Quantitative Profiling of Arabidopsis Polar Glycerolipids in Response to Phosphorus Starvation. Roles of Phospholipases Dζ1 and Dζ2 in Phosphatidylcholine Hydrolysis and Digalactosyldiacylglycerol Accumulation in Phosphorus-Starved Plants[1][W]

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Phosphorus is an essential macronutrient that often limits plant growth and development. Under phosphorus-limited conditions, plants undergo substantial alterations in membrane lipid composition to cope with phosphorus deficiency. To characterize the changes in lipid species and to identify enzymes involved in plant response to phosphorus starvation, 140 molecular species of polar glycerolipids were quantitatively profiled in rosettes and roots of wild-type Arabidopsis (*Arabidopsis thaliana*) and phospholipase D knockout mutants *pldζ1*, *pldζ2*, and *pldζ1pldζ2*. In response to phosphorus starvation, the concentration of phospholipids was decreased and that of galactolipids was increased. Phospholipid lost in phosphorus-starved Arabidopsis rosettes was replaced by an equal amount of galactolipid. The concentration of phospholipid lost in roots was much greater than in rosettes. Disruption of both *PLDζ1* and *PLDζ2* function resulted in a smaller decrease in phosphatidylcholine and a smaller increase in digalactosyldiacylglycerol in phosphorus-starved roots. The results suggest that hydrolysis of phosphatidylcholine by PLDζs during phosphorus starvation contributes to the supply of inorganic phosphorus for cell metabolism and diacylglycerol moieties for galactolipid synthesis.

Phosphorous is a macronutrient essential for plant growth and development (Raghothama, 1999). In acidic or calcareous soil, the availability of phosphorus for agriculture may be low due to low total phosphorus and/or to the formation of insoluble complexes (Vance et al., 2003). One remedy for the lack of phosphorus is to apply large quantities of fertilizers containing phosphate. However, this may lead to environmental pollution and is not economically sustainable (Withers et al., 2001). To improve plant performance under conditions of phosphorus deficiency, it is important to understand the mechanism by which plants respond to a low phosphorus environment (Hammond et al., 2004). Recently, substantial efforts have been put into defining two aspects of the plant response to phosphorus starvation. One is identification of phosphorus-responsive genes, which are potential candidates for genetic manipulation to improve plant performance under phosphorus-limited conditions (Hammond et al., 2003; Wu et al., 2003; Misson et al., 2005). The other is investigation of biochemical pathways involved in plant response to phosphorus starvation conditions. In particular, changes in glycerolipid metabolism under phosphorus-deficient conditions have drawn considerable attention (Benning et al., 1995; Härtel et al., 2000; Jouhet et al., 2003, 2004; Frentzen, 2004; Andersson et al., 2005).

Glycerolipids in plant membranes comprise principally phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidyl-Ser (PS), phosphatidic acid (PA), and phosphatidylglycerol (PG), and galactolipids, including monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). Each headgroup class of glycerolipids is composed of various molecular species, and the fatty acids or other hydrocarbon portions vary in chain length and unsaturation.

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To date, changes in the major head-group classes, PC, PE, PI, PG, MGDG, and DGDG, during phosphorus starvation have been described (Benning and Ohta, 2005; Nakamura et al., 2005), but detailed analysis of lipid alterations of the molecular species has not been reported. In addition, alterations of the minor lipid classes, such as PA, PS, lysophosphatidylcholine (lysoPC), lysophosphatidylethanolamine (lysoPE), and lysophosphatidylglycerol (lysoPG), have not been investigated. The advent of quantitative lipid profiling based on electrospray ionization tandem mass spectrometry (ESI-MS/MS) has made it feasible to determine the concentration of minor lipid classes and the concentration of individual molecular species (Welti et al., 2002; Welti and Wang, 2004). A comprehensive analysis of lipid species may shed light on the metabolic pathways that alter lipid species and plant response to phosphorus starvation.

Plants growing under phosphorus-limited conditions have a lower concentration of phospholipids and an increased concentration of galactolipids, mostly DGDG, than those growing under normal phosphorus conditions (Härtel et al., 2000; Benning and Ohta, 2005). The decrease in phospholipids presumably allows phosphorus to be used for other critical cell functions and also makes the lipid moiety diacylglycerol (DAG) available for galactolipid biosynthesis. Phospholipid-hydrolyzing enzymes, such as phospholipase D (PLD), and phospholipase C (PLC) have been proposed to be involved in the decrease in phospholipid content. The expression of one PC-hydrolyzing *PLC* was induced greatly in phosphorus-limited Arabidopsis (*Arabidopsis thaliana*), but the gene knockout of that *PLC* has no apparent effect on the lipid composition under normal and phosphorus-starved growth conditions (Nakamura et al., 2005). Of 12 Arabidopsis *PLD*s, *PLDα(3)*, *β(2)*, *γ(3)*, *δ*, *ε*, and *ζ(2)*, the expression of the two *PLDζ*s and particularly *PLDζ2* was induced most in phosphorus-deficient conditions (Misson et al., 2005; Li et al., 2006). In addition, the *PLDζ1* and *ζ2* double knockout displayed shorter primary roots and had a lower concentration of PA in roots than wild-type plants under the same phosphorus-limited conditions. Another study showed that *PLDζ2* was involved in lipid turnover in a phosphorus-limited condition (Cruz-Ramirez et al., 2006). These results suggest that *PLDζ1* and *ζ2* play a role in regulating root development in response to phosphorus limitation, but their roles, especially their potential additive roles, in the turnover of phospholipids and galactolipid accumulation during phosphorus starvation have not been investigated prior to this study (to our knowledge).

This study was undertaken to determine the changes in membrane lipid species and the role of *PLDζ1* and *PLDζ2* in the changes in Arabidopsis rosettes and roots in response to phosphorus starvation. Analysis of phospholipid and galactolipid species indicates that multiple lipid metabolic pathways are involved in plant response to phosphorus limitation. Comparative lipid profiling of wild type and *pldζ1*, *pldζ2*, and *pldζ1pldζ2* mutants defines the contribution of *PLDζ*s to the metabolism of phospholipids and galactolipids during phosphorus starvation.

RESULTS

Decrease in Phospholipids and Increase in Galactolipids during Phosphorus Starvation

To evaluate the effects of phosphorus starvation on plant growth and lipid composition, 3-d-old Arabidopsis seedlings on standard inorganic phosphate (P_i) agar plates (500 μM P_i) were transferred to 500 μM or phosphate-free agar plates for an additional 7 d. The growth of the aboveground portion of the plants, rosettes, was inhibited, while the root growth was stimulated (Fig. 1). The dry mass of phosphorus-starved rosettes was 35% less than that of normally grown rosettes, while the dry mass of phosphorus-starved roots was 38% more than that of normally grown roots (Fig. 1A). As a result, the dry weight ratio of roots to rosettes was increased 2-fold during phosphorus starvation (Fig. 1B). These data indicate that plants respond effectively to phosphorus starvation, and lipids from rosettes and roots under these phosphorus conditions were analyzed by ESI-MS/MS to investigate changes in lipid metabolism.

