Double Knockouts of Phospholipases Dζ1 and Dζ2 in Arabidopsis Affect Root Elongation during Phosphate-Limited Growth But Do Not Affect Root Hair Patterning[1]

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Root elongation and root hair formation are important in nutrient absorption. We found that two Arabidopsis (*Arabidopsis thaliana*) phospholipase Ds (PLDs), *PLDζ1* and *PLDζ2*, were involved in root elongation during phosphate limitation. PLDζ1 and PLDζ2 are structurally different from the majority of plant PLDs by having phox and pleckstrin homology domains. Both *PLDζ*s were expressed more in roots than in other tissues. It was reported previously that inducible suppression or inducible overexpression of *PLDζ1* affected root hair patterning. However, gene knockouts of *PLDζ1*, *PLDζ2*, or the double knockout of *PLDζ1* and *PLDζ2* showed no effect on root hair formation. The expression of *PLDζ*s increased in response to phosphate limitation. The elongation of primary roots in *PLDζ1* and *PLDζ2* double knockout mutants was slower than that of wild type and single knockout mutants. The loss of *PLDζ2*, but not *PLDζ1*, led to a decreased accumulation of phosphatidic acid in roots under phosphate-limited conditions. These results indicate that *PLDζ1* and *PLDζ2* play a role in regulating root development in response to nutrient limitation.

Plants encounter heterogeneous, constantly fluctuating environments and need many essential nutrients for survival and growth (Schachtman et al., 1998). Of them, phosphate plays essential roles in metabolic and regulatory reactions. Lack of phosphate in soil has profound effects on plant growth and development (Bieleski and Ferguson, 1983); for example, phosphate deprivation leads to root growth and architecture modifications that enable interactions with an increased volume of soil (Williamson et al., 2001; Ma et al., 2003).

Changes in lipid metabolism are involved in response to phosphate limitation. Upon phosphate starvation, plants increase levels of digalactosyldiacylglycerol in roots; this lipid class is believed to functionally replace acidic phospholipids (Härtel et al., 2000). A nonspecific phospholipase C, which hydrolyzes common membrane phospholipids, such as phosphatidylcholine to diacylglycerol, has been shown to increase in expression and activity during phosphate deprivation (Nakamura et al., 2005). This phospholipase C is hypothesized to provide diacylglycerol as a substrate for the synthesis for digalactosyldiacylglycerol (Nakamura et al., 2005).

Phospholipase D (PLD), which cleaves phospholipids to produce phosphatidic acid (PA) and a free head group such as choline, has been implicated in root hair growth and patterning (Ohashi et al., 2003). Ohashi and coworkers also imply that PLD and its product, PA, play a role in root elongation (Ohashi et al., 2003). In this work, we investigate the role of PLD in plant response to phosphate limitation. The *PLD* gene family has 12 members in Arabidopsis (*Arabidopsis thaliana*), designated as *PLDα1*, *PLDα2*, *PLDα3*, *PLDβ1*, *PLDβ2*, *PLDγ1*, *PLDγ2*, *PLDγ3*, *PLDδ*, *PLDε*, *PLDζ1*, and *PLDζ2* (Zhang et al., 2005). *PLD* previously has been proposed to play a pivotal role in many cellular processes, including signal transduction, membrane trafficking, cytoskeletal rearrangements, and membrane degradation (Wang, 2002). *PLDα1* and *PLDδ* were demonstrated to function in freezing and drought tolerance (Sang et al., 2001; Welti et al., 2002; Li et al., 2004; Zhang et al., 2004). PLDζ1

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and PLDζ2 are distinctively different from other PLDs; they have phox homology (PX) and pleckstrin homology (PH) domains that are also found in mammalian PLDs (Qin and Wang, 2002). PLDζ1 uses phosphatidylcholine selectively as a substrate (Qin and Wang, 2002), and *PLDζ1* gene function is implicated in mediating initiation and maintenance of root hairs (Ohashi et al., 2003). On the other hand, there is no detailed understanding of the function of *PLDζ1* in plant growth and development, particularly under stress conditions, nor have the functions of *PLDζ2* been identified. To understand the in planta roles of these *PLD* gene products, we isolated mutants defective in the expression of *PLDζ1* and *PLDζ2*, as well as double mutants lacking both *PLD*s. Analyses of the mutants revealed that *PLDζ1* and *PLDζ2* play a role in primary root elongation under low-phosphate conditions.

