Plastidic Phosphatidic Acid Phosphatases Identified in a Distinct Subfamily of Lipid Phosphate Phosphatases with Prokaryotic Origin*§

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Plastidic phosphatidic acid phosphatase (PAP) dephosphorylates phosphatidic acid to yield diacylglycerol, which is a precursor for galactolipids, a primary and indispensable component of photosynthetic membranes. Despite its functional importance, the molecular characteristics and phylogenetic origin of plastidic PAP were unknown because no potential homologs have been found. Here, we report the isolation and characterization of plastidic PAPs in Arabidopsis that belong to a distinct lipid phosphate phosphatase (LPP) subfamily with prokaryotic origin. Because no homolog of mammalian LPP was found in cyanobacteria, we sought an LPP ortholog in a more primitive organism, Chlorobium tepidum, and its homologs in cyanobacteria. Arabidopsis had five homologs of cyanobacterial LPP, three of which (LPPγ, LPPε1, and LPPε2) localized to chloroplasts. Complementation of yeast Δdpp1Δlpp1Δpah1 by plastidic LPPs rescued the relevant phenotype in vitro and in vivo, suggesting that they function as PAPs. Of the three LPPs, LPPγ activity best resembled the native activity. The three plastidic LPPs were differentially expressed both in green and nongreen tissues, with LPPγ expressed the highest in shoots. A knock-out mutant for LPPγ could not be obtained, although a lppε1lppε2 double knock-out showed no significant changes in lipid composition. However, lppγ homozygous mutant was isolated only under ectopic overexpression of LPPγ, suggesting that loss of LPPγ may cause lethal effect on plant viability. Thus, in Arabidopsis, there are three isoforms of plastidic PAP that belong to a distinct subfamily of LPP, and LPPγ may be the primary plastidic PAP.

The enzyme phosphatidic acid phosphatase (EC3.1.3.4) (PAP)3 catalyzes the dephosphorylation of phosphatidic acid (PA) to yield diacylglycerol (DAG). In membrane lipid metabolism, phosphatidic acid is synthesized by two sequential steps of acylation in the so-called Kennedy pathway (1). This pathway is common to the metabolism of three different classes of glycolipids, namely phospholipids (phosphatidylethanolamine and phosphatidyethanolamine), galactolipids, and triacylglycerol, and the last step in this pathway is catalyzed by PAP.

A model plant, Arabidopsis, has two pathways for glycerolipid biosynthesis, namely prokaryotic and eukaryotic pathways localized in the plastids and endoplasmic reticulum, respectively (2). In both pathways, PAP is considered to be involved in the committed step of membrane lipid biosynthesis. Therefore, PAP may be involved in lipid metabolism both in plastids and endoplasmic reticulum. Recently, a permease-like protein named trigalactosyldiacylglycerol (TGD1) was isolated and shown to be involved in the import of extraplastidic PA into plastids, suggesting that certain extraplastidic substrates may be imported for plastidic metabolism (3, 4). Galactolipid biosynthesis is indispensable for photosynthesis, and the importance of PAP in this process is illustrated by the fact that the first step after the two pathways meet is catalyzed by plastidic PAP (5–7).

Although information on plant PAP is scarce, native plastidic PAP activity has been studied in detail using isolated spinach chloroplast envelope (8, 9). Enzyme analysis of the purified inner or outer envelope indicates that plastidic PAP is tightly associated with the inner envelope (9). Its enzymatic features suggest two outstanding qualities that distinguish plastidic PAP from other PAPs. First, extraplastidic PAP is thought to be an acidic phosphatase (10), whereas plastidic PAP has a pH optimum in an alkaline range (8, 9). Second, Mg2+ inhibits plastidic PAP activity, whereas extraplastidic PAPs may variously require or be independent of Mg2+ (10, 11).

