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Rapid Paper

Phosphatidylglycerol is Essential for the Development of Thylakoid Membranes in *Arabidopsis thaliana*

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Phosphatidylglycerol is a ubiquitous phospholipid in the biological membranes of many organisms. In plants, phosphatidylglycerol is mainly present in thylakoid membranes and has been suggested to play specific roles in photosynthesis. Here, we have isolated two T-DNA tagged lines of *Arabidopsis thaliana* that have a T-DNA insertion in the *PGP1* gene encoding a phosphatidylglycerolphosphate synthase involved in the biosynthesis of phosphatidylglycerol. In homozygous plants of the T-DNA tagged lines, the *PGP1* gene was completely disrupted. The growth of these knockout mutants was dependent on the presence of sucrose in the growth medium, and these plants had pale yellow-green leaves. The leaves of the mutants had remarkably large intercellular spaces due to the reduction in the number of mesophyll cells. The development of chloroplasts in the leaf cells was severely arrested in the mutants. Mesophyll cells with chloroplast particles are only found around vascular structures, whereas epidermal cells are enlarged but largely conserved. The content of phosphatidylglycerol in the mutants was reduced to 12% of that of the wild type. These results demonstrate that *PGP1* plays a major role in the biosynthesis of phosphatidylglycerol in chloroplasts, and that phosphatidylglycerol is essential for the development of thylakoid membranes in *A. thaliana*.

Keywords: *Arabidopsis thaliana* — Chloroplast — Phosphatidylglycerol — Phosphatidylglycerolphosphate synthase — Thylakoid membrane.

Abbreviations: CL, cardiolipin; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PS, photosystem; RT-PCR, reverse transcription-PCR; TLC, thin-layer chromatography.

Introduction

Phosphatidylglycerol (PG) is a ubiquitous anionic phospholipid that constitutes the biological membranes of animals, plants and microorganisms. It is known that PG is synthesized by a common pathway in prokaryotes and eukaryotes (Dowhan 1997, Moore 1982, Shibuya 1992). As the first step of the biosynthesis of PG, the primary precursor glycerol 3-phosphate is acylated to lysophosphatidic acid by glycerol 3-phosphate acyltransferase, a step which is followed by a second acylation catalyzed by lysophosphatidic acid acyltransferase. The synthesized phosphatidic acid obtained by the two-step acylation of glycerol 3-phosphate is converted to CDP-diacylglycerol by CDP-diacylglycerol synthase and then to phosphatidylglycerolphosphate (PGP) by PGP synthase. The last step of PG biosynthesis is the dephosphorylation of PGP, a reaction which is catalyzed by PGP phosphatase (Joyard et al. 1998). PG is also used as a precursor for the biosynthesis of cardiolipin (CL). In eukaryotes, CL is exclusively present in the inner membranes of mitochondria (Bligny and Douce 1980).

Several mutants defective in the biosynthesis of PG have been isolated from bacteria, yeast, and mammalian cells with the aim of determining the roles of anionic phospholipids, PG and CL. A mutant of *Escherichia coli* possessing a disrupted *pgsA* gene for PGP synthase is defective in PG biosynthesis; this mutant contained undetectable levels of PG and CL (Kikuchi et al. 2000). This mutant has been shown to be viable if the gene for lipoprotein, located in the outer membrane requiring the diacylglycerol of PG for maturation, was disrupted. These findings demonstrated that PG and CL are dispensable for the viability of *E. coli*. A mutant of Chinese hamster ovary cells with a mutation in the *PGS1* gene encoding a PGP synthase resulted in a striking reduction of PG (Ohtsuka et al. 1993). In the mutant, respiratory electron transport and ATP synthesis in the mitochondria were impaired, and the morphology of the mitochondria was abnormal compared to that of the wild type (Kawasaki et al. 1999). Recently, a mutant of *Saccharomyces cerevisiae* was constructed; in this mutant, the expression of *PGS1* gene for PGP synthase, located in the mitochondria, can be decreased by exogenous addition of doxycyclin to growth medium (Ostrander et al. 2001). A decrease in anionic phospholipids caused by the suppression of *PGS1* expression leads to mitochondrial dysfunction, in particular, to the inhibition of the translation of protein components of the respiratory electron transport chain. These findings demon-

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strated that the anionic phospholipids PG and/or CL play important roles in mitochondria.

In plants, PG is mainly present in thylakoid membranes in chloroplasts and it is also found as a minor component of other membranes (Block et al. 1983, Yoshida and Uemura 1986). Major localization of PG in thylakoid membranes has suggested that PG plays a critical role in photosynthesis. The function of PG in photosynthesis has been studied with mutants of the cyanobacterium *Synechocystis* sp. PCC6803, which are defective in the biosynthesis of PG. The mutants were created by inactivation of the gene for CDP-diacylglycerol synthase or PGP synthase involved in the biosynthesis of PG (Hagio et al. 2000, Sato et al. 2000). These mutants can grow only in medium containing PG, and the photosynthetic activity of the mutants has been shown to dramatically decrease with a concomitant decrease in the PG content in thylakoid membranes. This decrease of photosynthetic activity has been attributed to a decrease in photosystem II (PSII) activity (Hagio et al. 2000). These findings have demonstrated that PG is essential for the growth of *Synechocystis* sp. PCC6803 and that PG plays an important role in PSII. Further analysis of one of these mutants elucidated that PG is indispensable for the PSII reaction center complex, in that PG maintains the structural integrity of the Q_B-binding site (Gombos et al. 2002). Recently, Jordan et al. (2001) reported that three PG molecules are bound to a reaction center complex of PSI. Two of the three molecules of PG are bound to the periphery of PSI complex, and the other molecule is bound to the central core of the PSI complex. Although the function of PG in PSI has not yet been clarified, the binding of PG to the PSI reaction center complex suggests that PG carries out an important function not only in PSII, but also in PSI.