RESULTS

PLDζ1 and *PLDζ2* Are Expressed Highly in Roots

The steady-state levels of *PLDζ1* and *PLDζ2* transcripts in various organs of 6-week-old soil-grown Arabidopsis plants were determined by real-time PCR (Fig. 1, A and B). Both *PLDζ1* and *PLDζ2* transcripts were detectable in inflorescences, flowers, siliques, stems, leaves, and roots, but the expression of *PLDζ1* was about 10-fold higher than that of *PLDζ2*. Both genes were expressed most highly in roots, but relative levels of *PLDζ1* and *PLDζ2* transcripts varied in siliques, flowers, stems, and leaves. Arabidopsis massively parallel signature sequencing (MPSS) data for *PLDζ* expression was obtained from http://mpss.udel.edu/at (Meyers et al., 2004). For *PLDζ1*, the MPSS expression profile was in general agreement with the real-time PCR data; *PLDζ1* expression level in roots was 5 times higher than that in leaves (Fig. 1C). *PLDζ2* was undetectable in normal organs, such as flowers, siliques, leaves, and roots, again consistent with the PCR data, indicating that *PLDζ2* expression was much lower than that of *PLDζ1*. Interestingly, the *PLDζ2* transcript accumulated to a high level in callus, but expression of *PLDζ1* was not detected in this undifferentiated tissue. Data from both the quantitative PCR and MPSS measurements indicate that the expression of *PLDζ1* and *PLDζ2* is differentially regulated.

Mutants Ablate the Expression of One or Both *PLDζ*s

To determine the physiological function of *PLDζ1* and *PLDζ2* in Arabidopsis, we isolated two sets of T-DNA insertional mutants for *PLDζ1* and *PLDζ2*: *pldζ1* and *pldζ2* in the Columbia (Col-0) ecotype, and *pldζ1-ws* and *pldζ2-ws* in the Wassilewskija (Ws) ecotype. *pldζ1* has the T-DNA inserted at the first exon and 231 nucleotides downstream of the ATG codon of *PLDζ1*, and *pldζ1-ws* has an insertion near the end of the first exon (Fig. 2A). The T-DNA insertions in *pldζ2* and *pldζ2-ws* are at the ninth exon (2,416 nucleotides downstream of the ATG codon) and the thirteenth exon, respectively (Fig. 2A). In each case, the presence and location of the T-DNA insertion were verified by junction PCR and sequencing, and homozygous lines were identified by PCR (Fig. 2B). In each line, the F_2 progeny segregated 3:1 for resistance to kanamycin, indicating a single insertion in the genome. The presence of a single insertion in each genome was further demonstrated by DNA gel-blot analyses of *pldζ1-ws* and *pldζ2-ws* (data not shown).

Double mutants in both Col-0 and Ws ecotypes were generated by crossing single *PLDζ1* and *PLDζ2* knockout mutants. Homozygous double mutants, *pldζ1pldζ2*, were identified in the F_3 generation. Loss of *PLDζ1* and *PLDζ2* gene expression in each single or double mutant was verified by reverse transcription