Like other higher plants, Arabidopsis is thought to have both soluble (PAP1) and membrane-bound (PAP2) PAP (10, 11). To our knowledge, however, there has been no report regarding native PAP activity in Arabidopsis. To date, four isoforms of

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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3 The abbreviations used are: PAP, phosphatidic acid phosphatase; DAG, 1,2-sn-diacylglycerol; GUS, β-glucuronidase; LPP, lipid phosphate phosphatase; PA, phosphatidic acid; RNAI, RNA interference; T-DNA, transferred DNA; TGD1, trigalactosyldiacylglycerol 1; WT, wild type.
Plastidic PAPs in Arabidopsis

membrane-bound PAP, designated lipid phosphate phosphatase (LPP) 1–4, have been reported that are homologous to mammalian LPP (12, 13). However, they are unlikely to be plastidic because no apparent transit peptide is predicted for chloroplast localization in any isoform using a localization prediction system such as TargetP (www.cbs.dtu.dk/services/TargetP/) and WOLFPsort (wolfpsort.seq.cbrc.jp/). Han et al. (14) recently cloned a soluble PAP, phosphatidate phosphohydrolase, for the first time in Saccharomyces cerevisiae; phosphatidate phosphohydrolase is not homologous to membrane-bound PAP. Arabidopsis has two homologs of phosphatidate phosphohydrolase, but they do not contain predictable transit peptides or membrane-spanning domains. Therefore, they are unlikely to be candidates for the plastidic PAP that is tightly associated with the inner envelope of chloroplasts. Taken together, this evidence suggests that Arabidopsis likely has additional LPPs that function as plastidic PAPs.

In an attempt to unravel the molecular characteristics and physiological function of plastidic PAP, we newly isolated a subfamily of LPP in Arabidopsis and their ancestral ortholog in the cyanobacterium Synecohystis sp. PCC6803 (Syn LPP). Our results showed that three of five isoforms in Arabidopsis (LPPγ, LPPε1, and LPPε2) were plastidic PAP. Among them, LPPγ may be the primary PAP in plasts.

EXPERIMENTAL PROCEDURES

Plant Materials—Arabidopsis thaliana Columbia-0 ecotype was used throughout the study. For plant growth, solidified Murashige and Skoog medium was used (15). T-DNA mutant lines were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Plasmid Construction and Transformation in Yeast—To measure PAP activity in vitro, LPPγ, LPPε1, LPPε2, and Syn LPP were amplified by PCR with specific primers and cloned into the NotI/PstI sites of pDO105 (16) to express them in the yeast mutant Δlpp1Δlpp1Δpha1 (14) that lacks the majority of PAP activity. The primers used here were: LPPγFw, GCGGCCGCGATGGACCTAATAC; LPPγRv, CTGCAGTTAATCACATTAGC; LPPε1Fw, GCGGCCGCGATGGACCTAATAC; LPPε1Rv, CTGCAGTTAATCAGATTCTTC; LPPε2Fw, GCGGCCGCGATGGACCTAATAC; LPPε2Rv, CTGCAGTTAATCAGATTCTTC; and SynLPFw, GCTGCAGTTAATCAGATTCTTC; SynLPPRv, GCTGCAGTTAATCAGATTCTTC. After transformation of the vector constructs, transformants were screened on solid SD medium without His, Trp, Leu, and Ura.

Enzyme Activity Assay—Each transformant grown in SD medium (without His, Trp, Leu, and Ura) was collected and disrupted by vortexing with glass beads. Total membrane proteins of each sample were collected by centrifugation (15,000 g, 10 min) and suspended in assay buffer (50 mM Tris-HCl, pH 7.0, 50 mM NaCl, 5% glycerol). Protein concentration in the mixture was incubated at 25 °C for 1 h. The reaction was stopped by vigorously vortexing with 0.5 ml of ethyl acetate and centrifuging twice at 1,500 × g for 5 min with 0.5 ml of 0.45% NaCl. The upper layer was dried and dissolved in 40 μl of chloroform/methanol (2:1 by v/v) for one-dimensional TLC analyses. The product solutions were developed in petroleum ether/ethyl ether/acidic acid (50:50:1 by v/v/v) for DAG isolation. Radioactive spots were identified by comigrating commercial lipid standards and quantitated by Image Plate (Fuji Photofilm, Tokyo, Japan) and Image Analyzer (Storm, Amersham Biosciences Bioscience, Piscataway, NJ). The same protocol was used for the activity assays of intact Arabidopsis chloroplasts.