In the chloroplasts of higher plants, it is known that PG is associated with light-harvesting complexes of PSII at the monomer interface (Murata et al. 1990, Tremolieres et al. 1994, Nussberger et al. 1993), and it is also required for the dimerization of the PSII reaction center (Kruse et al. 2000). These findings imply the involvement of PG in the assembly of the PSII complex. However, these findings were obtained from in vitro experiments; hence, they cannot be simply applied to account for the in vivo function of PG in chloroplasts. In order to understand the in vivo function of PG in the chloroplasts of higher plants, some mutants that contain a reduced amount of PG compared to that of the wild type have been isolated from *Arabidopsis thaliana*. For example, the *ats1* (*act1*) mutant is deficient in chloroplast glycerol 3-phosphate acyltransferase activity; this is the first enzyme required for the biosynthesis of PG, as well as for the biosynthesis of other glycerolipids in chloroplasts (Kunst et al. 1988). Although this enzyme is crucial for the biosynthesis of PG in chloroplasts, the content of PG in the mutant was only reduced to about 70% of that of the wild type, and no apparent change in phenotype was observed in the mutant. The content of PG in the *pho1* mutant, defective in the xylem loading of phosphate, was shown to be approximately 40% lower than that of the wild type (Poirier et al. 1991, Härtel et al. 1998). However, this decrease was not only limited to that of PG, but also included decreases in the contents of other phospholipids. Thus, it was not possible to clarify the specific function of PG by comparing the phenotypes of the mutant to those of the wild type. Xu et al. (2002) have recently isolated the *pgp1* mutant of *Arabidopsis* possessing a mutation in the *PGP1* gene for PGP synthase. The content of PG in the mutant was approximately 25% lower than that of the wild type. The mutant had pale green leaves and photosynthesis was slightly impaired, suggesting that PG plays an important role in chloroplast development and photosynthesis. However, the *pgp1* mutant was still capable of synthesizing a significant amount of PG in chloroplasts, although it did show reduced activity of PGP synthase in its chloroplasts, and the PG content was found to be decreased. Therefore, in order to understand the function of PG in chloroplasts, it appeared to be of interest to isolate the null mutant, which cannot synthesize PG in its chloroplasts. It was thought that if a mutant incapable of synthesizing PG in its chloroplasts could be isolated, it would provide an excellent system to clarify the function of PG in chloroplasts. Comparison of the phenotype of the mutant to that of the wild type would be likely to provide direct evidence for the function of PG in vivo.

Therefore, in the present study, we isolated and characterized *pgp1* knockout mutants in which the *PGP1* gene was disrupted by an insertion of T-DNA. The findings obtained in this study demonstrate that PG is indispensable for the development of thylakoid membranes in chloroplasts.

Results

Isolation of the pgp1 knockout mutants

In order to understand the function of PG in higher plants, we attempted to isolate T-DNA tagged lines in which a T-DNA is inserted into the *PGP1* gene encoding a PGP synthase involved in the biosynthesis of PG in *A. thaliana* (Müller and Frentzen 2001, Xu et al. 2002). We screened for the *PGP1* mutant in T-DNA tagged lines, which are stocked in the Kazusa DNA Research Institute, with the aid of a screening service provided by the same institute. Two independent tagged lines, KG10062 and KG12911, that possess a T-DNA insertion in the *PGP1* gene were isolated from approximately 50,000 T-DNA lines (Fig. 1). The *PGP1* gene of *Arabidopsis* is located on chromosome 2 and it is composed of six exons and five introns (Xu et al. 2002). Sequence analysis of the region of the *PGP1* gene containing T-DNA in the tagged lines revealed that a T-DNA was inserted 43 bp upstream from the predicted initiation codon ATG, with a deletion of 54 bp in the first exon in KG10062 (Fig. 1, upper figure) and 104 bp downstream from the ATG codon in KG12911 (Fig. 1, lower figure). These findings demonstrate that the coding region of the *PGP1* gene in the T-DNA tagged lines is interrupted by the T-DNA insertion, which causes a null mutation.