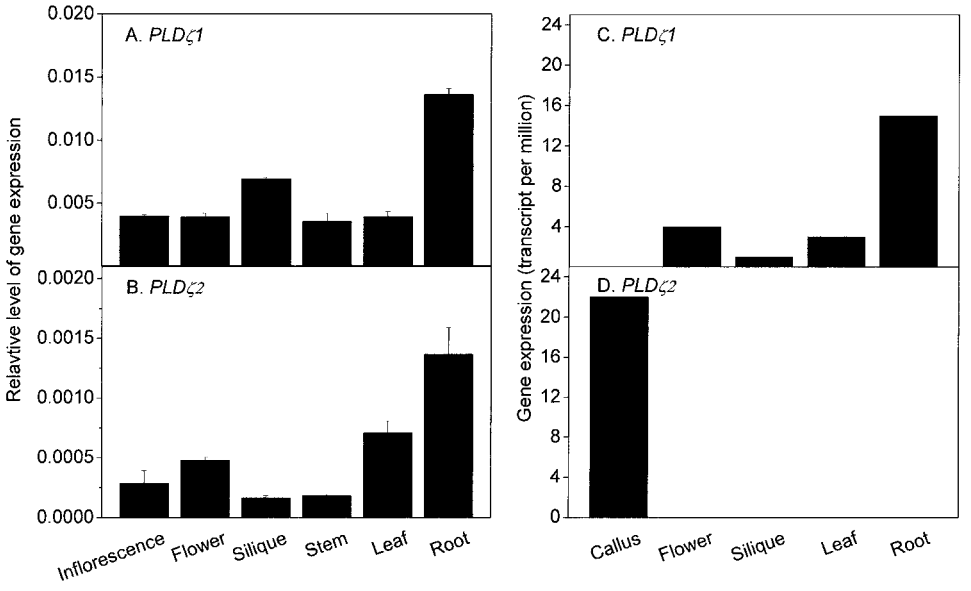

Figure 1. Expression levels of *PLDζ1* and *PLDζ2* in Arabidopsis tissues. Total RNA from tissues of 6-week-old, soil-grown plants were used for real-time PCR. A and B, PCR quantification of *PLDζ1* and *PLDζ2* transcripts, respectively. Transcript levels of each gene were expressed in relation to the *UBQ10* gene. Experiments for A and B were repeated with similar results. The bar represents SE (n = 3). C and D, Expression levels of *PLDζ1* and *PLDζ2* as detected by MPSS. The MPSS data were obtained from the Arabidopsis MPSS Web site (http://mpss.udel.edu/at; Meyers et al., 2004).