Under phosphorus-starvation conditions, the concentrations of phospholipids decreased and those of galactolipids increased, but the magnitude of alteration in lipid concentrations was more drastic in roots than in rosettes (Table I). Decreases in phosphorus-starved rosettes for PC, PE, and PG were 17%, 30%, and 28%, respectively, whereas the same lipids in phosphorus-starved roots decreased 51%, 65%, and 49%, respectively (Table I). In addition, concentrations of PI and PA did not decrease significantly in phosphorus-starved rosettes, but decreased 57% and 46%,

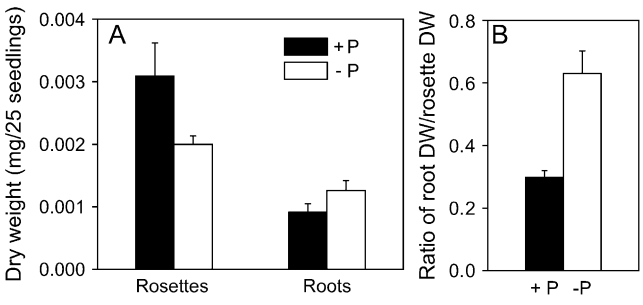

Figure 1. Effect of phosphorus on the growth of Arabidopsis rosettes and roots. Wild-type seeds (Columbia) were sterilized and germinated on 500 μM phosphate agar plates. Three-day-old seedlings were transferred onto five agar plates with 500 μM phosphate (+P) or five agar plates with 0 μM phosphate (−P). To each plate, 25 seedlings were transferred. After 7 additional days, the rosettes and roots were harvested for dry weight. A, Rosettes or roots from one agar plate were harvested as one sample for dry weight. B, Dry weight ratio of roots over rosettes. Five plates were measured for the ratio. Data are the means of five replicates ±SD ($n = 5$).

Table I. *Total concentration of each lipid class in normally grown and phosphorus-starved rosettes and roots*

Three-day-old seedlings were transferred to fresh 500 μM (+P) or 0 μM phosphate (−P) agar plates and cultured for an additional 7 d. The rosettes and roots were then harvested separately for lipid analysis by ESI-MS/MS. Data are means of five replicates ±SD (in superscript for clarity). "Significant difference" indicates that the lipid class was significantly altered by phosphorus starvation ($P < 0.05$) and the % difference is shown. PL, Phospholipids; GL, galactolipids.

	Rosettes			Roots		
	+ P	−P	Significant Difference	+P	−P	Significant Difference
	nmol/mg DW			*nmol/mg DW*		
PC	$28.0^{\pm1.7}$	$23.3^{\pm2.7}$	−17%	$21.4^{\pm0.41}$	$10.4^{\pm0.42}$	−51%
PE	$21.3^{\pm1.3}$	$15.0^{\pm2.5}$	−30%	$29.9^{\pm1.3}$	$10.3^{\pm1.2}$	−65%
PI	$5.93^{\pm0.60}$	$4.94^{\pm1.03}$		$4.97^{\pm0.45}$	$2.12^{\pm0.30}$	−57%
PS	$1.40^{\pm0.25}$	$1.93^{\pm0.45}$		$1.88^{\pm0.51}$	$1.75^{\pm0.48}$	
PA	$0.47^{\pm0.04}$	$0.36^{\pm0.11}$		$1.30^{\pm0.40}$	$0.70^{\pm0.11}$	−46%
PG	$29.6^{\pm2.4}$	$21.5^{\pm3.5}$	−28%	$1.97^{\pm0.44}$	$1.01^{\pm0.35}$	
LysoPC	$0.06^{\pm0.001}$	$0.04^{\pm0.007}$		$0.04^{\pm0.013}$	$0.02^{\pm0.001}$	−47%
LysoPE	$0.12^{\pm0.008}$	$0.08^{\pm0.011}$	−37%	$0.19^{\pm0.012}$	$0.07^{\pm0.011}$	−64%
LysoPG	$0.01^{\pm0.0108}$	$0.01^{\pm0.007}$		$0.002^{\pm0.001}$	$0.008^{\pm0.0019}$	
MGDG	$63.3^{\pm3.17}$	$71.6^{\pm7.13}$		$1.56^{\pm0.59}$	$1.61^{\pm0.24}$	
DGDG	$15.2^{\pm1.1}$	$26.1^{\pm2.0}$	72%	$0.84^{\pm0.30}$	$8.95^{\pm1.08}$	965%
Total PL	$86.7^{\pm4.19}$	$67.0^{\pm9.90}$	−23%	$61.8^{\pm1.68}$	$26.4^{\pm3.22}$	−57%
Total GL	$78.5^{\pm3.87}$	$97.7^{\pm8.76}$	24%	$2.4^{\pm0.89}$	$10.6^{\pm1.26}$	340%
Total lipid	$165^{\pm7.6}$	$165^{\pm18.4}$		$64^{\pm2.3}$	$37^{\pm2.3}$	−42%

respectively, in roots (Table I). Moreover, DGDG increased 10-fold in phosphorus-starved roots, but only 72% in rosettes (Table I). These data indicate that the availability of phosphorus has more impact on root than rosette lipid metabolism and that large decreases in root phospholipid concentration are correlated with a large increase in root galactolipid concentration.

Galactolipids Replace Phospholipids Quantitatively in Phosphorus-Starved Rosettes

The concentration of phospholipids in rosettes decreased 23% when 3-d-old seedlings were starved for phosphorus for 7 d, and the concentration of galactolipids increased 24% to replace the phospholipids (Table I). In normally grown rosettes, the total concentration of phospholipids, including PC, PE, PI, PG, PS, and PA, was 86.7 nmol/mg dry weight; the total concentration of galactolipids, including MGDG and DGDG, was 78.5 nmol/mg dry weight (Table I). Thus, the total concentration of lipids, including phospholipids and galactolipids, was 165 nmol/mg dry weight in normally grown rosettes (Table I). In phosphorus-starved rosettes, however, the total phospholipids and galactolipids were 67.0 and 97.7 nmol/mg dry weight, respectively, but the total concentration of lipids was still 165 nmol/mg dry weight (Table I). Although the total lipid concentration was unchanged in phosphorus-starved rosettes as compared to normally grown rosettes, the percentage of phospholipids in the total lipid dropped from 52 to 41. The homeostatic response, in which membrane lipid concentrations are maintained, highlights the important role of membranes in cellular function.

Roots respond to phosphorus starvation differently from rosettes not only in terms of growth (Fig. 1) but also in terms of lipid alterations (Table I). In phosphorus-starved roots, the concentration of total phospholipids, including PC, PE, PI, PA, PS, and PG, decreased 35 nmol/mg dry weight, while the concentration of total galactolipids, including MGDG and DGDG, only increased 8 nmol/mg dry weight (Table I). Thus, in roots, galactolipids do not quantitatively replace phospholipids as they do in rosettes during phosphorus starvation.

Individual Molecular Species Respond Differently to Phosphorus Starvation

The molecular species of eight lipid classes, including PI, PG, PS, PE, PC, PA, DGDG, and MGDG, were profiled in rosettes and roots under normal and phosphorus starvation conditions (Fig. 2). There are two general tendencies for the alteration of individual lipid species among the lipid species. First, the concentrations of the major molecular species in phospholipid classes, except for PS, were lower in phosphorus-starved rosettes and roots than in normally grown ones (Fig. 2, A and C), and the differences were greater in roots than in rosettes. Second, the concentrations of most molecular species of galactolipids were higher in phosphorus-starved rosettes and roots than in normally grown rosettes and roots (Fig. 2, A and C). These differences constitute the lipid class differences already described, in which phospholipids are decreased and galactolipids are increased during phosphorus starvation. These alterations likely allow maintenance of functional membranes during phosphorus starvation.

One exception from the general tendency in species' alteration under the two phosphorus conditions is PS species. The concentrations of most PS species with very long chain fatty acids were higher in phosphorus-starved rosettes than normally grown rosettes (Fig.

