A defect in atToc159 of Arabidopsis thaliana causes severe defects in leaf development

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Plastid protein import 2 (ppi2), a mutant of Arabidopsis thaliana, lacks a homologue of a component of the translocon at the outer envelope membrane of chloroplasts (Toc), designated Toc159 of the pea. Toc159 is thought to be essential for the import of photosynthetic proteins into chloroplasts. In order to investigate the effect of protein import on the plant development, we examined the morphologies of the developing leaves and the shoot apical meristems (SAM) in the ppi2 plants. Our histological analysis revealed that the development of leaves is severely affected in ppi2, while the structure of SAM is normal. Abnormalities in leaves became obvious in the later stages of leaf development, resulting in the generation of mature leaves with fewer mesophyll cells and more intercellular spaces as compared with the wild type. Palisade and spongy tissues of the mature leaves were indistinguishable in ppi2. Replication of chloroplast DNA was also suggested to be impaired in ppi2. Our results suggest that protein import into chloroplasts is important for the normal development of leaves.

Key words: chloroplast biogenesis, leaf development, plastid protein import

INTRODUCTION

The plastid is an organelle that is present only in plant cells. It is thought to be essential for the viability of plants because of its functions, ranging from photosynthesis to biosynthesis of amino acids (Galili, 1995; Lam et al., 1995; Radwanski and Last, 1995; Singh and Shaner, 1995), lipids (Ohlrogge and Browse, 1995), starch (Martin and Smith, 1995), and hormones (Seo and Koshiba, 2002; Weber, 2002; Helliiwell et al., 2001). The majority of proteins that reside in chloroplasts are encoded in the nuclear genome, and synthesized in the cytosol. These nuclear-encoded proteins are imported into the chloroplast via the cooperative action of multimeric complexes in the outer and inner chloroplast envelope membranes, designated the Translocon at the outer envelope membrane of chloroplasts (Toc) complex, and the Translocon at the inner envelope membrane of chloroplasts (Tic) complex (Jarvis and Soll, 2001). The Toc complex in the pea consists of three major components, i.e., Toc75, Toc34, and Toc159 (Cline, 2000). Toc159 is an integral membrane protein exhibiting GTPase activity, and it functions as a receptor of transit-peptide of chloroplast-localized proteins (Kessler et al., 1994). Bauer et al. (2000) analyzed a genetic mutant of Arabidopsis, designated ppi2, which lacks atToc159, a homologue of Toc159. The authors demonstrated that the ppi2 mutation caused defects in chloroplast development, and was seedling-lethal on soil. The concentration of several photosynthetic chloroplast proteins was decreased in the ppi2 plants, because of the reduction in the import of these proteins into the chloroplasts, and the reduction in the level of expression of the appropriate genes (Bauer et al., 2000). It was also demonstrated that a couple of nonphotosynthetic proteins were present in the chloroplasts of the ppi2 plants at the same level as in the wild type. Besides that, it has been indicated that atToc159 is not essential for the biogenesis of plastids in roots (Yu and Li, 2001). Therefore, atToc159 is suggested to be involved in the import of photosynthetic proteins but not involved in the import of nonphotosynthetic proteins into chloroplasts.

It is also suggested that chloroplasts are required for the proper development of plants. It has been demonstrated that several chloroplast proteins encoded in nuclear genomes are involved both in the biogenesis of chloroplasts, and in plant development. For example, defects in genes encoding chloroplast-targeted proteins such as PALE CRESS (PAC) of Arabidopsis thaliana (Reiter et al., 1994; Tirlapur et al., 1999), PGP1 of A. thaliana (Hagio et al., 2002), DAG of Antirrhinum majus...
and defects in genes such as *DEFECTIVE CHLOROPLASTS AND LEAVES (DCL)* of *Lycopersicon esculentum* (Keedie et al., 1996), and *CLO-ROPLASTOS ALTERADOS 1 (CLA1)* in *A. thaliana* (Mandel et al., 1996) impair the development of chloroplasts and mesophyll cells. The *vd1* mutant of *Nicotiana tabacum* and the *crl* mutant of *A. thaliana*, which also encode plastid-targeted proteins, display defects in the development of leaves, roots, flowers, and chloroplasts (Wang et al., 2000; Asano et al. 2004). Based on the localization of these proteins in the chloroplasts, it has been proposed that putative signals, or some metabolites that are generated by the plastids, are required for the proper development of the plants. These genes are nuclear-encoded, and therefore, the protein products of these genes must be imported into the chloroplasts by protein import machineries that reside in the envelope membrane of the chloroplasts. However, the effects of chloroplast protein import on plant development have not been extensively investigated.

