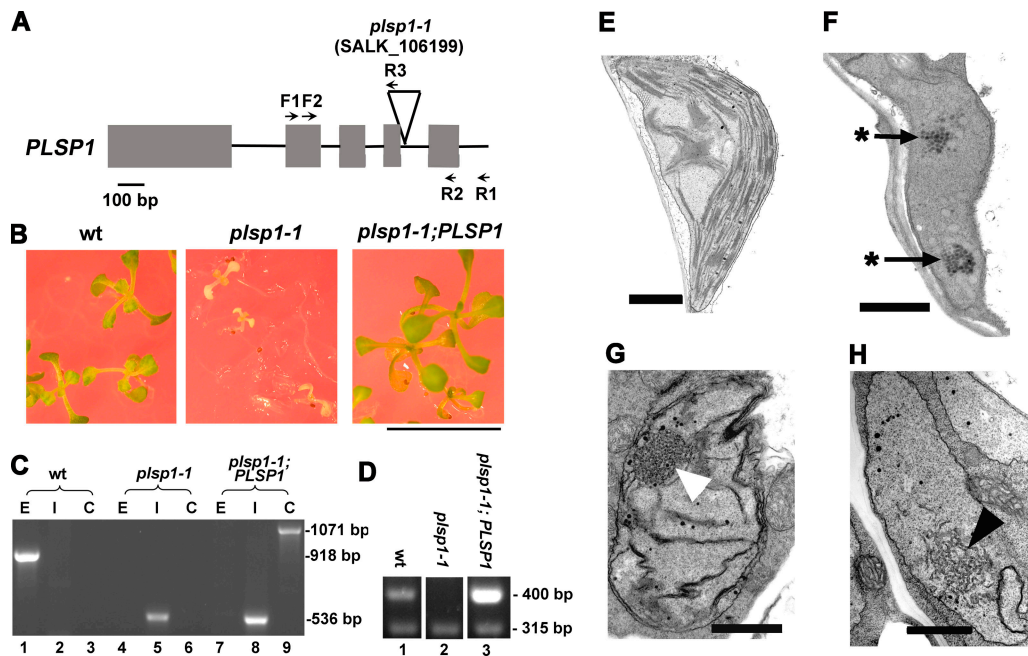


Figure 2. Disruption of *PLSP1* results in a seedling lethal phenotype and reduction of the development of plastid internal membranes. (A) Structure of *PLSP1*. Gray boxes and solid lines indicate exons and introns, respectively. The position of the T-DNA insertion in *plsp1-1* was determined experimentally and is indicated with a white arrowhead. Positions of primers used for genomic and RT-PCR are indicated with black arrows. Primers and inserted T-DNA indicated with arrows/arrowhead are not to scale. (B) Seedlings of wild-type (*wt*), *plsp1-1*, and *plsp1-1;PLSP1* plants grown on MS media supplemented with 1% sucrose for 2 wk. Bar, 1 cm. (C) Genomic PCR of wild-type and mutant *A. thaliana* seedlings. E, I, and C indicate reactions specific to amplify endogenous *PLSP1*, the T-DNA insertion, and the transgene used to complement the mutation, respectively. Primers that were used are listed as follows: F2 and R1 for E, F2 and R3 for I, and primers from 35S promoter (forward) and nopaline synthase terminator (reverse) for C. (D) RT-PCR analyses of wild-type and mutant *A. thaliana* seedlings. Each reaction contained two sets of primers: one for *PLSP1* cDNA (F1 and R2) that produces a 400-bp fragment and another for cDNA derived from 18S RNA that produces a 315-bp fragment. Images from different portions of the same gel are in separate boxes. (E and F) Plastids in 2-wk-old wild-type (E) and homozygous *plsp1-1* (F) cotyledons. Plastoglobules are indicated with asterisks. (G and H) Plastid in 7-wk-old wild-type (G) and homozygous *plsp1-1* (H) etiolated seedlings. White and black arrowheads indicate PLB and disorganized membrane structure, respectively. (E–H) Bars, 1 μ m.

stromal targeting domain followed by a signal sequence that directs the protein to the thylakoid and is cleaved by the thylakoidal processing peptidase (Yuan et al., 1994). In contrast to most proteins that were examined but similar to Toc75, OE33 was found to accumulate in *plsp1-1* plants mainly as an apparent intermediate form that lacks the first but retains the second targeting domain (Fig. 3, lane 14). This result indicates that either Plsp1 is directly involved in the maturation of OE33 as a thylakoidal processing peptidase or that Plsp1 is required for the proper function of a thylakoidal processing peptidase that is distinct from Plsp1.