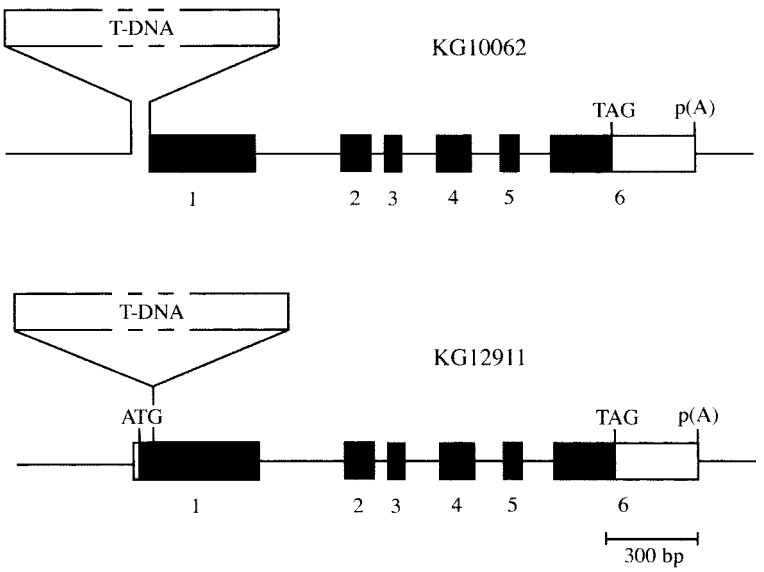

Fig. 1 Structure of the *PGP1* gene and the T-DNA insertion sites in the KG10062 and KG12911 lines. Boxes numbered from 1 to 6 and the filled areas in the boxes indicate exons and coding regions, respectively. The translation initiation codon (ATG), the translation termination codon (TAG), and the polyadenylation site [p (A)] are shown. In the KG10062 line, a region including the initiation codon is deleted and a T-DNA is inserted into that region.

Expression of the PGP1 gene

To confirm the null mutation of the *PGP1* in the isolated T-DNA tagged lines, we performed a reverse transcription-PCR (RT-PCR) analysis. Fig. 2 shows the results of the RT-PCR analysis, with total RNAs extracted from the leaves of 3-week-old plants. In the case of the wild-type and the heterozygous plants of the T-DNA tagged line of KG10062, a fragment corresponding to the *PGP1* cDNA was amplified. Hence, the fragment was not amplified in the homozygous plants of the T-DNA tagged line. Similarly, the fragment was also amplified in the heterozygous plants of the T-DNA tagged line of KG12911, but not in the homozygous plants of that line (data not shown). As a control, the level of expression of the gene for α-tubulin, which is transcribed in roots, leaves and flowers (Ludwig et al. 1987), was also tested by RT-PCR analysis using the same RNA samples. In all tested plants, a fragment corresponding to cDNA for α-tubulin was amplified at the same level. These data demonstrate that the *PGP1* gene was completely disrupted by T-DNA insertion and that it was not expressed in the homozygous plants of the T-DNA tagged lines.

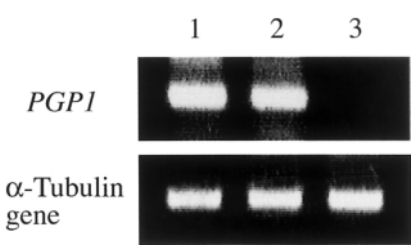

Fig. 2 Expression of the *PGP1* gene. In the upper panel, the level of *PGP1* mRNA was analyzed by RT-PCR with total RNAs prepared from the wild-type plant (lane 1), and heterozygous (lane 2) and homozygous (lane 3) plants of the *pgp1* mutant. The level of mRNA for α-tubulin was also analyzed as a control using the same total RNAs (lower panel).

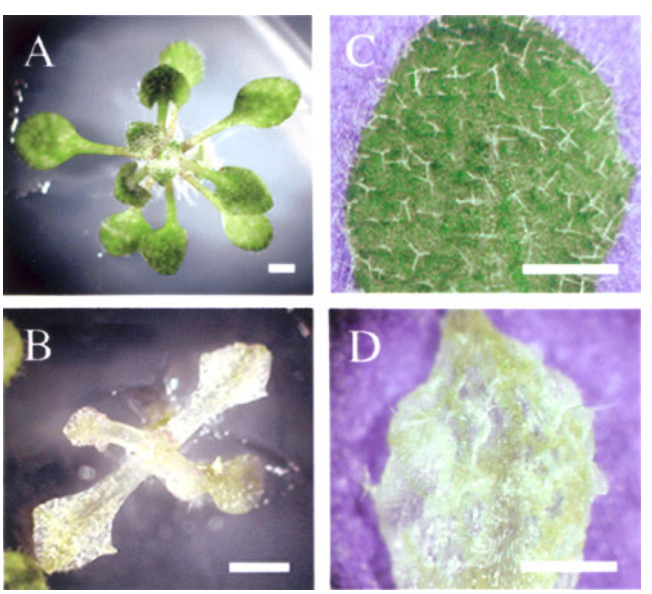

Fig. 3 Phenotypes of heterozygous and homozygous plants of the *pgp1* mutant. The heterozygous and homozygous plants of the *pgp1* mutant were grown for 3 weeks. (A) and (B) Seedlings of the heterozygous (A) and homozygous (B) plants. (C) and (D) Leaves of the heterozygous (C) and homozygous (D) plants. Bars in (A) and (B) = 2 mm; bars in (C) and (D) = 1 mm.