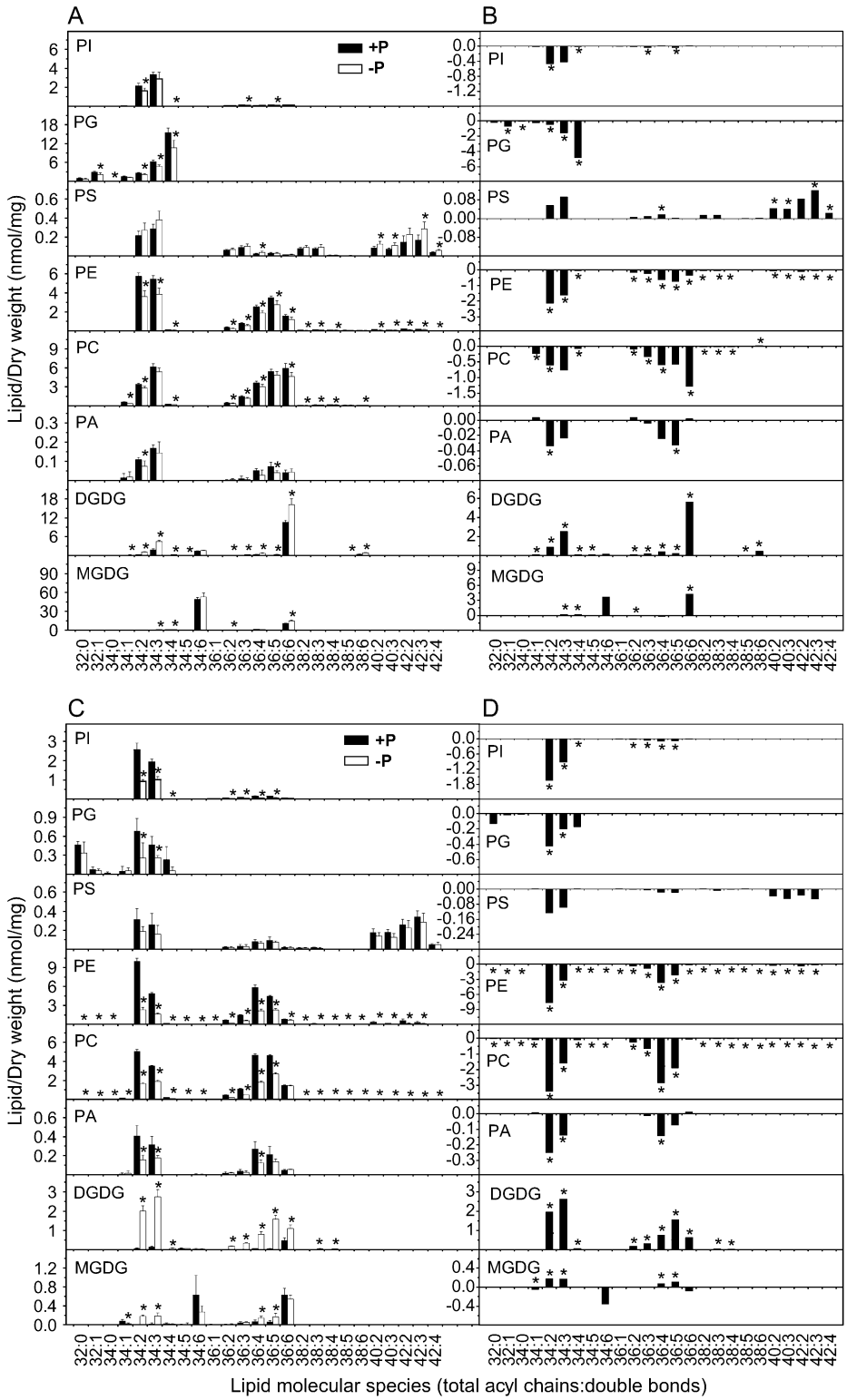

Figure 2. Concentration of individual molecular species in lipid classes of PI, PG, PS, PE, PC, PA, DGDG, and MGDG in rosettes and roots of wild-type Arabidopsis under 500 μM and 0 μM phosphate conditions. Three-day-old seedlings were transferred to the indicated concentration of phosphate plates, 500 μM phosphate (+P) or 0 μM phosphate (−P), respectively. After 7 additional days, the rosettes and roots were harvested for lipid analysis by ESI-MS/MS. A, Individual lipid molecular species in rosettes under two phosphorus conditions. B, Alteration in individual lipid species in rosettes between two phosphorus conditions. C, Individual lipid molecular species in roots under two phosphorus conditions. D, Alteration in individual lipid species in roots between two phosphorus conditions. Data are expressed as mean ± SD ($n = 5$). *, Difference in that particular lipid species under two phosphorus conditions is significant ($P < 0.05$).

2A). This exception may reflect the strategies by which plants cope with phosphorus starvation. PS is only a minor lipid, constituting 3% of total phospholipid in rosettes. Higher concentrations of individual PS species may serve as signaling molecules to modulate the survival of stressed cells (Salomoni et al., 1998; Kim et al., 2000; Neshatg et al., 2000; Yu et al., 2004).

To show the altered concentrations of each individual species, the concentration of each species in normally grown rosettes or roots was subtracted from that

of phosphorus-starved rosettes or roots (Fig. 2, B and D). In rosettes and roots, the major molecular species, such as 34:2, 34:3, 36:4, 36:5, and 36:6, were generally lower in phospholipids during phosphorus starvation (Fig. 2). The increase in these species, but not the plastid-derived 34:6 species, in DGDG (Fig. 2) suggests that hydrolysis of phospholipids supplies DAG moieties for DGDG synthesis during phosphorus starvation.

The Endoplasmic Reticulum-Plastidic Pool Contributes to the Accumulation of DGDG during Phosphorus Starvation

Lipids in Arabidopsis are synthesized through distinguishable routes, the prokaryotic pathway and the eukaryotic pathway. The prokaryotic pathway is localized on the plastid inner envelope, and the eukaryotic one is localized on the endoplasmic reticulum (ER; Somerville et al., 2000; Wallis and Browse, 2002). DGDG can be considered as being in three pools: the plastidic pool is derived from the prokaryotic pathway and is located in the plastid, the ER-extraplastidic pool is derived from the eukaryotic pathway and is located outside the plastid, while the ER-plastidic pool is derived from the eukaryotic pathway but is located inside the plastid. The 34-carbon DGDGs are derived from both the prokaryotic (mainly 18:3-16:3 DGDG, here called 34:6, and 18:3-16:0 DGDG, here one of two major 34:3 species) and eukaryotic pathways (mainly 16:0-18:3, which is the second major 34:3 species), while 36-carbon DGDGs are derived from the eukaryotic pathway (Härtel et al., 2000; Somerville et al., 2000). It has been reported that the DGDG that accumulates during phosphorus starvation contains considerable 16:0 (Härtel et al., 2000). This information, coupled with the finding that the *act1* mutant that lacks functional prokaryotic galactolipid synthesis is able to increase DGDG concentrations upon phosphorus deprivation to an extent similar to wild-type plants (Härtel et al., 2000), suggests that the DGDG accumulated during phosphorus starvation is part of the ER-extraplastidic pool and that the accumulated 16:0-containing species must be ER-derived species, such as 16:0-18:3 DGDG. However, a potential role for the plastidic pool as well as the ER-plastidic pool in the DGDG accumulation during phosphorus starvation in wild-type plants was not ruled out.