All proteins examined in this study were synthesized as larger precursors with transit peptides and imported into the organelle via the general pathway. Our data show that their stromal targeting sequences were properly cleaved, indicating that *plsp1-1* plastids retain the ability to import proteins and also contain the stromal processing peptidase at the normal functional level.

Maturation of Toc75 appears to occur in the envelope membranes. Thus, if Plsp1 is directly responsible for this process, it should be located at the envelope membranes. Recently, however, Plsp1 was found in the thylakoidal membrane proteome (Peltier et al., 2004). To confirm its suborganellar localization, we subjected radiolabeled Plsp1 to *in vitro* chloroplast import assays. As shown in Fig. 4 A, the 32.6-kD Plsp1 precursor protein (lane 1) was processed to 25 kD (lane 2), which was

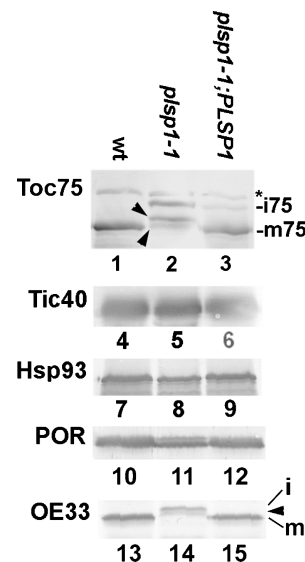


Figure 3. Disruption of *PLSP1* results in the accumulation of unprocessed forms of Toc75. Protein extracts of etiolated *A. thaliana* seedlings were analyzed by immunoblotting using antisera against the indicated plastidic proteins. m75, i75, and a 90-kD protein nonspecifically detected by the antisera (indicated with an asterisk) are indicated on the right side of the top row. Two protein bands slightly larger than 75 kD that were detected in *plsp1-1* plants are indicated with arrowheads (lane 2). Bands corresponding to the intermediate and mature forms of OE33 and also a minor band larger than the mature form are indicated with the letters i, m, and an arrowhead, respectively, in the bottom row.

integrated into membranes and was not exposed to the surface of the organelle, as indicated by its resistance to carbonate extraction (lane 6) and also to thermolysin treatment (lane 12). Trypsin is a protease that can reach the surface of the inner membrane (Jackson et al., 1998). Interestingly, trypsin treatment of chloroplasts containing the imported Plsp1 resulted in the production of a partially degraded protein of ~22 kD along with the intact mature protein (Fig. 4 A, lane 18). The ratio of the two proteins was ~1:1, which was consistent among three independent experiments and did not change by increasing the concentration of trypsin up to 10 times (not depicted). The Plsp1 precursor was completely degraded after direct incubation with trypsin (Fig. 4 A, lane 13). However, when chloroplasts containing the imported protein were treated with trypsin in the presence of a detergent, a 22-kD protein was produced (Fig. 4 A, lane 14). These data imply that after Plsp1 was incorporated into chloroplasts, it formed a structure in which most lysine and arginine residues in the protein were highly protected even when the lipid bilayer was disrupted. Under the current conditions, a peripheral inner membrane protein that was located in the intermembrane space, Tic22 (Kouranov et al., 1998), was recovered in the supernatant after alkaline treatment (Fig. 4 A, lane 23) and was completely digested by trypsin (Fig. 4 A, lane 36). An outer membrane protein, DGD1 (Froehlich et al., 2001), was susceptible to thermolysin, whereas a stroma-facing inner membrane protein, m110N (Jackson et al., 1998), was resistant to trypsin (Fig. 4 A, lanes 44 and 52, respectively). Together, these results suggest that Plsp1 was targeted to at least two subcompartments: at the location where trypsin can reach (i.e., at the envelope membranes) and at the location where trypsin cannot reach (i.e., at the thylakoid). This idea was supported by fractionation analysis (Fig. 4 B). Imported Plsp1 was detected mainly in the thylakoid but also in envelope fractions in a ratio of ~6:1 (Fig. 4 B, lanes 1–4). In contrast, envelope proteins Toc75-IV and Tic110 were detected almost equally in the thylakoid and in envelope fractions (Fig. 4 B, lanes 5–12), and light harvesting chlorophyll *a/b*-binding protein was recovered in the thylakoid but not in the envelope fraction (Fig. 4 B, lanes 13–16).