Characterization of phenotypes of the pgp1 knockout lines

In order to understand the function of *PGP1* gene in *Arabidopsis*, the phenotypes of the T-DNA tagged lines were investigated. The homozygous plants of the T-DNA tagged lines were able to grow on agar plates with exogenously supplied sucrose, but not on those without sucrose, demonstrating that the homozygous plants cannot grow photoautotrophically. The growth of the homozygous plants was slower than that of the wild-type plants, and the homozygous plants ceased to grow 3 weeks after germination. Fig. 3 shows the pictures of the heterozygous and homozygous plants of the T-DNA tagged line of KG10062, which were grown for 3 weeks. Compared with the heterozygous plants having well-developed green leaves with a smooth surface, the homozygous plants had pale yellow-green leaves with a rough surface (Fig. 3A, B). In addition, the leaves of the homozygous plants were almost transparent and smaller than those of the heterozygous plants (Fig. 3C,

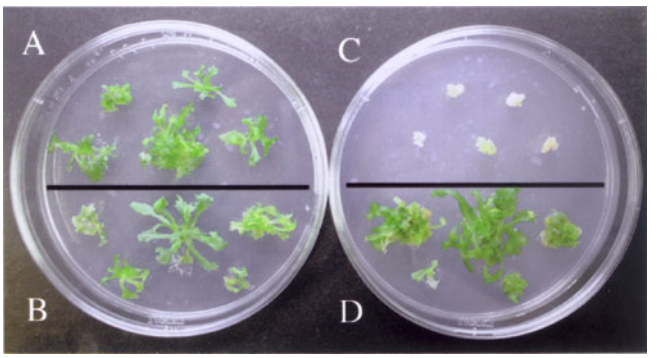

Fig. 4 Complementation of the *pgp1* mutant with *PGP1* cDNA. Calli derived from the heterozygous (A and B) and homozygous (C and D) plants of the *pgp1* mutant were transformed with either pBI121 (A and C) or pBI121-*PGP1* (B and D). The transformed calli were incubated at 23°C for 3 weeks in the presence of 100 μg ml^{-1} hygromycin, 250 μg ml^{-1} kanamycin and 20 μg ml^{-1} carbenicillin.

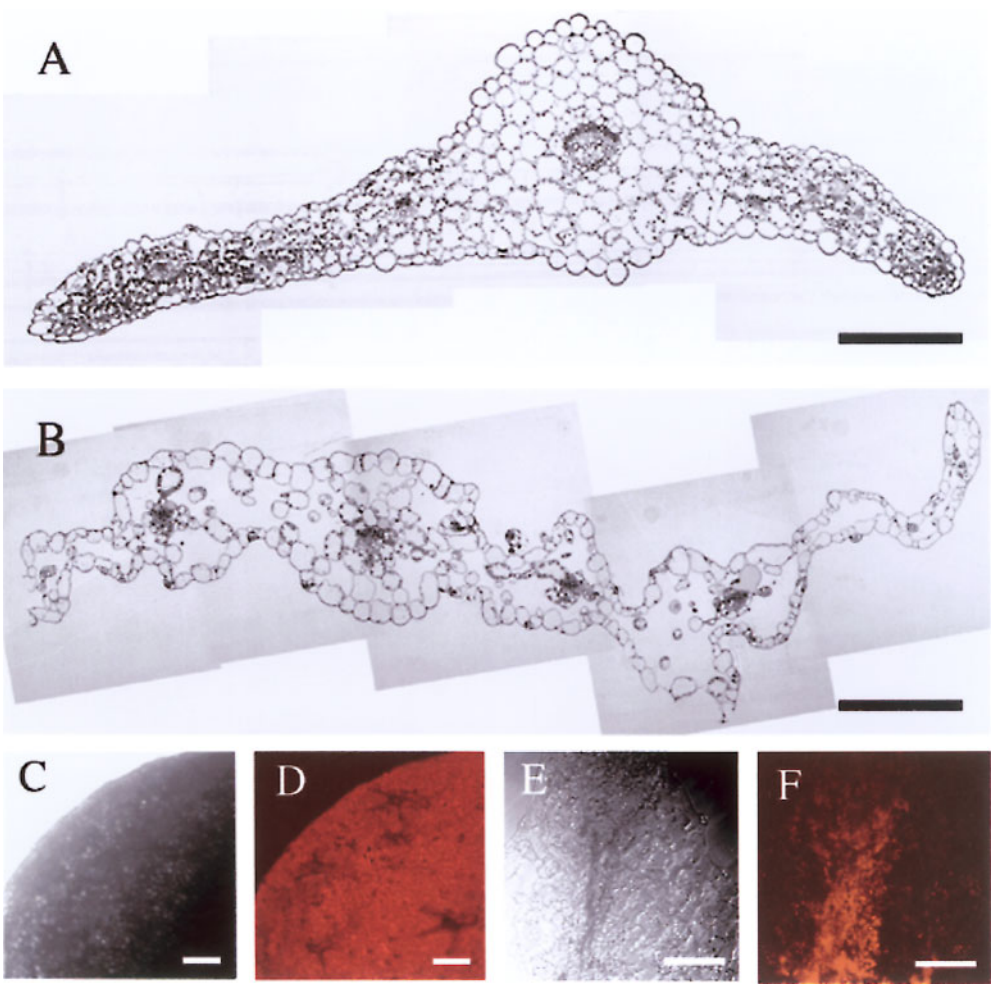

Fig. 5 Microscopic analysis of the wild type and the *pgp1* mutant. (A) and (B) Leaf section of the wild type (A) and the mutant (B). (C) and (E) Image of the differential interference micrograph of the wild type (C) and the mutant (E). (D) and (F) Red fluorescence from chlorophylls in the leaves of the wild type and the *pgp1* mutant. Bars in (A) and (B) = 0.1 mm; bars in (C) and (D) = 250 μm; bars in (E) and (F) = 100 μm.