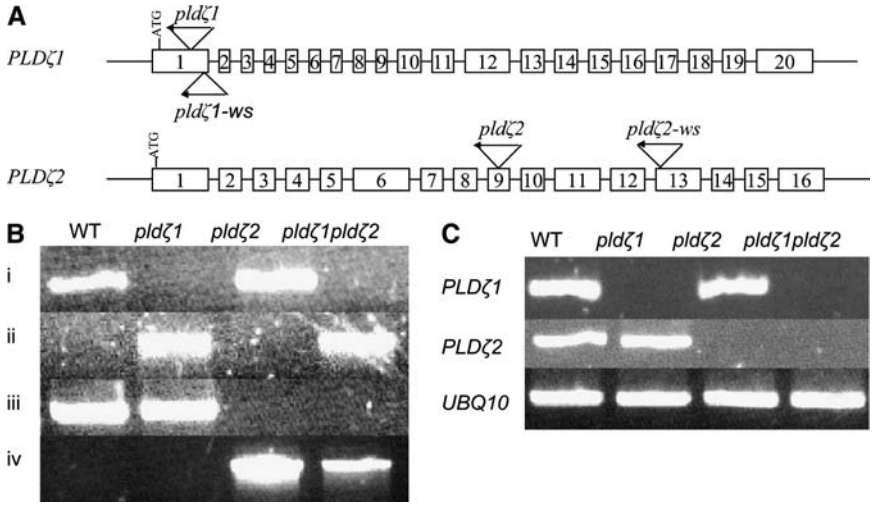

Figure 2. T-DNA insertion positions and loss of gene expression in *PLDζ* single and double mutants. A, *PLDζ1* and *PLDζ2* genes with the sites of the T-DNA insertions in Col-0 ecotype, *pldζ1* and *pldζ2*, and Ws ecotype, *pldζ1-ws* and *pldζ2-ws*, indicated. Exons are shown as white boxes, not drawn to scale. B, PCR confirmation of T-DNA insertions in *PLDζ* genes and of the homozygosity of the mutations. Genomic DNA was isolated from soil-grown plant leaves. i, Products produced using two *PLDζ1*-specific primers, 1a and 1b. The lack of the *PLDζ1* DNA band in *pldζ1* and *pldζ1pldζ2* demonstrates that the mutant is homozygous; the presence of T-DNA made the fragment too large to be amplified under these PCR conditions. The presence of the T-DNA insert is confirmed using a left-border (LB) primer and a *PLDζ1* primer (1a) as shown in ii. iii and iv, Primer combinations 2a and 2b, and 2a and LB, respectively, are used to confirm the T-DNA insertion in *pldζ2* and *pldζ1pldζ2*. C, Verification of the loss of *PLDζ* transcripts in *pldζ1*, *pldζ2*, and *pldζ1pldζ2* by RT-PCR. Total RNA was isolated from leaves of soil-grown plants. RT-PCR analyses were carried out with primers specific for *PLDζ1* and *PLDζ2* genes. Gene-specific primers, 1c and 1d, were used to detect *PLDζ1* mRNA, and primers 2c and 2d were used to detect *PLDζ2* mRNA. *PLDζ1* and *PLDζ2* gene-specific primers failed to amplify a band in *pldζ1* and *pldζ2* mutants. The sequence of the gene-specific primers, 1a, 1b, 1c, 1d, 2a, 2b, 2c, and 2d, is listed in Table I.

(RT)-PCR (Fig. 2C). Arabidopsis plants deficient in *PLDζ1*, *PLDζ2*, or both *PLDζ*s grew and developed normally under regular laboratory growth conditions. These mutants were also compared with wild-type plants in response to hormones abscisic acid and auxin indole-3-acetic acid, and to salt (NaCl) and osmotic (mannitol) stresses, and no apparent difference in plant growth and development was observed between these genotypes.

Loss of *PLDζ1* and *PLDζ2* Reduces Primary Root Elongation, But Promotes Lateral Root Elongation under Low-Phosphate Conditions

Because *PLDζ1* and PA have been implicated as having roles in root function (Ohashi et al., 2003), we investigated the potential effect of *PLDζ* mutations on roots. Seeds were germinated on Murashige and Skoog agar media, and 3-d-old seedlings were transferred to fresh agar media that either contained the standard amount of phosphate (500 μM) or low phosphate (25 μM). Root growth patterns and elongation were examined. In 500 μM phosphate, there was no difference in primary root elongation among wild-type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* plants (Fig. 3A). With limited phosphate, wild type and the single mutants *pldζ1* and *pldζ2* still displayed no difference in primary root growth (Fig. 3B). However, primary root elongation of the double mutant *pldζ1pldζ2* was retarded; the primary root elongation between the fourth and seventh days after transfer was reduced 33% compared to wild type (Fig. 3, B and C). In contrast, lateral roots grew longer in the double mutant than in wild-type plants in low phosphate (Fig. 3, B and D). There was no significant difference in lateral root length among wild type and single mutants at either low or standard phosphate levels (Fig. 3, B and D), and the numbers of lateral roots were also similar among wild type, single, and double mutants (data not shown). The phenotypes of single and double *pldζ* mutants in the Col-0 and Ws backgrounds were the same, so only data obtained from *pldζ1* and *pldζ2* (Col-0 background) are presented hereafter. Taken together, the results suggest that *PLDζ1* and *PLDζ2* promote primary root growth but inhibit lateral root elongation when plants are challenged with low-phosphate levels. The fact that the root alteration occurs only in double, but not in single, mutants indicates that the *PLDζ*s have overlapping functions in primary root elongation.

In addition to alterations in root elongation, a decrease in the dry weight of shoots has been observed in response to phosphate starvation (Williamson et al., 2001). Plants at lower phosphate showed lower rosette growth, but higher root growth, than those grown on standard Murashige and Skoog medium (Fig. 3, E and F). The root-to-rosette growth ratios, as measured by