Histochemical GUS Staining Analysis—To produce transgenic plants harboring the promoter:GUS construct, ~1,000 bp of the upstream region of genomic sequences from the start codons for LPPγ, LPPε1, and LPPε2 were cloned into the PstI/Sall sites for LPPγ and LPPε2 and HindIII/Sall sites for LPPε1 of pBl101 and introduced into Arabidopsis plants via Agrobacterium-mediated transformation. Primers used here were as follows: pLPPγFw, CTGCAGCTCTCAACATCTCGGATATTTCATGT; pLPPγRv, CTGCAGATCTGATAGTCCTGACGTCTCTCTTT; and pLPPε1Fw, AGACATCAATACTTTACATCCTAAGAGGTTGTACATCCTACAGATTCTTC; pLPPε1Rv, GCTGCAGATCTGATAGTCCTGACGTCTCTCTTT; pLPPε2Fw, CTGCAGCTCTCAACATCTCGGATATTTCATGT; pLPPε2Rv, GCTGCAGATCTGATAGTCCTGACGTCTCTCTTT; and SynLPPFw, GCTGCAGATCTGATAGTCCTGACGTCTCTCTTT; SynLPPRv, GCTGCAGATCTGATAGTCCTGACGTCTCTCTTT. Transformants harboring each construct were subjected to histochemical GUS staining as described (18).

Fractionation of Plant Tissues—Intact Arabidopsis chloroplasts were isolated essentially according to Douce and Joyard (19) with minor modifications as described by Yamamoto et al. (20). To obtain microscopic and soluble fractions, the remaining supernatant in chloroplast isolation was first centrifuged at 10,000 × g, and the obtained supernatant was further centrifuged at 1,000,000 × g for 30 min. The obtained pellet here was defined as microscopic fraction, and supernatant was regarded as soluble fraction.

Antibody Production and Western Blot Analysis—To avoid any cross-detection (especially between LPPε1 and LPPε2), specific peptide antigens were synthesized as follows: LPPγ NH2-KDLEDVTVGGGIGG; LPPε1, NH2-RKDLVTGGGGG; and LPPε2, NH2-RGEDFQAL-COOH (Operon Biotechnologies, Tokyo, Japan). Polyclonal antibodies were raised in rabbits (Suka Flat, Saitama, Japan).

For Western blot analyses, 200 μg of proteins were separated by SDS-PAGE (12.5% acrylamide) and transferred to a PROTORAN nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Each LPP was detected with the respective antibody at a 1:500 dilution using a secondary antibody coupled to alkaline phosphatase. Preimmune sera gave no signal. To
detect markers for chloroplast, microsome, and soluble frac-
tions, antibodies (1:1,000 dilution) for light-harvesting chloro-
phyll-binding protein, plasma membrane aquaporin (21), and
NADPH-dependent thioredoxin reductase A were used.

**Lipid Analysis**—Total lipids were extracted by the method of
Bligh and Dyer (22). Quantification of each lipid class and their
fatty acid composition was conducted as described by Naka-
mura et al. (23).

**Production of LPPγ-overexpressing Transformants and**
**Complementation of lpp/Ipγ Homozygous Mutant**—To
overexpress LPPγ in planta, full-length LPPγ fragment was
obtained from pDO105-LPPγ vector by Nol/Pstl digestion
and cloned into Nol/Pstl sites of pZErO-2 vector. The constructed
vector (pBI121-LPPγ) was introduced into WT Arabidopsis via
the Agrobacterium-mediated method. Transformed plants
were selected by kanamycin, and expression levels of LPPγ were
measured by Northern blotting (5 μg of RNA used). One of the
transformants with the highest overexpression level of LPPγ,
designated LPPγOX#5, was crossed to Nol/Pstl sites of pZErO-2 vector. Then the
cloned LPPγ fragment was excised by XbaI/BamHI to ligate it
to the XbaI/BamHI sites of pBI121 vector. The constructed
vector (pBI121-LPPγ) was introduced into WT Arabidopsis via
the Agrobacterium-mediated method. Transformed plants
were selected by kanamycin, and expression levels of LPPγ were
measured by Northern blotting (5 μg of RNA used). One of the
transformants with the highest overexpression level of LPPγ,
designated LPPγOX#5, was crossed to LPPγ/Ipγ heterozy-
gous mutant, and F2 generation was used for complementation
assessment.

**Northern Blot Analyses**—Northern blot analyses were done
as described previously (23).

**In Vitro Pollen Tube Germination**—Pollen tube germination
of LPPγ/Ipγ heterozygous mutant and WT was conducted as
described previously (24).