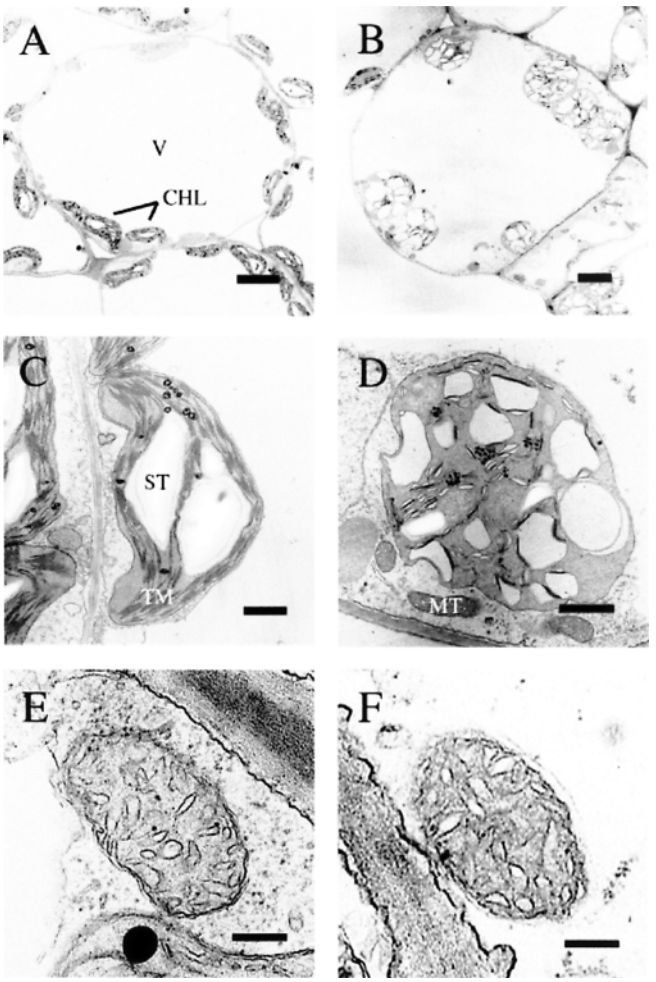

Fig. 6 Ultrastructure of mesophyll cells, chloroplasts, and mitochondria of the wild type and the *pgp1* mutant. Mesophyll cells (A and B), chloroplasts (C and D), and mitochondria (E and F) of the wild type (A, C and E) and the *pgp1* mutant (B, D and F). CHL, chloroplast; MT, mitochondrion; ST, starch; TM, thylakoid membrane; V, vacuole. Bars in (A) and (B) = 10 μm; bars in (C) and (D) = 2 μm; bars in (E) and (F) = 0.5 μm.

D). Similar characteristics were observed in the homozygous plants of the other T-DNA tagged line KG12911 (data not shown). These results suggest that *PGP1* plays an important role in the development and growth of *Arabidopsis*. Because we did not find any significant differences of the phenotypes between the homozygous plants of the T-DNA tagged lines KG10062 and KG12911, KG10062 was chosen for further experiments. Hereafter, the homozygous plants of KG10062 are referred to as the *pgp1* mutants.

Since the same changes in the phenotypes were observed in two independent T-DNA tagged lines with a T-DNA insertion in the *PGP1* gene, it is very likely that the changes in the phenotypes were caused by the insertion of T-DNA into the *PGP1* gene. However, it is still possible that an unknown mutation in other genes resulted in the changes in the phenotypes of the *pgp1* mutants. To exclude this possibility, we transformed calli derived from the *pgp1* mutant and those from heterozygous plants with the plasmid pBI121-*PGP1* containing *PGP1* cDNA. As a control, calli were also transformed with the vector plasmid pBI121, and shoots were induced on shoot-inducible medium. Fig. 4 shows the results of shooting of transgenic calli. In heterozygous plants, the calli transformed with pBI121 or pBI121-*PGP1* developed green transgenic shoots on the shoot-inducible medium (Fig. 4A, B). Similarly, in homozygous plants, the calli transformed with pBI121-*PGP1* generated green transgenic shoots on the shoot-inducible medium (Fig. 4D). However, the calli derived from the homozygous plants and transformed with pBI121 did not generate green transgenic shoots (Fig. 4C). These results clearly demonstrate that the *pgp1* mutant was complemented by the transformation with *PGP1* cDNA, and that the changes in phenotypes of the *pgp1* mutant were caused by the T-DNA insertion into the *PGP1* gene.

To elucidate the relationship between the disruption of the *PGP1* gene and the mutant growth characteristic in detail, we observed the leaf structure under a microscope. Fig. 5A, B shows leaf sections of the wild type and the *pgp1* mutant. Unlike the wild-type leaves, the *pgp1* mutant leaves had remarkably large intercellular spaces due to the reduction in the number of mesophyll cells. Mesophyll cells with chloroplast particles are only found around vascular structures, whereas epidermal cells are enlarged but largely conserved. These findings demonstrate that the *PGP1* gene plays an important role in leaf morphogenesis.