Here, our data show that concentrations of most DGDG species were higher in phosphorus-starved rosettes than in normally grown rosettes (Fig. 2, A and B). There was 11 nmol/mg dry weight more newly formed DGDG in phosphorus-starved rosettes than normally grown rosettes (Table I). Of this, 34% came from 34-carbon DGDG and 61% from 36-carbon DGDG (Table II). In addition, there was 8 nmol/mg dry weight more DGDG in phosphorus-starved roots than in normally grown roots (Table I), of which 57% came from 34-carbon DGDG and 42% from 36-carbon DGDG (Table II). DGDGs with 36-carbons can only originate from the eukaryotic pathway (Somerville et al., 2000). Among 34-carbon DGDGs, 34:6 (18:3-16:3 DGDG) and 34:5 (mostly in the form of 18:2-16:3 DGDG, with a minor amount of 18:3-16:2 DGDG) originate from the prokaryotic pathway, while 34:4, 34:3, 34:2, and 34:1 could represent species that originate from either the prokaryotic or eukaryotic pathways (Somerville et al., 2000). From the data in Table II, it is clear the eukaryotic pathway, here called the ER-plastidic pool, contributes to the DGDG accumulation during phosphorus starvation. The fact that neither 34:6-DGDG nor 34:5-DGDG increased during phosphorus starvation in rosettes and roots (Fig. 2) suggests that newly formed DGDG might not originate from the plastidic pool.

Table II. *Percentage of 34- and 36-carbon individual molecular species in newly accumulated DGDG during phosphorus starvation*

	Rosettes[a]	Roots[b]
	%	%
34 Total	34.3	56.8
34:1	0.7	0
34:2	8.2	24.0
34:3	23.8	32.2
34:4	0.9	0.7
34:5	0.7	0
34:6	0	0
36 Total	61.3	42.3
36:1	0	0
36:2	0.7	2.0
36:3	1.8	4.0
36:4	3.7	9.2
36:5	2.2	18.8
36:6	52.9	8.3

[a]The percentage of individual DGDG species in 11 nmol/mg dry weight (Table I), which was the total DGDG increase in phosphorus-starved rosettes compared to rosettes grown at the standard phosphorus condition. [b]The percentage of individual DGDG species in 8 nmol/mg dry weight (Table I), which was the total DGDG increase in phosphorus-starved roots compared to roots grown at the standard phosphorus concentration.

PLDζ1 and *ζ2* Contribute to PC Hydrolysis and DGDG Accumulation in Phosphorus-Starved Roots

PLD is a major family of phospholipid-hydrolyzing enzymes in plants. Arabidopsis has 12 *PLD*s, and their levels of expression differed in response to phosphorus starvation (Fig. 3). The mRNA level for most *PLD*s, *α2*, *α3*, *β1*, *β2*, *δ*, and *ε*, did not change. The expression level of three *PLDγ*s decreased, whereas that of *PLDα1*, *ζ1*, and *ζ2* increased. The most drastic change is *PLDζ2*, and its mRNA level increased more than 10-fold in phosphorus-starved rosettes and roots. These results are consistent with the microarray data in which *PLDζ2* was drastically induced by phosphorus starvation (Misson et al., 2005). PLDζ2 is structurally similar to PLDζ1, and both are distinctly different from other PLDs by having phox homology and pleckstrin homology domains that are found in mammalian PLDs (Qin and Wang, 2002).

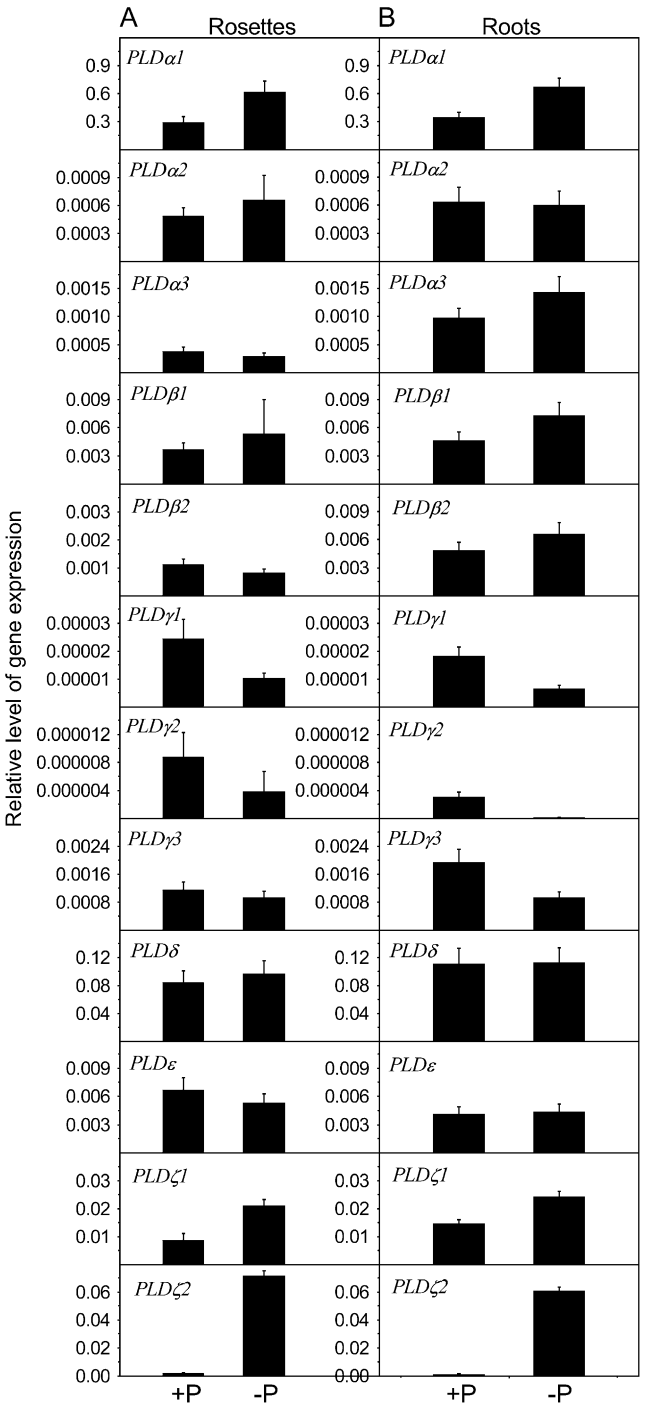

Figure 3. Expression of 12 *PLD* genes in rosettes (A) and roots (B) of wild type under 500 μM phosphate and 0 μM phosphate conditions. Three-day-old seedlings were transferred to the indicated concentration of phosphate, 500 μM phosphate (+P) or 0 μM phosphate (−P), respectively. After 7 additional days, the rosettes and roots were harvested for total RNA isolation and real time PCR. The levels of expression are expressed relative to the expression level of *UBQ10*. Values are the mean of three replicates ±SD ($n = 3$).