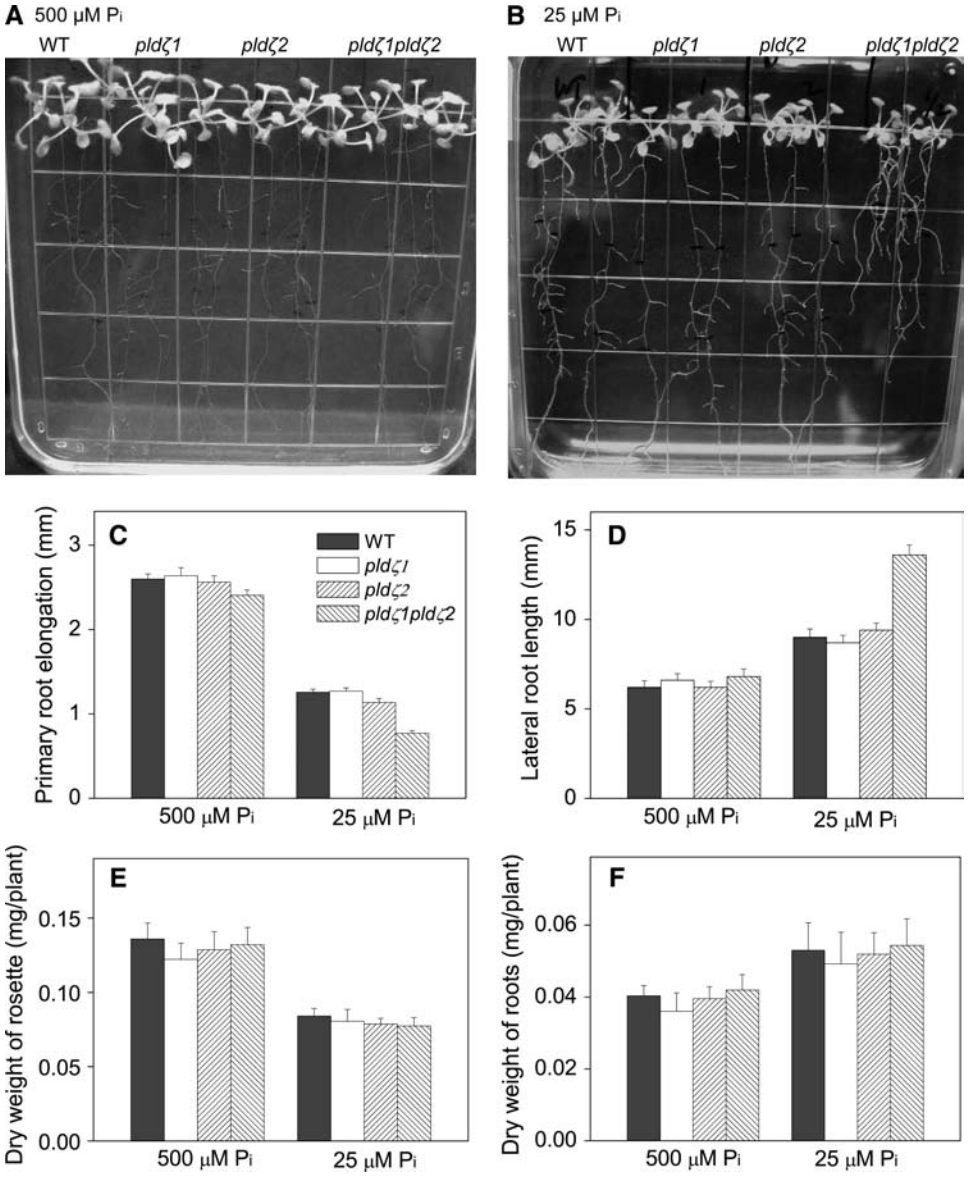

Figure 3. Effect of *PLDζ* mutants on root growth under two phosphate conditions. A and B, Seedlings of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* grown for 7 d in 500 and 25 μM phosphate agar media, respectively. Seedlings were transferred to the indicated media 3 d after germination. C, Elongation of primary roots from the fourth to the seventh day (mm/3 d) after transfer onto 500 or 25 μM phosphate agar plates. D, Elongation of lateral roots from the first to the seventh day (mm/7 d) after transfer. Data for primary roots were obtained from 20 seedlings in five agar culture plates. Data for lateral roots were obtained from 20 seedlings, four lateral roots per seedling for each genotype. Values are means ± SE. E and F, Dry weights of rosettes and roots, respectively, of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under the two phosphate conditions. Seedlings were grown on 500 μM phosphate agar plates for 3 d and then transferred to agar plates containing 500 and 25 μM phosphate for an additional 7 d before harvesting for dry weight measurement. Values are means ± SE ($n = 5$); each was performed on 20 seedlings of each genotype per treatment.