In this study, we investigated effects of the *ppi2* mutation on the developmental processes of leaves. Plants of the *ppi2* mutant were observed to be albino, and were seedling-lethal on soil (Bauer et al., 2000). However, to our knowledge, there is no detailed description of the *ppi2* leaf morphology. Our results indicated that severe defects occurred in the development of mesophyll cells in the late stages of leaf development in *ppi2*, while the development of mesophyll cells was normal in the early stages of leaf development. These data suggested that the proliferation of mesophyll cells was impaired in the late stages of leaf development in *ppi2*. Our data also suggested that the replication of chloroplast DNA during chloroplast development was suppressed.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**  The Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) Heynh. was the wild type used in this study. A transferred-DNA (T-DNA) insertion mutant of *ppi2* (CS11072) was obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, Ohio. Seeds of *A. thaliana* were sown in soil, or on Murashige and Skoog (MS) medium plates containing 0.2% Gelrite (Wako Pure Chemical Industries Ltd., Osaka, Japan). The seeds were placed at 4°C in the dark for 2 days, and then grown at 22°C under a 16/8 hour light/dark cycle for phenotypic analysis or under continuous light.

**Microscopy**  Plastid sections were prepared and observed, as previously described by Hamada et al. (2000). Scanning electron microscopy was performed, as described by Semiarti et al. (2001). Genomic DNA of chloroplasts were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope.
Fig. 3. Structures of leaf lamina in ppi2 and wild type plants. Cross sections of rosette leaves (the first leaves) of wild type (A) and the ppi2 mutant (B). (C) A magnified image of the area indicated by a rectangle in (A). (D) A magnified image of the areas indicated by a rectangle in (B). Representative chloroplasts in the mesophyll cells in the wild type are indicated by arrowheads in (C). Bars = 1 mm.

Fig. 4. Sections of developing leaf primordia and shoot apical meristem. Longitudinal sections of shoot apical meristem (SAM) and leaf primordia adjacent to SAM of wild type (B, D, F) and ppi2 (C, E, G). Developmental stages in (B) to (G) are indicated schematically in (A). Note that palisade layers are distinguishable in (D) to (G), and spongy layers are distinguishable in (F) and (G). Bars = 10 µm.
dole (DAPI), and observed, as described by Kuroiwa and Fujie (1992).

RESULTS

Morphological abnormalities in leaves of the *ppi2* mutant As shown in Fig. 1B, the *ppi2* plants growing on MS medium had several leaves (Lee et al., 2003). While the shapes of the cotyledons were relatively normal, the rosette leaves were severely distorted (Fig. 1C and 2F). The leaf lamina was narrow in width, and the leaf had irregular margins (Fig. 1B, C). Examination of the leaf morphology by scanning electron microscopy (SEM) revealed that the morphology of the cotyledon was also distorted in the *ppi2* plants (Fig. 2B). The shapes of the epidermal cells in cotyledons and the rosette leaves of the *ppi2* plants were less complex compared to those of the wild type (Fig. 2D, H).

Examination of cross sections of the rosette leaves of the *ppi2* plants revealed that the thickness of the leaf lamina was uneven. The *ppi2* leaf lamina contained fewer cells and more intercellular spaces as compared with the wild type (Fig. 3B). Palisade and spongy tissues were not obvious in the *ppi2* leaf lamina (Fig. 3B, D). In some areas of the leaves, no mesophyll cells were present between the adaxial and abaxial epidermis (Fig. 3B, D). These results indicate that the proper development of leaves is impaired in *ppi2*. We also investigated the root morphology of the *ppi2* mutants by propidium iodide staining, however, no morphological abnormality

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![Fig. 5. DAPI staining of cross sections of developing leaf primordia, shoot apical meristems, and matured leaves. Developmental stages in (B) to (G) are indicated schematically in (A). (B) and (C) Cells in leaf primordia in area I shown in (A). (D) and (E) Cells in developing leaves in area II shown in (A). (F) and (G) Cells in developing leaves in area III shown in (A). (H) and (I) Cells in matured leaves. Bars = 10 µm. Arrowheads indicate representative chloroplast DNA.](image-url)
was observed (data not shown).