To investigate the role of *PLDζ*s in phosphorus starvation, the phospholipids and galactolipids of the single mutants, *pldζ1* and *pldζ2*, and double mutant, *pldζ1pldζ2*, were profiled. Under normal phosphorus conditions, there was no difference in the concentrations of various lipid classes examined among the roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* (Fig. 2; Supplemental Figs. S1–S3). However, under phosphorus-starved conditions, the total PC concentration was significantly higher and DGDG concentration was significantly lower in phosphorus-starved roots of *pldζ1pldζ2* than in wild type (Fig. 4; Table III). In roots of phosphorus-starved plants, the PC concentration was 1.4 nmol/mg dry weight higher in *pldζ1pldζ2*

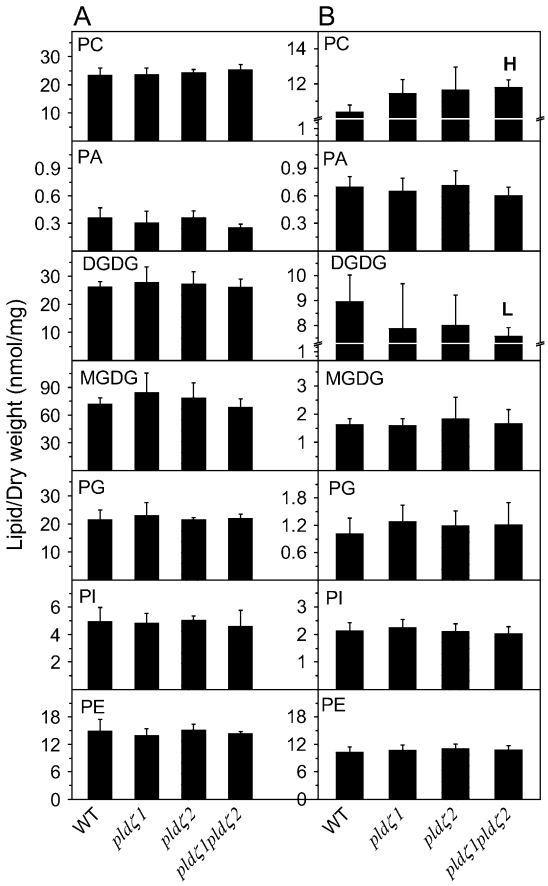

Figure 4. Concentration of PC, PA, DGDG, MGDG, PG, PI, PS, and PE in rosettes and roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under the phosphorus starvation condition. Three-day-old seedlings in each genotype were transferred to the phosphorus starvation condition (0 μM phosphate). After 7 additional days, the rosettes and roots were harvested for lipid analysis by ESI-MS/MS. A, Concentration of eight lipid classes in rosettes of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under the phosphorus starvation condition. B, Concentration of eight lipid classes in roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under the phosphorus starvation condition. Data were expressed as mean ± SD ($n = 5$). The marker H indicates that the PC concentration was significantly higher in *pldζ1pldζ2* than in wild type ($P < 0.05$). The marker L indicates that the DGDG concentration was significantly lower in *pldζ1pldζ2* than in wild type ($P < 0.05$).

Table III. *Concentrations of PC and DGDG in Arabidopsis rosettes and roots under phosphorus-starved conditions in wild type, pldζ1, pldζ2, and pldζ1pldζ2 mutants*

The data were expressed as average ± SD (n = 5). SD is in superscript for clarity.

	Rosettes				Roots			
	Wild Type	*pldζ1*	*pldζ2*	*pldζ1pldζ2*	Wild Type	*pldζ1*	*pldζ2*	*pldζ1pldζ2*
	nmol/mg DW				*nmol/mg DW*			
PC/+P	$28.0^{\pm1.7}$	$30.3^{\pm3.2}$	$29.7^{\pm3.1}$	$31.2^{\pm2.7}$	$21.4^{\pm0.4}$	$20.4^{\pm1.5}$	$21.1^{\pm1.5}$	$21.9^{\pm1.8}$
PC/−P	$23.3^{\pm2.7}$	$23.5^{\pm2.6}$	$24.6^{\pm1.3}$	$25.4^{\pm1.9}$	$10.4^{\pm0.4}$	$11.4^{\pm0.8}$	$11.6^{\pm1.3}$	$11.8^{\pm0.4,\ a}$
DGDG/+P	$15.2^{\pm1.1}$	$17.8^{\pm3.4}$	$17.3^{\pm1.3}$	$17.6^{\pm1.3}$	$0.84^{\pm0.30}$	$0.90^{\pm0.30}$	$0.70^{\pm0.22}$	$0.65^{\pm0.12}$
DGDG/−P	$26.1^{\pm2.0}$	$27.7^{\pm5.6}$	$27.2^{\pm4.4}$	$26.0^{\pm2.9}$	$8.95^{\pm1.08}$	$7.87^{\pm1.81}$	$8.00^{\pm1.23}$	$7.55^{\pm0.35,\ b}$

[a]The concentration was significantly higher in *pldζ1pldζ2* than in wild type ($P < 0.05$), and the difference between them is 1.4 nmol/mg dry weight. [b]The concentration was significantly lower in *pldζ1pldζ2* than in wild type ($P < 0.05$), and the difference between them is 1.4 nmol/mg dry weight.

plants than in wild type, while the DGDG concentration was 1.4 nmol/mg dry weight lower in *pldζ1pldζ2* plants than in wild-type plants (Table III). The reciprocal alterations of PC and DGDG suggest that *PLDζ*s contribute to the PC hydrolysis and DGDG accumulation in roots during phosphorus starvation. This result implicates PC as a substrate of PLDζs in vivo; this substrate specificity is consistent with earlier in vitro data that show that PLDζ1 selectively hydrolyzes PC (Qin and Wang, 2002). This also suggests that the *PLDζ*s contribute to DGDG accumulation at the expense of PC in phosphorus-starved roots.

Although the single mutants *pldζ1* and *pldζ2* did not show a significant difference in the concentrations of PC and DGDG in phosphorus-starved root, there was a tendency for PC to be higher and DGDG to be lower than wild type (Fig. 4). These concomitant changes indicate that the effects of *PLDζ1* and *PLDζ2* on PC hydrolysis and DGDG accumulation are additive.

Analysis of lipid molecular species showed that the concentrations of most PC species, except for 36:6-PC and 36:2-PC, are significantly higher in phosphorus-starved roots of *pldζ1pldζ2* double mutants than in those of wild-type plants (Fig. 5). Conversely, the concentration of most DGDG species, except for 36:6-DGDG and 36:5 DGDG, are lower in phosphorus-starved roots of *pldζ1pldζ2* double mutants than in wild type. In lipid classes other than PC and DGDG, the molecular species did not show major differences in the mutant plants as compared to wild-type plants (Fig. 2; Supplemental Figs. S1–S3). At the species level, single mutants *pldζ1* and *pldζ2* displayed significant differences from the wild type in some PC and DGDG species (Fig. 5); *pldζ1* had a higher concentration of 36:6-PC, whereas *pldζ2* had lower concentrations of 34:2-DGDG and 36:6-DGDG. These data further support the hypotheses that *PLDζ1* and *PLDζ2* have additive effects on PC hydrolysis and that PLDζ-mediated hydrolysis of PC contributes to DGDG accumulation in phosphorus-starved roots.