The development of chloroplasts in the leaf cells was severely arrested in the *pgp1* mutant. Fig. 5C–F shows the leaves of the wild type and the *pgp1* mutant, as observed both by differential interference microscopy and by fluorescence microscopy. In the wild-type plant, strong red fluorescence originating from the chlorophylls was emitted from the entire area of leaves, suggesting a normal development of chloroplasts in these leaves. In contrast, in the leaves of the *pgp1* mutant, weak fluorescence from the chlorophyll was detected only around the veins. This result is consistent with the above microscopic observation that mesophyll cells are developed only around the vascular structures. Fig. 6A, B shows the ultrastructure of cells from the wild type and the *pgp1* mutant. In the wild-type cells, many well-developed chloroplasts located along the plasma membrane were observed. These chloroplasts had well-developed thylakoid membranes and large starch granules. In contrast, in the *pgp1* mutant, chloroplasts containing smaller starch granules were observed instead of well-developed chloroplasts. The number of chloroplasts in a single cell of the *pgp1* mutant was almost the same as that of chloroplasts in a single wild-type cell, suggesting that *PGP1* was not involved in the regulation of the number of chloroplasts in *Arabidopsis* cells. Fig. 6C, D shows the structures of chloroplasts in the wild type and the *pgp1* mutant. The chloroplasts in the wild-type cells contained well-developed thyla-

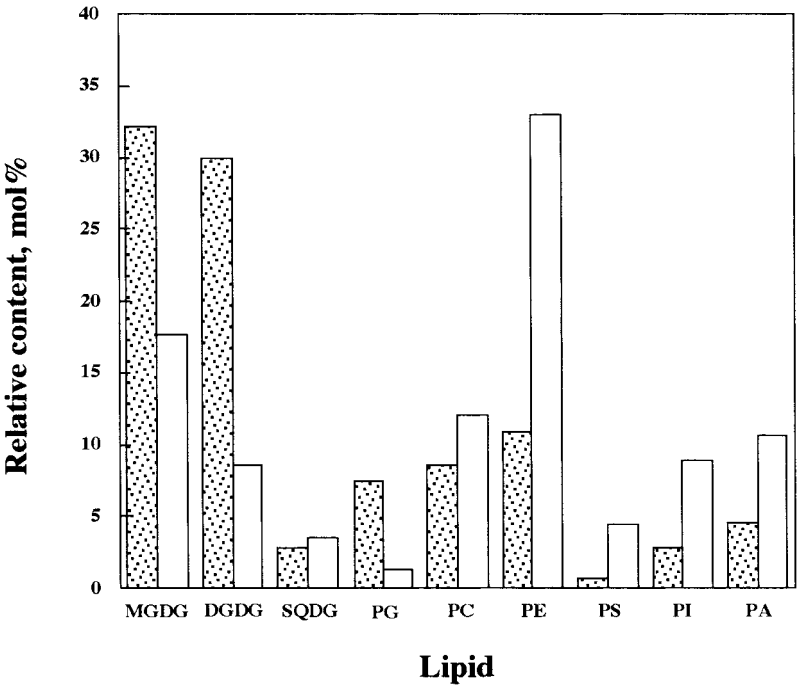

Fig. 7 Lipid composition of the wild type and the *pgp1* mutant. Total lipids extracted from the leaves of 3-week-old plants were separated into different lipid classes by two-dimensional TLC. Each lipid class separated on the TLC plate was derivatized to fatty acid methyl esters and quantified by gas chromatography. Dotted and open bars represent the wild type and the mutant, respectively. The content of total lipids in the wild type and the mutant was 3.3±0.4 and 2.0±0.1 μg (mg FW)$^{-1}$, respectively. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid. The values are the averages from two independent experiments.

koid membranes with a granum structure. However, in the *pgp1* mutant, the chloroplasts contained a few swollen thylakoid membranes without a granum structure. The chloroplasts in the *pgp1* mutant showed slight enlargement with many vacuolated structures. These findings demonstrate that *PGP1* is required for the development of chloroplasts, in particular, for the formation of thylakoid membranes.

It is already known that PG is utilized for the biosynthesis of CL, which is exclusively present in the inner membranes of mitochondria. Defects in PGP synthase in both yeast and mammalian cells leads to structural changes and to the functional impairment of the mitochondria in these cells (Chang et al. 1998, Ohtsuka et al. 1993). Thus, it is reasonable to assume that the disruption of *PGP1* also affects mitochondrial function, in addition to affecting the development of chloroplasts. In order to investigate whether or not mitochondria were also affected in the *pgp1* mutant, we analyzed the ultrastructure of the organelles. Fig. 6E, F shows mitochondria of the wild type and the *pgp1* mutant. No apparent morphological differences were observed in the mitochondria between the wild type and the *pgp1* mutant. This finding suggests that the *PGP1* gene is not essential for the function of the mitochondria.