the dry weights, were greater in all genotypes in 25 μM than in 500 μM phosphate (Fig. 3, E and F). However, there was no difference in total rosette and root weights among wild type, single mutants, and double mutants, indicating that the total root volume for the nutrient absorption is not impaired by the loss of *PLDζ*s. The *pldζ1pldζ2* plants had shorter primary roots, but longer lateral roots, in 25 μM phosphate.

Ablation of *PLDζ1* and *PLDζ2* Does Not Affect Root Hair Patterning

Elongation of root hairs is a response to a low-phosphate environment (Williamson et al., 2001), and *PLDζ1* was implicated in root hair initiation and patterning (Ohashi et al., 2003). In Arabidopsis, the root epidermis is composed of two types of cell files, hair cell files and hairless cell files, of which only hair cell files produce root hairs (Dolan et al., 1993). Mutation of *GLABRA2* (*GL2*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) caused hairless cell files in the root epidermis to produce root hairs (Cristina et al., 1996). TTG1 was suggested to regulate *GL2* positively, and GL2 regulates *PLDζ1* negatively by binding to its promoter region (Ohashi et al., 2003). When expression of *PLDζ1* was suppressed by an inducible promoter, root hairs developed from random positions and appeared to be expanded and globular (Ohashi et al., 2003). On the other hand, when *PLDζ1* expression was increased, root hairs were initiated from both types of cell files, and the hairs were frequently branched and swollen (Ohashi et al., 2003). These results led to the hypothesis that *PLDζ1* is involved in both initiation and maintenance of root hair development.

To investigate whether the loss of *PLDζ*s affected root hairs, 3-d-old seedlings in standard agar plates

were transferred to fresh standard and low-phosphate media. The root hair pattern, root hair length, and root hair density were recorded (Fig. 4). Neither single mutants *pldζ1* and *pldζ2* nor double mutant *pldζ1pldζ2* exhibited root hair patterning (Fig. 4A) or root hair growth that differed from wild type under standard phosphate conditions (Fig. 4, B and C). The pattern of root hair initiation and growth was examined from seedling to flowering stages, and no apparent difference was observed between wild type and *PLDζ* mutants under normal growth conditions. At the low-phosphate level, some root hairs of the *pldζ1pldζ2* double mutants appeared slightly globular at the tip (Fig. 4A), but the overall root hair pattern in low-phosphate conditions was not affected by the loss of the *PLDζ*s. The same subtle phenotype was observed in *PLDζ* single and double mutants in the Ws background (data not shown).

Transcripts of *PLDζ*s Increase during Phosphate Deprivation

One potential explanation for the discrepancy between the lack of changes in root hair patterning that we observed in the *PLDζ* knockout lines and the much more dramatic phenotype affecting root hair patterning, observed by Ohashi et al. (2003) when expression of *PLDζ1* was suppressed by an inducible promoter, is that other *PLD*s in Arabidopsis may compensate when *PLDζ1*, *PLDζ2*, or both are permanently lost. Therefore, expression of other *PLD* genes was quantified with real-time PCR in wild type and single and double mutants to determine whether there was any compensation at the transcriptional level. First, we examined whether loss of one *PLDζ* affected the expression of the other *PLDζ* or other *PLD*s. Loss of *PLDζ2* did not affect the expression of *PLDζ1* in roots (Fig. 5B), but the *PLDζ1* transcript level was about 20% higher in *pldζ2* than in wild-type rosettes under either standard or low-phosphate conditions (Fig. 5A). Loss of *PLDζ1* did not affect the expression of *PLDζ2* in roots and rosettes at either phosphate level (Fig. 5, C and D). The level of *PLDζ1* expression was higher than that of *PLDζ2* in young seedlings on agar plates, and this result is consistent with that of plants grown in soil (Figs. 1 and 5). However, PLD*ζ1* expression is highest in roots of 6-week-old, soil-grown plants (Fig. 1), but this is not the case in 10-d-old, agar plate-grown seedlings (Fig. 5). The difference in growth conditions and developmental stage might cause this discrepancy. In addition, rosettes used in Figure 5 contained all parts above roots, in particular the apical meristematic tissues that were not included in the soil-grown plants (Fig. 1). Thus, the difference in tissue types might also account for the discrepancy.