Lysophospholipids Decrease in Phosphorus-Starved Tissues

Lysophospholipids are biologically active in a range of important cellular signaling pathways. In addition, they are involved in lipid metabolism, possibly serving as carriers to transfer acyl chains from ER to chloroplast (Mongrand et al., 2000). However, due to their low abundance, the concentration of lysophospholipids was not been determined previously in response to phosphorus starvation. The concentrations of lysoPC and lysoPE decreased by 33% and 37%, respectively, in phosphorus-starved rosettes, and the decrease was 47%

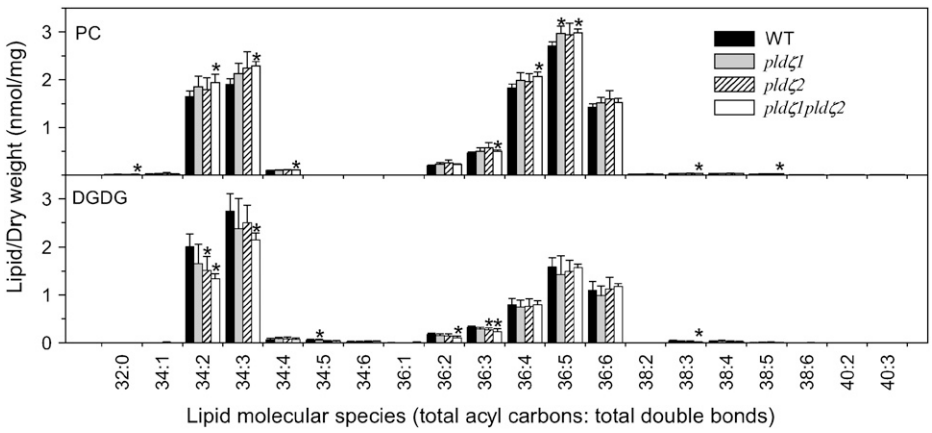

Figure 5. Concentration of individual species in lipid classes of PC and DGDG in roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under phosphorus starvation condition. Three-day-old seedlings in each genotype were transferred to the phosphorus starvation condition (0 μM phosphate). After 7 additional days, the rosettes and roots were harvested for lipid analysis by ESI-MS/MS. Data were expressed as mean ± SD ($n = 5$). *, Difference in that particular lipid species is significant as compared with wild-type roots ($P < 0.05$).

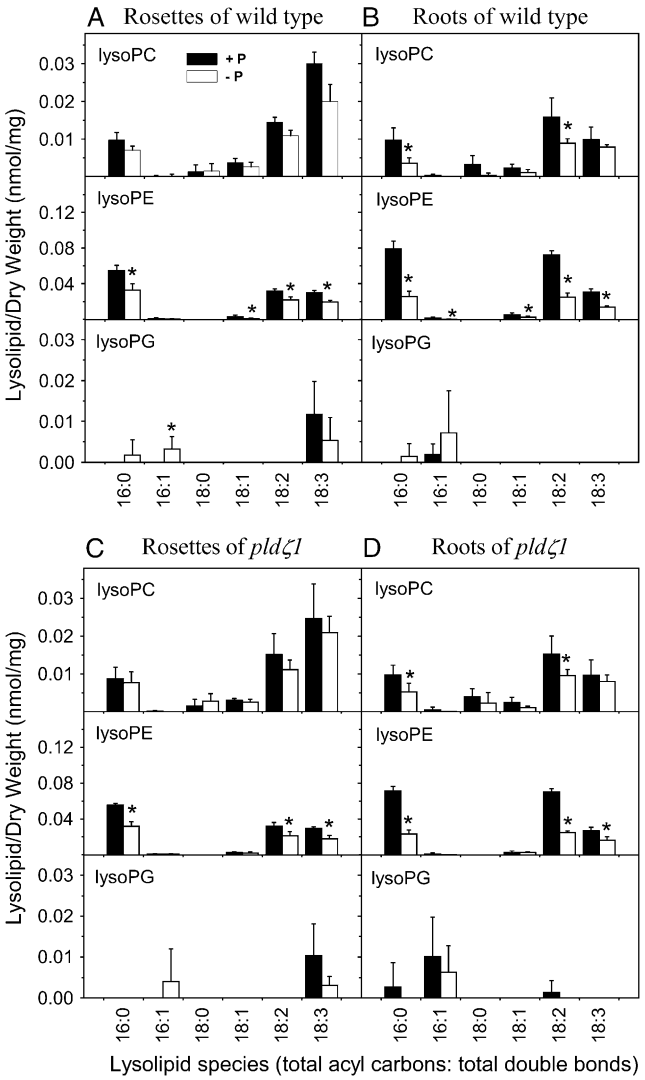

Figure 6. Concentration of individual molecular species in lipid classes of lysoPC, lysoPE, and lysoPG in rosettes and roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under 500-μM and 0-μM phosphate conditions. Three-day-old seedlings were transferred to the indicated plates, containing 500 μM phosphate (+P) or 0 μM phosphate (−P). After 7 additional days, the rosettes and roots were harvested for lipid analysis by ESI-MS/MS. A and B, Rosettes and roots of wild type, respectively. C and D, Rosettes and roots of *pldζ1*, respectively. E and F, Rosettes and roots of *pldζ2*, respectively. G and H, Rosettes and roots of *pldζ1pldζ2*, respectively. Data are expressed as mean ±SD ($n = 5$). *, Lipid species is significantly different in the two phosphate conditions ($P < 0.05$).

and 64%, respectively, in phosphorus-starved roots (Table I). However, lysoPG concentrations in rosettes and roots were similar under both phosphorus conditions. Mutation of *PLDζ1* and *PLDζ2* has no significant effect on lysophospholipid concentrations under normal or phosphorus-starved conditions (Fig. 6).

DISCUSSION

During phosphorus starvation, plants accumulate galactolipid (Härtel et al., 2000), mostly DGDG, to compensate for the loss of phospholipids (Table I). DGDG could be synthesized from DAG that results from phospholipid hydrolysis and/or from de novo synthesis. Our lipid profiling analysis demonstrates that the concentration of galactolipids increased to compensate quantitatively for the decreased concentration of phospholipids in phosphorus-starved rosettes (Table I). The lower concentration of particular molecular species in phospholipids occurred simultaneously with the higher concentration of corresponding species in galactolipids during phosphorus starvation (Fig. 2). In roots, the amount of DAG from phospholipid hydrolysis is more than sufficient to provide the DAG moieties used for galactolipid biosynthesis during phosphorus starvation. These alterations suggest that the DAG moieties derived from phospholipid hydrolysis are used in galactolipid biosynthesis.

In addition, the profiling of lipid species shows that the DAG species of DGDG that increased resemble most closely the PC species that decreased (Fig. 2). This result

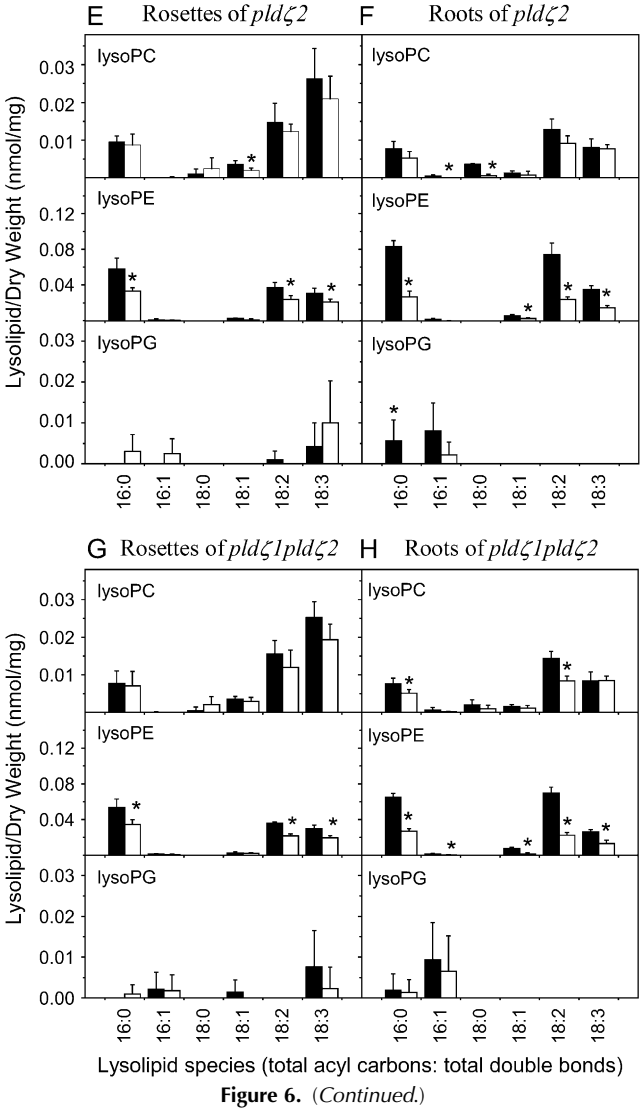

Figure 6. (*Continued.*)