Impairment of PG biosynthesis in the pgp1 knockout mutant

In order to assess the changes in the content of PG in the *pgp1* mutant, lipids were extracted from the leaves of 3-week-old plants and analyzed. Fig. 7 shows the lipid composition of the total lipids of the wild type and the *pgp1* mutant. Although the relative content of PG in the leaves of the wild type was 8%, the content of PG in the leaves of the mutant was only 1%. As regards the other phospholipids, especially phosphatidylethanolamine and phosphatidylserine, the contents increased, whereas those of monogalactosyldiacylglycerol and digalactosyldiacylglycerol decreased. Since glycolipids are present in chloroplast membranes, the relative proportion of mesophyll cells in leaves reflects to that of the glycolipids in total lipids extracted from leaves. Thus, the decrease in the contents of monogalactosyldiacylglycerol and digalactosyldiacylglycerol is consistent with the finding that there are small numbers of mesophyll cells in the mutant leaves and only a few thylakoid membranes were present in the chloroplasts of the mutant. However, the content of sulfoquinovosyldiacylglycerol slightly increased although it is also present in chloroplast membranes. The increase of the sulfoquinovosyldiacylglycerol can be considered as a compensatory phenomenon that maintains the content of negatively charged lipids, PG and sulfoquinovosyldiacylglycerol, as observed in many photosynthetic organisms (Benning et al. 1993, Güler et al. 1996, Essigmann et al. 1998). These results demonstrate that the *PGP1* gene plays a major, but not exclusive role in the biosynthesis of PG. Because the

reduction of PG content in the mutant was remarkable, it is likely that PGP synthase encoded by the *PGP1* gene was predominantly involved in the biosynthesis of PG in the leaves.

Discussion

In plants, PG carries out important functions, not only in mitochondria, but also in chloroplasts. The importance of PG in chloroplasts can be assumed based on its presence in chloroplast membranes (Block et al. 1983). The *pgp1* knockout mutant of *Arabidopsis* obtained in this study had significantly reduced level of PG. The mutant had abnormal leaf morphology, undeveloped thylakoid membranes, and it required sucrose for growth. The latter requirement of this mutant must be due to the deficiency of the thylakoid membranes in the chloroplasts. Without the thylakoid membranes, NADPH and ATP, required for CO_2 fixation in chloroplasts, cannot be synthesized by the light reactions of photosynthesis. The findings obtained in this study clearly demonstrate that PG is essential for the differentiation of chloroplasts with well-developed thylakoid membranes.

In the *pgp1* knockout mutant, the PG content dramatically decreased compared to that of the wild-type plant. Although the determination of the exact amount of PG in subcellular localization is practically impossible under our growth conditions of the *pgp1* mutant, it can be inferred that the large decrease in PG content in the *pgp1* mutant is ascribed to the suppression of the development of thylakoid membranes due to the defect in the biosynthesis of PG. However, the mutant still contained a significant amount of PG, although the *PGP1* gene was completely disrupted and no expression of the *PGP1* gene was detected. The finding suggests that there is another biosynthetic pathway of PG that does not depend on the *PGP1* gene. It is already known that the biosynthesis of PG in higher plants takes place in plastids, mitochondria, and endoplasmic reticulum (Moore 1974, Moore 1982, Mudd and Dezacks 1981). Müller and Frentzen (2001) have recently reported that there are two genes for PGP synthase in the *Arabidopsis* genome. When the gene corresponding to the *PGP1* gene was expressed in *S. cerevisiae*, the expressed protein was associated with the mitochondrial fraction, and high PGP synthase activity was detected in the same fraction, thus indicating that PGP synthase encoded by the *PGP1* gene was localized in the mitochondria of the yeast cells. The expression of the other gene resulted in an increase in PGP synthase activity in the microsomal fraction, and it was suggested that the expressed protein was localized in the endoplasmic reticulum. The mitochondrial localization of PGP1 in the yeast cells raised the possibility that PGP1 is located in the mitochondria in *Arabidopsis* cells, in addition to its presence in the chloroplasts. If it is located in both organelles, the disruption of *PGP1* must cause a defect in the biosynthesis of PG in both chloroplasts and mitochondria. However, we observed dramatic changes in the phenotypes of chloroplasts, but not in those of mitochondria, in the *pgp1* mutant. It is therefore possible that the defect in the biosynthesis of PG in mitochondria, but not that in chloroplasts, is compensated by the transportation of PG from the endoplasmic reticulum, where PG is synthesized by the PGP synthase encoded by the second gene. Another possibility would be that PGP1 is located only in chloroplasts, and that one more gene for PGP synthase located in the mitochondria is present in the *Arabidopsis* genome. We searched in a database of the *Arabidopsis* genome and found a third gene encoding a polypeptide homologous to PGP1, in addition to the two genes described above. It will be of interest to investigate whether or not the gene encodes a PGP synthase located in the mitochondria. Characterization of the cDNA corresponding to the gene is now in progress.

In this study, we were able to isolate a *pgp1* knockout mutant of *Arabidopsis* that has significantly reduced level of PG. We demonstrated that PG is essential for the development of thylakoid membranes and hence the sustainable photoautotrophic growth. This is the first report that gives direct evidence for the essential function of PG in higher plants. The *pgp1*-knockout mutant obtained in this study provides a useful experimental system for understanding of the role of PG in chloroplasts.

Materials and Methods

Plant materials and growth conditions

All plants used as experimental materials were of the Columbia ecotype *A. thaliana*. Plants were grown on 1% (w/v) agar-solidified MS medium (Murashige and Skoog 1962) containing 1% (w/v) sucrose under continuous light (30 μmol photons m^{-2} s^{-1}) at 23°C. Plants of T-DNA tagged lines were grown in the presence of 20 μg ml^{-1} hygromycin.