What does the dual targeting of Plsp1 mean? Tranel et al. (1995) showed that the level of Toc75 transcript peaked in young tissues that undergo rapid plastid development and declined as the plant matured, whereas the amount of Toc75 protein remained almost constant during leaf development. In addition, the intermediate but not the mature form of Toc75 was the predominant product in import assay *in vitro* under conditions in which mature tissues were used as a source of chloroplasts (Fig. 1, lane 2). These observations imply that the maturation of Toc75 takes place in developing plastids more actively than in mature chloroplasts. The location of Plsp1 may depend on the developmental stage of plastids, and Plsp1 may have multiple functions. In premature chloroplasts where internal membranes are not fully developed yet, Plsp1 may catalyze complete maturation of Toc75 at envelope membranes. In contrast, in mature chloroplasts, Plsp1 may be mainly located at the thylakoidal membrane and process thylakoidal proteins.

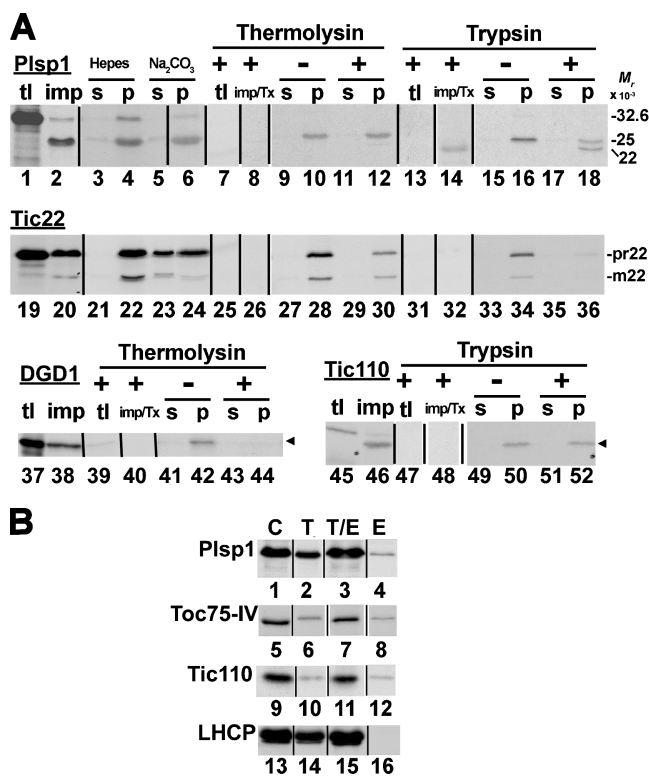


Figure 4. Plsp1 is targeted to chloroplast membranes *in vitro*. (A) Radiolabeled precursors (tl; 10% input) were incubated with pea chloroplasts. Chloroplasts were either directly analyzed (imp) or lysed and separated into supernatant (s) and pellet (p) fractions as described previously (Inoue and Keegstra, 2003). Alternatively, chloroplasts containing imported proteins were treated without or with thermolysin or trypsin (0.1 μ g protease/1 μ g chlorophyll-equivalent chloroplasts) and separated into soluble (s) and pellet (p) fractions or treated with proteases with the presence of 1% Triton X-100 (imp/Tx). The translation products directly treated with proteases are also shown. Imported DGD1 and m110N are indicated with arrowheads in the bottom two panels. (B) Imported radiolabeled proteins (C) were further fractionated by centrifugation to thylakoids (T) or total membrane (T/E) and envelope (E) fractions as described previously (Baldwin et al., 2005). Black lines indicate grouping of images of different exposures from different gels (A) or that of images from different portions of the same gel (B).

How is Plsp1 involved in the biogenesis of plastid internal membranes? There are at least two potential mechanisms. The first one depends on Toc75. The translocation machinery containing immature Toc75 may not be able to properly import proteins that are required for the development of internal membranes. The second one is independent of Toc75. Plsp1's function as a thylakoidal processing peptidase may be vital for the biogenesis of internal membranes.