is in agreement with a previous study that suggests that the acyl composition of DGDG is consistent with its synthesis from DAG moieties derived from PC hydrolysis (Nakamura et al., 2005). However, our data also reveal a substantial decrease in certain PE species that resemble DGDGs (Fig. 2). In particular, the decrease in PC of certain species, such as 34:3 in rosettes and roots, does not account quantitatively for the increase in the same DGDG species, but a combination of the loss of both the PE and PC species is more than sufficient to provide the DAG needed for DGDG synthesis (Fig. 2). These results suggest that in addition to PC hydrolysis, PE hydrolysis also contributes to the increase of DGDG under phosphorus-starved conditions.

The release of DAG by phospholipid hydrolysis could be catalyzed by PLD and PLC (Fig. 7). We show here that disruption of *PLDζ*s resulted in a higher PC concentration with a concomitant lower DGDG concentration in phosphorus-starved roots. In addition, PLDζs were drastically induced by phosphorus starvation. Thus, we conclude that PLDζs play a role in hydrolyzing PC in phosphorus-starved roots. PLD activity produces PA that could be hydrolyzed further by purple acid phosphatases to generate DAG and inorganic phosphorus (Fig. 7). DAG may be used directly or further deacylated to produce free fatty acids for lipid reconstruction; DAG from both pathways may contribute to DGDG synthesis in phosphorus-starved roots. As a result, the accumulated DGDG could replace phospholipids and the released inorganic phosphorus could provide invaluable phosphorus for phosphorus-starved plants.

The PLDs in plants are composed of two types, C_2-PLDs, such as PLDα1, α2, α3, β1, β2, γ1, γ2, γ3, δ, and ε, and phox homology/pleckstrin homology-PLDs, such as PLDζ1 and ζ2 (Qin and Wang, 2002). The C_2 domain binds to Ca^{2+} and the activities of PLDα1, β1, γ1, and δ have been demonstrated to be Ca^{2+} dependent (Zhang et al., 2005); the activity of PLDζ1 has been demonstrated to be Ca^{2+} independent (Qin and Wang, 2002). Recently, a Ca^{2+}-independent PLD-like activity was detected in phosphorus-starved oat (*Avena sativa*; Andersson et al., 2005). In light of the gene expression data in which *PLDζ*s' expression increased drastically in phosphorus-starved Arabidopsis roots, this Ca^{2+}-independent PLD activity in oats comes most likely from a PLDζ-like enzyme. In addition, radioactive tracing experiments indicate that, in plasma membrane from phosphorus-deficient oat, [^{14}C]PC degradation markedly increased, and both [^{14}C]PA and [^{14}C]DAG were formed (Andersson et al., 2005). Moreover, PLDζ1 was demonstrated to selectively hydrolyze PC (Qin and Wang, 2002). Taken together, it is most likely that the PLD pathway is utilized to generate DGDG for the replacement of phospholipids, and through this pathway, inorganic phosphorus is released to cope with phosphorus starvation in roots.

The disruption of both PLDζ genes does not abolish the loss of PC under phosphorus-starved conditions (Supplemental Figs. S1–S3), suggesting that the degradation of phospholipids involves other enzyme reactions. These enzymes could be other PLDs and/or PLCs. Besides PLDζs, Arabidopsis has 10 more PLD genes, and their roles in coping with phosphorus starvation remains to be determined. In addition, Arabidopsis has a family of six putative PC-hydrolyzing PLCs (NPLCs), and the expression of *NPLC-4* was drastically induced during phosphorus starvation (Nakamura et al., 2005). NPLC-4 hydrolyzes PC to DAG in vitro, but knockout of *NPLC-4* did not change the levels of PC and DGDG during phosphorus starvation (Nakamura et al., 2005). It is possible that other *NPLC*s compensate for loss of *NPLC-4* function in the knockout plants, so at this point, the role of NPLC-4 or other NPLCs in phospholipid hydrolysis under phosphorus-limited conditions is still unclear.

In addition, 29 purple acid phosphatases have been identified from an Arabidopsis protein database (Li

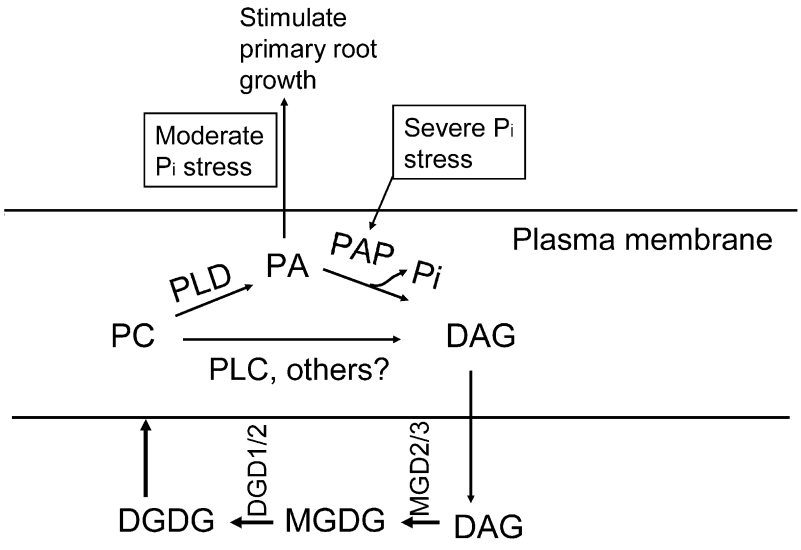

Figure 7. Possible involvement of *PLDζ1/2* in the PC hydrolysis and DGDG accumulation during phosphorus starvation. In this scheme, DAG is shown to be generated by three pathways, the PLD pathway, the PLC pathway, and the de novo pathway. In the PLD pathway, PC is hydrolyzed by PLDζs to PA, and then PA is further hydrolyzed by purple acid phosphatase (PAP) to DAG and inorganic phosphorus under severe phosphorus-limited conditions. Under moderate phosphorus deficiency conditions, *PLDζ1* and *PLDζ2* might function to modulate root growth for better nutritional absorption by increasing PA to stimulate root growth. In the PLC pathway, PC is hypothesized to be hydrolyzed to DAG. DAG can be used as a substrate by MGD2/3 to produce MGDG, from which DGDG is synthesized by DGD1/2.

et al., 2002). Of them, 11 have been verified so far to be induced during phosphorus starvation (Misson et al., 2005). For the synthesis of DGDG, three MGDG synthases (MGD1–3) and two DGDG synthases (DGD1/2) have been identified. During phosphorus starvation, expression of MGD2/3 and DGD1/2 are induced, activating the galactolipid biosynthetic pathway leading to DGDG accumulation (Kelly and Dörmann, 2002). This pathway is crucial for DGDG accumulation because the *dgd1dgd2* double knockout mutants show undetectable accumulation of DGDG even under phosphorus starvation conditions (Kelly et al., 2003). The induction of *PLDζ*s during phosphorus starvation synchronized well with that of purple acid phosphatases, MGD2/3 and DGD1/2, suggesting that these enzymes may function coordinately in the alteration of membrane lipid composition during phosphorus starvation conditions.