**RESULTS**

A **Strategy to Identify Plastidic PAPs in Arabidopsis**—To
search for plastidic LPP isoforms in *Arabidopsis*, a clue was
obtained from cyanobacteria. *Synechocystis sp.* PCC6803 has no
mammalian LPP homolog, although it has membrane-bound
PAP activity *in vivo* (data not shown). Because cyanobacteria
are a potential ancestor of chloroplasts, it is possible that cya-
obacteria have a distinct type of LPP that might have been
inherited in chloroplasts of higher plants. Based on this hypoth-
esis, we first tried to identify a cyanobacterial PAP. Because a
direct BLAST search using known LPP sequences was unsuccess-
ful, we identified an ancestral LPP in a more primitive or-
ganism, *Chlorobium tepidum*, for which the genome has been
sequenced. Using a Chlorobium LPP sequence as the BLAST
query, we found one homolog, sll0545, in *Synechocystis sp.*
PCC6803 (SynLPP). Interestingly, when *Arabidopsis* homologs
of this SynLPP were sought for, the known *Arabidopsis* LPP1–4
isozymes were not detected, but five LPP candidates were newly
found (Table 1). These candidate genes were named LPPβ (At4g22550), LPPγ (At5g03080), LPPδ (At3g58490), LPPε (At5g0920), and LPPε (At5g66450). We gave the known LPP isoforms (LPP1–4) tentative names, LPPα1–α4. These putative
protein products have a molecular mass of 23–31 kDa, except
for LPPδ, which is 46.1 kDa. Apart from LPPε1 and LPPε2, which share extremely high amino acid identity (94%), the
sequence identity of these proteins varies from 28 to 47%.

**The Newly Identified LPPs in Arabidopsis and Synechocystis**
**Belong to a Distinct LPP Subgroup**—The active sites of LPPs
contain three conserved domains in which certain essential res-
ides have been identified by site-directed mutagenesis (25, 26).
These domains are highly conserved among the newly identi-
fied LPP candidates in Synechocystis and *Arabidopsis* (Fig. 1A),
although other regions show variability (data not shown).

We constructed a phylogenetic tree using the protein sequences of the three conserved domains (Fig. 1B). The known
*Arabidopsis* LPPα1–α4 isoforms are indeed close to mamma-
lian and yeast LPP. By contrast, the cyanobacterial LPP candi-
dates are distant from mammalian and yeast LPP but much
closer to the newly identified *Arabidopsis* LPP candidates.
These data suggest that these LPP candidates may be prokary-
otic type LPPs that are distinct from the known LPPα1–α4,
which are categorized as the eukaryotic type. The predicted
amino acid sequences of these LPP candidates showed that
three of them, LPPγ, LPPε1, and LPPε2, had putative transit
peptides (Table 1). Because plastidic PAP activity was detected
exclusively at inner envelopes, the relevant protein(s) may have
transit peptide(s) targeted to chloroplasts. In this view, further
studies were done for LPPγ, LPPε1, and LPPε2 as potential
candidates for plastidic PAP.

**Three Newly Identified Arabidopsis LPPs Localize to**
**Chloroplasts**—To assess whether the three *Arabidopsis* LPPs
with putative transit peptides localize to chloroplasts, we raised
specific antibodies and analyzed their localization by Western
blotting. Because LPPε1 and LPPε2 share extremely high amino
acid identities, antigens for LPPε1 and LPPε2 were synthetically
prepared for specific 10-amino acid sequences (LPPε1, NKDLVTTGGL; LPPε2, RDGEDRFQAL), respectively. For
LPPγ antigen, C-terminal 14 amino acid residues of LPPγ were
employed (ARAARKDMDSAKSD). WT *Arabidopsis* plants
were fractionated as intact chloroplasts (19, 20), microsome,
and soluble fraction (23) and subjected to Western blotting. As
can be seen in Fig. 2, all three candidates were enriched in the
purified chloroplast fraction, although the overall protein level
of each isoform was so low as to be undetectable in crude
extract, microsome, and soluble fractions. These antibodies

**TABLE 1**

| Name   | AGI code | Protein accession number | Amino acid | Molecular mass | Transit peptide* |
|--------|----------|--------------------------|------------|----------------|-----------------|
| LPPβ   | At4g22550| NP_193986                | 214        | 23,371         | No              |
| LPPγ   | At5g03080| NP_195928                | 227        | 25,736         | Yes             |
| LPPδ   | At3g58490| NP_191408                | 417        | 46,151         | No              |
| LPPε1  | At3g50920| NP_190661                | 280        | 30,578         | Yes             |
| LPPε2  | At5g66450| NP_201446                | 287        | 31,512         | Yes             |

* Putative transit peptides were predicted by the TargetP and WOLFPSORT programs.