Materials and methods

Chloroplast protein import and Lep assays

Preparation of radiolabeled precursor proteins and *in vitro* chloroplast protein import assays were performed as previously described (Inoue and Keegstra, 2003). For the Lep assay, radiolabeled proteins were incubated with the bacterial recombinant enzyme (gift from R. Dalbey, Ohio State University, Columbus, OH) at 37°C in a total volume of 10 μ l for 10 min. The reaction was stopped by the addition of 10 μ l of 2 \times SDS-PAGE sample loading buffer (0.1 M Tris-HCl, pH 8.0, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 5% 2-mercaptoethanol) and incubation in boiling water for 5 min. Proteins were separated by SDS-PAGE and analyzed by fluorography.

Plant materials and growth conditions

A T-DNA insertional mutant of *PLSP1* (*plsp1-1*) was identified from SALK_106199 seeds (Alonso et al., 2003) that were obtained from the *Arabidopsis* Biological Resource Center. Surface-sterilized seeds of wild-type and mutant *A. thaliana* (ecotype Columbia) plants were sown on MS media (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.8% Phytagar (Invitrogen), and seedlings were grown under 12-h light/dark cycles with white fluorescent light of $80 \mu\text{mol m}^{-2}\text{s}^{-1}$ at 25°C for 2 wk. For the growth of etiolated tissue, the 2-wk-old seedlings were transferred to a 500-ml Erlenmeyer flask containing 100 ml MS liquid media supplemented with 1% sucrose and were grown in the dark at 25°C with a rotation at 45 rpm for an additional 5 wk before they were harvested and analyzed.

Molecular analysis of mutant plants

Genotypes of *A. thaliana* plants were examined by PCR using primers specific to *PLSP1* (Fig. 2 A). The exact location of the T-DNA insertion in *plsp1-1* plants was determined by DNA sequencing of the PCR product. For RT-PCR analysis, cDNA was prepared from total RNA from *A. thaliana* seedlings using Superscript II and random primers (Invitrogen). PCR amplifications of part of *Plsp1* cDNA were performed with a set of gene-specific primers (Fig. 2 A). Control amplification of cDNA that was derived from 18S-RNA was performed according to the manufacturer's instructions (Ambion).

Cloning of *Plsp1* cDNA and complementation of *plsp1-1* plants

The coding sequence of *Plsp1* that was isolated from *A. thaliana* seedlings was subcloned into a pGEM-T Easy vector (Promega) as described previously (Inoue and Potter, 2004). For complementation of homozygous *plsp1-1* plants, the *Plsp1* coding sequence was subcloned into vector pBIG-HYG (Becker, 1990) and transformed to heterozygous *plsp1-1* plants by the *Agrobacterium tumefaciens*-mediated infiltration method (Bechtold et al., 1993). Transformants were selected based on their resistance to hygromycin, and their genotypes were further examined by genomic PCR using primers specific to *PLSP1* (Fig. 2 A).

Protein extraction and immunoblot experiments

Plant materials were ground to powder in liquid N₂ by a mortar and pestle, and proteins were extracted with buffer (0.1 M Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 5% 2-mercaptoethanol, and 1 mM PMSF). Protein concentration was determined with BSA as a standard as described previously (Bradford, 1976). 15 μg of proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories), which was incubated with antisera against various plastid proteins. Reactive proteins were detected using secondary antisera conjugated with alkaline phosphatase and substrates, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Bio-Rad Laboratories). Antisera against pea OE33 were prepared as described previously (Ettinger and Theg, 1991). Those against pea Toc75 (Tranel et al., 1995), pea Hsp93 (Constan et al., 2004), and *A. thaliana* Tic40 (Chou et al., 2003) were gifts from K. Keegstra (Michigan State University, East Lansing, MI), and those against wheat POR were obtained from B. Pogson (Australian National University, Canberra, Australia).

Image acquisition

For transmission EM, ultrathin sections were examined with a Philips electron microscope (EM400; FEI Company) at an accelerating voltage of 80 kV, and their images were taken on films and enlarged on a Durst enlarger (model S-45; Durst Image Technology) at the University of California, Davis (Electron Microscopy Laboratory, Department of Pathology and Laboratory Medicine, School of Medicine). Pictures of *A. thaliana* seedlings were taken with a digital camera (Coolpix 4500; Nikon). Images of ethidium bromide-stained DNA agarose gels were obtained by using the Alphamager Imaging System (Alpha Innotech Corporation). Films that were exposed to protein gels containing radiolabeled proteins were developed with X-ray film developer 100 Plus (All-Pro Imaging) and scanned with Precision Scan LTX (Hewlett Packard). All images were processed in Photoshop 7.0 (Adobe).

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