**Histological analysis of the developing leaves and shoot apical meristems in ppi2 plants** To determine when the developmental abnormality became obvious in the development of leaves in ppi2, we investigated the longitudinal sections of shoot apices. As shown in Fig. 4, the structures and morphologies of shoot apical meristems (SAM) and leaf primordia adjacent to SAM were the same as those of the wild type (Fig. 4B, C, area I). The inner structure of the leaf primordia of the ppi2 plants was similar to that of the wild type until the developmental stage, at which palisade tissue became morphologically distinguishable (Fig. 4D, E, area II). After this stage, however, the structure of mesophyll in the ppi2 plants became different from that of the wild type, i.e., cells corresponding to palisade and spongy tissue were morphologically indistinguishable, and fewer cells and more intercellular spaces were present in the mesophyll cells of the ppi2 mutants (Fig. 4F, G, area III). These results demonstrated that the structure of SAM and leaf primordia at the early developmental stages were not affected in ppi2.

**Copy number of chloroplast DNA in ppi2 plants** To investigate whether the copy number of chloroplast DNA (cpDNA) is normally increased during the leaf development in ppi2, sections of mature leaves and shoot apices were stained with DAPI, and were examined under a microscope. In the wild type, small dots that emitted fluorescence were observed in the cytosol of leaf primordia cells at the early developmental stage (Fig. 5B, area I). These dots became larger in the late developmental stages (Fig. 5D and F, area II and III), indicating that the copy number of cpDNA started to increase before the developmental stage shown in area II in Fig. 5C. In the early developmental stage of the ppi2 plants, the size of the fluorescing dots was the same as in the wild type (Fig. 5C, area I). However, the dots in the ppi2 plants did not become larger in the late developmental stages (Fig. 5E, G, area II and III). While some of these dots in ppi2 may represent mitochondrial DNA, this result suggests that the replication of cpDNA is suppressed from the early developmental stage in leaves in ppi2.

**DISCUSSION**

We have shown in the ppi2 plants that the mutation causes severe defects in leaf morphology (Fig. 2F). This result suggests that protein import into chloroplasts is important for the normal development of leaves. Several reports have demonstrated that defects in the nuclear-encoded genes prevent early stage chloroplast development, and also prevent leaf development (Reiter et al., 1994; Tirlapur et al., 1999). Based on the localization of these gene products in chloroplasts, it is suggested that the integrity of chloroplasts is important for the normal development of leaves. Defects in leaf morphology in ppi2 plants may be a result of the suppression of chloroplast development, which is caused by a defect in the protein import into chloroplasts.

Our results also showed that mesophyll in ppi2 contained fewer cells and more intercellular spaces as compared with wild type (Fig. 3B, D, and Fig. 4G). This result suggested that the proliferation of mesophyll cells was partially suppressed, or that the frequency of cell death was increased in ppi2. The results of trypane blue staining of the ppi2 plants indicated that the number of dead cells in the mesophyll was not increased in ppi2 (data not shown). Consequently, the proliferation of mesophyll cells may require intact chloroplasts and/or chloroplast products.

In ppi2, the development of mesophyll cells was not affected in the early stages (Fig. 4). This result suggests that the plastid protein import apparatus is not required in the early developmental stages of leaves. However, three homologues of Toc159 are present in A. thaliana (Bauer et al., 2000). These Toc159 homologues may be responsible for protein import into the chloroplasts in the early developmental stages of leaves. Alternatively, chloroplast function may not be required for leaf development in the early stages. Our results also showed that the gross morphology of cotyledons was relatively normal in ppi2 (Fig. 2B), suggesting that the development of cotyledons requires atToc159 to a lesser extent as compared with rosette leaves. Toc159 homologues other than atToc159 might be major components of the Toc complex in cotyledons.

The structure of SAM and roots were also normal in ppi2 (Fig. 4C), suggesting that homologues of Toc159 other than atToc159 may function in SAM and roots. Alternatively, plastid function may not be required for the integrity of SAM and roots. However, this is not likely to be the case in roots because the VDL gene of tobacco and the CRL gene of A. thaliana, which encode plastid-localized proteins, have been demonstrated as essential for the development of roots (Wang et al., 2000; Asano et al., 2004). These results suggest that the integrity of plastids is important for root development.

Our results suggest that the replication of cpDNA is inhibited in the mesophyll cells of ppi2 (Fig. 5). It is unclear at present whether nuclear-encoded genes are involved in the replication of cpDNA during chloroplast development. However, the involvement of a product of a nuclear-encoded gene, designated plastid envelope DNA binding protein (PEND), has been suggested in the pea (Sato et al., 1998). The presence of chloroplast-localized DNA polymerase protein in rice has also been demonstrated (Kitamura et al., 2002). The replication of cpDNA during chloroplast development may require products
that are encoded in the nuclear genome, and the import of such proteins might be impaired in ppi2.

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REFERENCES