**References**

1. K. Motohashi and T. Hisabori, unpublished observations.
gave single band in chloroplast fraction (supplemental Fig. S1). These results suggest that LPPγ, LPPε1, and LPPε2 were all localized mainly to the chloroplasts.

The Three Plastidic LPPs Are Differentially Expressed in Green and Nongreen Tissues—We next analyzed whether the three plastidic LPPs differed in their expression levels. Semi-quantitative reverse transcription-PCR using cDNA prepared from shoots showed that LPPγ expression was predominant, whereas expression of LPPε1 and LPPε2 was low (Fig. 3A), in agreement with the result of the Massively Parallel Signature Sequencing data base (www.mpss.udel.edu/at/GeneAnalysis.php/). This suggests that LPPγ is the most predominantly expressed isoform of plastidic PAP in shoots.

To analyze the spatial expression pattern of these three LPPs, histochemical staining with GUS was carried out using promoter::GUS fusion transformants. The three LPP isoforms were all expressed in leaves. However, there was a difference in the staining pattern in that pLPPγ::GUS was stained mainly in vascular tissues, whereas pLPPε1::GUS and pLPPε2::GUS were stained at meristematic ends of young leaves and cotyledons (Fig. 3, B–G). Furthermore, a differential GUS staining pattern was also observed in nonphotosynthetic organs; LPPγ, but not LPPε1 or LPPε2, showed strong GUS staining in flowers (Fig. 3, H–P). Detailed analysis showed that the staining pattern of LPPγ changed dramatically during flower development. In the juvenile buds, the whole carpel was strongly stained (Fig. 3H). This staining was then consolidated to the pistils as buds matured (Fig. 3, I and J). When filament elongation occurred, dense staining in whole anthers suddenly emerged (Fig. 3K). This staining then moved to filaments as female gametophytes became fertile (Fig. 3, L–N). In the roots, the three LPPs were strongly expressed at root tips and branch points (Fig. 3, Q–S). Thus, although the three isoforms of LPP were cooperatively expressed in leaves and roots, only LPPγ was expressed strongly in floral organs. These results suggest that the function of LPPγ may be nonredundant to the other two isoforms.

Plastidic LPPs and Synechocystis LPP Encode Functional PAPs—Because LPPs are all membrane-integrated proteins, these candidates were expressed in a yeast mutant line...
The introduction of these LPPs rescued the temperature-sensitive phenotype of Arabidopsis mutants (8, 9). Because Arabidopsis chloroplastic PAP activity has not been described previously, chloroplast membranes were isolated from Arabidopsis and characterized. As shown in Fig. 5, the PAP activity in chloroplast membranes showed a broad pH optimum from 6.0 to 8.0, and the activity was inhibited by the addition of Mg^{2+}, in agreement with previous reports using spinach chloroplasts. Furthermore, when these enzymatic features were compared with those of the three prokaryotic LPP isoforms, they closely resembled that of LPPγ.

Delta_lpp1Delta_lpp1Delta_pah1, which has significantly lower total PAP activity (14). When the plastidic LPP candidates as well as that of Synechocystis were introduced by transformation, membrane-bound PAP activity of the mutant yeast recovered to the wild type level, indicating that these LPPs indeed have PAP activity in vivo (Fig. 4A). Because the yeast mutant has a temperature-sensitive growth phenotype, we evaluated whether the temperature sensitivity could be rescued by the introduction of these LPPs. Transformation of SynLPP, LPPγ, and LPPε2 rescued, at least partially, the mutant yeast phenotype, suggesting that these candidates also have PAP activity in vivo (Fig. 4B).

Next, the enzymatic features of these LPPs were characterized with respect to pH optimum and Mg^{2+} dependence. Enzyme analysis of membrane proteins isolated from transformed yeast indicated that the optimal pH was 7.0 for LPPε1 and LPPε2, but LPPγ showed a broader pH optimum, from ~6.0 to 8.0 (Fig. 5). These enzymes were all inhibited by the addition of Mg^{2+}, which is a feature unique to chloroplastic PAP activity (8, 9). Because Arabidopsis chloroplastic PAP activity has not been described previously, chloroplast membranes were isolated from Arabidopsis and characterized. As shown in Fig. 5, the PAP activity in chloroplast membranes showed a broad pH optimum from 6.0 to 8.0, and the activity was inhibited by the addition of Mg^{2+}, in agreement with previous reports using spinach chloroplasts. Furthermore, when these enzymatic features were compared with those of the three prokaryotic LPP isoforms, they closely resembled that of LPPγ. These results suggest that LPPγ may be the primary PAP in chloroplasts.