Screening of the pgp1 knockout mutant

We screened for the lines possessing a T-DNA insertion in the *PGP1* gene from the T-DNA-tagged lines, which are stocked in the Kazusa DNA Research Institute. The screening was performed according to the screening system implemented at the same institute. The lines were screened by PCR and Southern hybridization analysis. Two primer sets, 5′-TCAGATCCGGTCTGGCTTCGTTAAT-3′ and 5′-CGGCTAAAGACCAAACAGATAGTCC-3′, and 5′-AAGAAAATG-CCGATACTTCATTGGC-3′ and 5′-CGGCTAAAGACCAAACAGA-TAGTCC-3′ were used for the PCR. The position of the T-DNA insertion was determined by sequencing the amplified DNAs. T_2 seeds of the T-DNA-tagged lines of *A. thaliana*, KG10062 and KG12911, were obtained from the Kazusa DNA Research Institute. Hygromycin-resistant plants were selected from T_2 plants of each line. Heterozygous and homozygous plants that had a T-DNA insertion in the *PGP1* gene were selected from hygromycin-resistant plants by checking the insertion of T-DNA into the *PGP1* gene with PCR. Heterozygous plants showed the same phenotypes as those of the wild type, but the homozygous plants had pale yellow-green leaves and they ceased to grow at 3 weeks after germination, without making flowers. T_3 seeds were collected from the heterozygous plants after self-fertilization and the T_3 plants were used for the experiments.

RNA analysis

Total RNA was extracted from the leaves of approximately 3-

week-old *Arabidopsis* plants, according to the instructions of the RNA extraction Kit (RNeasy Plant Mini Kit, QIAGEN). RT-PCR was performed with an RT-PCR kit (Superscript One-Step System, Life Technologies) using the following two primers: 5′-ACTGGCTT-GACGGCTATCTTGCCCG-3′ and 5′-CGGCTAAAGACCAAACA-GATAGTCC-3′. Total RNA (250 ng) was used for the RT-PCR analysis. As a control, the expression of the gene for α-tubulin was analyzed with the same RNA preparations using the following two primers: 5′-CTACTGAGAGAAGATGCGAG-3′ and 5′-CAACATCTCCTCGGT-ACATC-3′.

Complementation of the pgp1 knockout mutant

The *Arabidopsis PGP1* cDNA was amplified by PCR using the primers 5′-GCTCTAGAATGCTCAGATCCGGTCT-3′ and 5′-GCTC-TAGACTACTTCATTAGTACTTTCC-3′. An *Arabidopsis* cDNA library was constructed with λgt_{11} and cDNAs synthesized from poly $(A)^+$ RNAs of leaves and roots. Lambda phage DNA prepared from the library was used as the template. The eight-nucleotide sequence 5′-GCTCTAGA-3′, including an *Xba*I site, was added to the 5′-end of each primer. The amplified cDNA was subcloned into a pGEM-T easy vector (Promega) and its nucleotide sequence was determined. The plasmid containing *PGP1* cDNA was digested with *Xba*I and was ligated into the *Xba*I site of a Ti plasmid, pBI121 (Clontech). The resultant plasmid, designated as pBI121-*PGP1*, was used for the transformation of calli derived from the *pgp1* mutant by the agrobacterium-mediated method (Akama et al. 1992). The calli were also transformed with pBI121, and the obtained transformants were used as negative controls. The transformed calli were selected and grown on a plate containing shoot-inducible medium supplemented with 100 μg ml^{-1} hygromycin, 250 μg ml^{-1} kanamycin and 20 μg ml^{-1} carbenicillin.

Microscopic analysis

For microscopic analysis, leaf segments of 3-week-old *Arabidopsis* plants were fixed in 70 mM cacodylate buffer (pH 7.4) containing 2.5% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde. The samples were post-fixed with 1% (v/v) osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in a resin (Epon 812, TAAB Laboratories Equipment). Sections of approximately 0.5 μm were prepared and observed by a microscope (BX-10, Olympus). For the electron microscopic observation, ultrathin sections were stained with 2% uranyl acetate for 20 min, and followed by lead citrate for 10 min. The resultant specimens were observed with a transmission electron microscope (H-800, Hitachi).

To observe red fluorescence from the chlorophylls in the leaves, a confocal laser scan microscope (LSM410, Carl Zeiss) was used. The fluorescence was excited with a Kr/Ar laser at 488 nm and the fluorescence was detected with a long-pass 590-nm filter set.

Lipid analysis

Leaves collected from 3-week-old *Arabidopsis* plants were immediately frozen in liquid nitrogen and the lipids were extracted as described by the method of Bligh and Dyer (1959). The lipid extract was analyzed by two-dimensional thin-layer chromatography (TLC) according to the method as described by Inatsugi et al. (2002). The first dimension was developed with acetone/benzene/methanol/water/acetic acid (80 : 30 : 20 : 10 : 1.4, by volume) and the second dimension was performed with chloroform/methanol/acetic acid/water (85 : 12.5 : 12.5 : 2, by volume). Lipids separated on the TLC plates were detected under UV light after spraying the plates with 0.01% primuline in acetone/water (4 : 1, by volume). Each spot of lipid was scraped off and was subsequently subjected to methanolysis. The resultant fatty acid methylesters were analyzed by gas chromatography (GC-17, Shimadzu), as described previously (Wada and Murata 1989).

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