These results, together with our previous data (Li et al., 2006), show that *PLDζ1* and *PLDζ2* both function in regulating root growth and lipid turnover during phosphorus shortage. Three phosphorus conditions have been examined so far. Under a standard growth condition (500 μM P_i), disruption of *PLDζ1*, *PLDζ2*, or both did not result in the differences in the concentrations of PC, PA, DGDG, and root growth in contrast to wild type. In addition, the basal level of *PLDζ2* expression was very low. Under a phosphorus-limited condition (25 μM P_i), disruption of both *PLDζ1* and *PLDζ2* results in a lower concentration of PA in roots, a retarded primary root growth, and an unchanged concentrations of PC and DGDG in roots (Li et al., 2006). PA has been implicated in promoting root growth; an inhibitor of PA formation decreased root elongation and lateral root formation (Gomez-Merino et al., 2005). Under the phosphorus-free condition (0 μM P_i), disruption of both *PLDζ1* and *PLDζ2* results in a lower concentration of PC with a corresponding increased concentration of DGDG in roots. However, *PLDζ1* and *PLDζ2* mutants exhibit no alteration in the concentration of PA or primary root elongation (data not shown). These results indicate that, under moderate phosphorus deficiency conditions (25 μM P_i), *PLDζ1* and *PLDζ2* might function to modulate root growth for better nutritional absorption by increasing PA to stimulate root growth (Fig. 7). PA may stimulate root growth by mediating signal transduction, membrane trafficking, and/or cytoskeletal rearrangements (Anthony et al., 2004; Mishra et al., 2006; Wang et al., 2006). The binding of PA to phosphoinositide-dependent protein kinase 1 is involved in root growth (Anthony et al., 2004). During severe phosphorus starvation (0 μM P_i), however, *PLDζ1* and *PLDζ2* might function to regulate lipid turnover between phospholipids and galactolipids for efficient use of internal phosphorus stores (Fig. 7). Thus, PLDζs play signaling and metabolic roles in plant response to different severities of phosphorus deficiency.

In conclusion, we show that phospholipids are quantitatively replaced by galactolipids in phosphorus-starved Arabidopsis rosettes. DGDG is formed from DAG from the ER-extraplastidic and ER-plastidic pools, but not from the prokaryotic, plastidic pathway. In addition, our data show both *PLDζ1* and *PLDζ2* are positive regulators of plant adaptation to phosphorus-limited conditions. Furthermore, the modes of PLD action are different in response to different severities of phosphorus deficiency (Fig. 7). The additive functions of PLDζs constitute a phosphorus-deficiency-response pathway through which PC is hydrolyzed to release inorganic phosphorus and DAG to increase DGDG synthesis in phosphorus-starved plants.

MATERIALS AND METHODS

Mutant Isolation

PLD knockout mutants, *pldζ1* and *pldζ2*, were identified from SALK T-DNA lines (Alonso et al., 2003) of Arabidopsis (*Arabidopsis thaliana*), ecotype Columbia-0, through analysis of the SiGnAL database at http://www.signal.salk.edu/cgi-bin/tdnaexpress. Seeds were obtained from The Ohio State University Arabidopsis Biological Resource Center (ABRC). Homozygous mutant plants were isolated using the T-DNA left-border primer and gene-specific primers (Li et al., 2006). The double mutant, *pldζ1pldζ2*, was generated by crossing the single knockout mutants. All mutants were verified by PCR and reverse transcription-PCR (Li et al., 2006).

Plant Growth and Phosphate Treatments

For plant growth, seeds were surface-sterilized and germinated on modified Murashige and Skoog medium, 1% Suc, 1% agar, 47 mM MES, pH 6.0. Plants were grown vertically under 200 μmol m^{-2} s^{-1} light with 12-h-day/12-h-night cycles, and the day/night temperature was controlled at 23°C/20°C. The modified MS medium contained 1.25 mM KNO_3, 1.5 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.5 mM KH_2PO_4, 75 μM FeEDTA, 50 μM H_3BO_3, 10 μM MnCl, 2 μM $ZnSO_4$, 1.5 μM $CuSO_4$, and 0.075 μM $(NH_4)_6Mo_7O_{24}$. Three-day-old seedlings on normal 500 μM phosphate medium were transferred onto either 500 μM (+P) or 0 μM (−P) phosphate medium and grown for an additional 7 d. Rosettes and roots were harvested from the seedlings grown on both media. Dry weight and lipid composition were determined, and RNA was isolated.

Lipid Profiling

The processes of lipid extraction, lipid analysis, and lipid quantification were performed as described (Welti et al., 2002; Wanjie et al., 2005). Briefly, for each replicate sample, rosettes or roots from 25 seedlings in one petri dish were collected and immersed immediately into 3 mL of hot isopropanol with 0.01% butylated hydroxytoluene at 75°C to inhibit lipolytic activities. The tissues were extracted with chloroform:methanol (2:1, v/v) five times with 30 min of agitation each time. The remaining plant tissues were dried under 105°C oven overnight and then weighed. The weights of these dried, extracted tissues are the dry weights of the samples. Lipid samples were analyzed on an electrospray ionization triple quadrupole mass spectrometer (API 4000; Applied Biosystems). The molecular species of phospholipids and galactolipids were quantified in comparison to the two internal standards using a correction curve determined between standards (Wanjie et al., 2005). Five replicates of each treatment for each phenotype were processed and analyzed. The Q-test for discordant data was performed on the total lipid data, and based on this test the data for one of the five replicates were occasionally discarded (Shoemaker et al., 1974). Paired values were subjected to the Student's *t* test to determine the statistical significance.

Real-Time PCR

Total RNA was isolated using a rapid cTAB method (Stewart and Via, 1993), and RNA was precipitated using 2 M LiCl overnight at 4°C. RNA integrity was checked on 1% (w/v) agarose gel prior to DNase I digestion. Eight micrograms of total RNA were digested with RNase-free DNase I according to the manufacturer's instructions (Ambion). The absence of

genomic DNA contamination was subsequently confirmed by PCR, using RNA without reverse transcription. For reverse transcription, first-strand cDNA was synthesized from 1 μg of total RNA using iScript cDNA synthesis kit (Bio-Rad) in a total reaction volume of 20 μL according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time PCR amplification of a control gene encoding *UBQ10* (At4g05320), and the *UBQ10* gene Ct (threshold cycle) value was 20 $\pm$ 0.5. Only cDNA preparations that yielded similar Ct values for the control genes were used for determination of *PLD* gene expression. The primer sequences for *UBQ10* and 12 *PLD* genes were same as shown in the previous report (Li et al., 2006). PCRs were performed with MyiQ sequence detection system (Bio-Rad) using SYBR Green to monitor dsDNA synthesis. Each reaction contained 7.5 μL of 2$\times$ SYBR Green Master Mix reagent (Bio-Rad), 1.0 ng cDNA, and 200 nM of each gene-specific primer in a final volume of 15 μL. The following standard thermal profile was used for all PCRs: 95°C for 3 min; and 50 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_112553 for PLDζ1 and NM_111436 for PLDζ2.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Effect of phosphorus starvation on lipid species in *pldζ1* knockout tissues.

Supplemental Figure S2. Effect of phosphorus starvation on lipid species in *pldζ2* knockout tissues.

Supplemental Figure S3. Effect of phosphorus starvation on lipid species in *pldζ1pldζ2* double knockout tissues.

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