Mutation Analysis of Plastidic PAP Suggests That LPPγ Is an Indispensable Enzyme—We next isolated T-DNA-tagged mutants of these LPPs (Fig. 6, A and B). The knock-out mutants of LPPε1 (lpe1:SALK_000157) and LPPε2 (lpe2: SALK_055964) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH), and homozygous mutants were successfully isolated (Fig. 6A). In addition, a
double knock-out line (lpp1lpp2) was also produced (Fig. 6B) as a precaution against the possibility that these two isoforms might be functionally redundant because of their high amino acid sequence similarity. However, the result of mutant analyses showed that even the lpp1lpp2 double mutant revealed no observable phenotypical alteration under normal growth condition. Furthermore, lipid analyses of the mutant lines showed no significant differences in either lipid composition or fatty acid composition (data not shown). These results suggest that loss of LPP1 and LPP2 has no observable impact on the bulk of glycerolipid metabolism.

Regarding the knock-out line for LPPγ, three T-DNA tag lines were available in the Arabidopsis Biological Resource Center. However, only CS846388 had T-DNA insertion within the LPPγ open reading frame (Fig. 6A). Although the homozygous mutant was isolated, the homozygous line could not be obtained despite screening 100 F2 plants of the heterozygous mutant, suggesting that loss of LPPγ might cause lethal effect on plant viability.

Therefore, we investigated whether lppγ/lppγ homozygous mutant can be isolated under the ectopic overexpression of LPPγ. First, we generated LPPγ-overexpressing transformant lines driven by the cauliflower mosaic virus 35S promoter (35S::LPPγ) and obtained the LPPγOX#5 line, which showed more than 10 times higher expression levels than WT (Fig. 6C). The LPPγOX#5 line showed no visible phenotype nor had any changes in lipid contents (supplemental Fig. S2B) and fatty acid composition (data not shown). Then this transformant was crossed with LPPγ/lppγ heterozygous plants, and F2 seeds were subjected to genotype analysis by PCR amplification. Because LPPγ is a gene with single exon, the size of PCR product will be the same whether it is amplified from endogenous LPPγ sequence or the ectopically introduced vector (35S::LPPγ). Therefore, we judged the genotype of LPPγ by amplifying open reading frame + 500 bp upstream region of LPPγ. In the F2 generation of the 35S::LPPγ introduced LPPγ/lppγ heterozygous plants, PCR analysis identified a certain plant in which full open reading frame is amplified but not open reading frame + 500 bp, indicating that this plant was lppγ/lppγ homozygous mutant (Fig. 6D). This plant showed no differences in phenotypes from WT, indicating that overexpression of LPPγ in planta can complement the lethal effect of LPPγ knock out.

To further investigate the lethality of LPPγ knock out, we observed female and male gynoecophytes of LPPγ/lppγ. Silique of LPPγ/lppγ had no significant amount of aborted ovules or wrinkled seeds, suggesting that lppγ/lppγ is unlikely to be embryonic lethal. On the other hand, in vitro pollen tube germination experiments showed that pollen from LPPγ/lppγ had a significantly lower germination rate compared with that of WT (WT, 100/301; LPPγ/lppγ, 24/297). These results indicate that loss of LPP affects normal pollen tube germination.
An independent experiment was designed to generate RNAi-suppressed LPPγ knock-out/knock-down lines because complete knock-out of LPPγ caused lethal effect. We isolated 30 independent transformants harboring LPPγ-RNAi. The expression level of LPPγ was decreased to some extent in most of them, with LPPγ-RNAi#5 and #8 showing the least expression level (~15% of WT; supplemental Fig. S2A). However, these lines showed no impact on lipid composition (supplemental Fig. S2B), suggesting that a partial suppression (~15% of WT) may be dispensable for plant viability. Considering that we could not obtain any transformants that showed further decrease in LPPγ expression, those with a strongly suppressed line (below 15%) might become lethal and therefore unable to be obtained by screening. Thus, the results of mutant analyses suggest that LPPγ is an indispensable PAP for plant viability, whereas LPPε1 and LPPε2 are unlikely to contribute significantly to the lipid metabolism, at least under normal growth conditions.

**DISCUSSION**

The phylogenetic tree constructed by comparison of the three conserved domains of the LPP family (LPP motifs) showed that LPPs are clearly divided into two subfamilies: either LPP isoforms in human, yeast, and Arabidopsis or those in cyanobacteria and their homologs in Arabidopsis. This classification suggests that the LPP isoforms may be categorized either as eukaryotic or prokaryotic LPPs, respectively. The fact that prokaryotic PAP is detected only in bacteria and higher plants leads us to presume that prokaryotic PAP might have been introduced into higher plants by endosymbiosis, thereby yielding plastidic PAP. Because the prokaryotic PAPs found in Arabidopsis and cyanobacteria have very low amino acid similarity to known eukaryotic LPPs, they were undetectable by a conventional homology search approach. However, three LPP domains are highly conserved in all Arabidopsis prokaryotic LPPs. Site-directed mutagenesis of these domains in yeast or mammalian LPP suggests that these domains are critical for enzymatic activity (25, 26), and together they may form an active site because they all face the same side of the membrane in every case studied so far (27). However, using a deduced membrane topology of these LPPs that consider membrane-spanning regions, domain 3 of prokaryotic LPP in Arabidopsis faces the opposite side (predicted by SOSUI program, available at bp.nuap.nagoya-u.ac.jp/sosui/). Considering that these LPPs also exhibited significant PAP activity both in vitro and in vivo, it is likely that domain 3 is not a part of the active site but might have an alternate function. This idea is also supported by the evidence that SynLPP showed significant PAP activity in vitro and perfectly complemented the temperature-sensitive phenotype of mutant yeast in vivo, even though it partially lacks the highly conserved residues (Ser and Arg) in domain 3. Therefore, it is of enzymological interest to determine the functions of each domain by comparatively analyzing both eukaryotic and prokaryotic LPP families.

The three prokaryotic LPPs in Arabidopsis, LPPγ, LPPε1, and LPPε2, all localized to the chloroplast. In each protein, membrane-spanning regions were identified by the SOSUI program, suggesting that these enzymes are integral membrane proteins. This is in good agreement with a previous report on spinach chloroplasts indicating that chloroplastic PAP activity is tightly associated with the inner envelope (9). Considering that all three LPPs have apparent transit peptide sequences and that the previous report detected no significant PAP activity in purified outer envelope (9), these LPPs are likely to be integrated in the inner envelope of chloroplasts. As for the topological orientation of these LPPs, it seems consistent that PAP faces the stromal side of the inner envelope because glycerol-3-phosphate acyltransferase is a stromal protein (28) and lysophosphatidic acid acyltransferase activity faces the stromal side of the inner envelope (29). In addition, a recent report on TGD1 (4) suggests that it might deliver PA as far as the stromal side of the inner envelope. However, monogalactosyldiacylglycerol synthase 1, an enzyme that acts subsequent to PAP, faces the intermembrane space, raising the question of how the substrate DAG penetrates the inner envelope from the stromal side to the opposite side (4). The reaction steps leading from PA to monogalactosyldiacylglycerol are rather rapid and thought to be highly regulated because chloroplastic PAP activity is negatively regulated by the product DAG (30), and PA induces monogalactosyldiacylglycerol synthase activity (31). Therefore, it is important to elucidate the membrane topology of the chloroplastic LPPs. However, to do so, one must first determine which regions of LPPs are involved in substrate binding or catalytic activity, because they are integral membrane proteins that have several exposed regions on each side of the membrane. Because the conventional idea that the three LPP domains form an active site is not applicable for plastidic LPPs, future studies are required to identify the essential regions of the enzyme and determine its topology.

The comparison of expression levels among the three plastidic LPPs revealed that LPPγ expression was highest in shoots. This result was supported by the data distributed by the Massively Parallel Signature Sequencing data base that LPPγ is expressed at more than 10 times higher levels than the other isoforms. Histochemical GUS staining showed that all these LPPs were expressed in leaves. However, there was a difference in the staining pattern in that LPPγ was expressed in vascular tissues, whereas LPPε1 and LPPε2 were expressed at the meristematic end of young leaves and cotyledons. On the other hand, GUS staining was detected in flowers only for LPPγ, which showed a dynamic GUS staining pattern during flower development. Although there are plenty of examples showing specific expression in flowers, the distinctiveness of the LPPγ pattern is that it was expressed temporally and spatially in both male and female gametophytes. These results suggest that LPPγ may be a predominant plastidic PAP in photosynthetic organs and play a unique role in development of floral organs. The lethal effect by LPPγ knock-out might reflect partly the functional importance of this isoform in floral organs.

The expression of recombinant LPP in the yeast mutant Δdpp1Δlpp1Δpah1 showed significant recovery of total PAP activity for the four LPPs analyzed. Furthermore, the tempera-
ture-sensitive phenotype of the mutant yeast was, at least in part, recovered by the introduction of some of these LPPs. These results suggest that the newly identified LPPs function as PAPs both in vitro and in vivo. Enzymatic characterization of these LPPs showed that their PAP activities were inhibited by Mg^{2+}. The effective inhibitory concentration of Mg^{2+} was consistent with that reported in isolated spinach chloroplasts (8, 9). However, this is in contrast to data for the eukaryotic LPPs in Arabidopsis (LPPα1 and α2) in that Mg^{2+} does not inhibit PAP activity and even activates it (12). This suggests that the enzymatic properties of plastidic LPPs are distinct from known Arabidopsis LPPs but similar to those of intact chloroplast membranes isolated from spinach and Arabidopsis. Furthermore, the pH profiles for the PAP activity of LPPα1 and LPPα2 had a sharp peak at pH 7, whereas that for LPPγ showed a broader maximal activity from pH 6 to 8, which corresponds well to that of isolated Arabidopsis chloroplasts. The fact that the inhibitory effect by Mg^{2+} and optimal pH were also similar between LPPγ and Arabidopsis chloroplasts again suggests that LPPγ activity predominates among the three chloroplastic LPPs. Although analysis with the isolated spinach chloroplast envelope characterized chloroplastic PAP as an alkaline phosphatase (8, 9), this might not be applicable to other species because chloroplastic PAP in Arabidopsis showed a broader pH optimum from slightly acidic to weakly alkaline under our assay condition.

To study the function of the three plastidic LPPs in vivo, T-DNA tagged mutants were isolated. Although homozygous mutants of LPPα1 and LPPα2 were isolated successfully and a double knock-out mutant was also produced, no significant changes in lipid composition or fatty acid composition of each lipid class were observed even in the double knock-out mutant. By contrast, the homozygous mutant of LPPγ was not obtained even by self-crossing the heterozygous mutant (n = 100). However, the lppγ/lppγ homozygous mutant was isolated only under ectopic overexpression of LPPγ in planta, suggesting that LPPγ is an indispensable enzyme for plant viability. Although it is currently unknown why the loss of LPPγ is lethal, one possible interpretation is that LPPγ plays a crucial role in lipid metabolism. Because suppression of TGD1 severely affects the eukaryotic pathway (3, 4), a significant portion of eukaryotic lipid may be imported into chloroplasts in a form of plastid. In addition, crossing of tgd1 with act1, in which prokaryotic lipid metabolism is abolished (32), resulted in embryonic lethal phenotype (4). As mentioned in the introduction, plastidic PAP is considered to be involved in both eukaryotic and prokaryotic pathways because PA is the major extraplastidic lipid to be incorporated into plastids (4). Therefore, it is expected that knock-out of plastidic PAP also shows lethality as was observed in act1tgd1 (4). The reason lppγ/lppγ was not embryonic lethal but showed possible defects in pollen elongation might be that in addition to the primary involvement in leaf PAP activity, LPPγ plays crucial roles in a certain process during male gametophyte development. The strong GUS staining in floral organs and results of in vitro pollen tube germination experiment support this idea. Because lppγ/lppγ homozygous mutant is not available and RNAi knock-down strategy was unsuccessful so far, the remaining question is how LPPγ is involved in lipid metabolism in leaves as well as flowers. In this regard, it may be useful in future study to selectively express individual genes in specific tissues using the gene induction systems such as the GVG system (33) or the XVE system (34). Any phenotypical changes in leaves or floral organs may extend our understanding of the function of LPPγ in vivo.

In summary, we isolated one cyanobacterial PAP in Synecho-
cystis sp. PCC6803 (SynLPP) and three homologous plastidic PAPs in Arabidopsis (AtLPPγ, LPPα1, and LPPα2) that belong to a distinct subfamily (prokaryotic type) of LPPs. Characterization of the three plastidic PAP isoforms suggests their differentiated function, with which LPPγ may predominate over LPPα1 and LPPα2. Further studies are expected to uncover the full scope of PAP in Arabidopsis.

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