Next, the transcript levels of the other 10 *PLD* genes were monitored in *pldζ1*, *pldζ2*, and *pldζ1pldζ2* mutants to determine whether their expression in roots and rosettes was significantly altered relative to wild-type plants. At the standard phosphate level, the transcript level of *PLDα1* was slightly higher in the single and double knockout mutants than in wild type in both roots and rosettes (Fig. 5E). The *PLDα2* transcript level in double mutants was higher than in wild type in rosettes, but not in roots (Fig. 5F). The transcript levels of the rest, *PLDα3*, *PLDβ1*, *PLDβ2*, *PLDγ1*, *PLDγ2*, *PLDγ3*, *PLDδ*, and *PLDε*, were not significantly different from wild-type levels or the difference was very subtle (Fig. 5, G–N). With low phosphate, the *PLDα1* transcript levels of wild type and mutants were similar in rosettes and roots, and *PLDα2*, *PLDα3*, *PLDβ1*, and *PLDβ2* expression levels were higher than wild-type

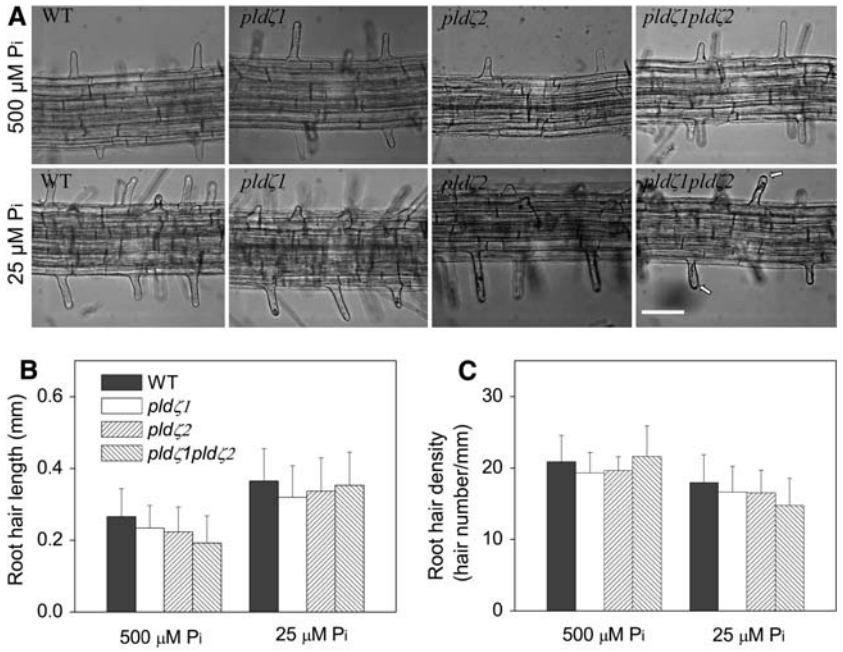

Figure 4. Morphology, length, and density of root hairs from wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under two phosphate conditions. A, Top images are root hairs of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2*, respectively, under standard phosphate (500 μM) conditions. Bottom images are root hairs of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2*, respectively, under low-phosphate (25 μM) conditions. Arrows indicate slightly globular root hair tips. Scale bars represent 100 μm. B and C, Root hair length and root hair density of the *PLDζ* mutant lines were similar to wild type. Plants were grown for 3 d on agar plates containing 500 μM phosphate and then transferred to agar plates containing either 500 or 25 μM phosphate. Twenty-four hours after the transfer, images of the root hairs were captured. Root hairs growing in the region 3 to 4 mm above the root tips were measured (B) and root hair numbers within the region 3 to 4 mm from the root tips were counted (C). Data are based on the measurements of root hairs on 20 seedlings; values are means ± SE.

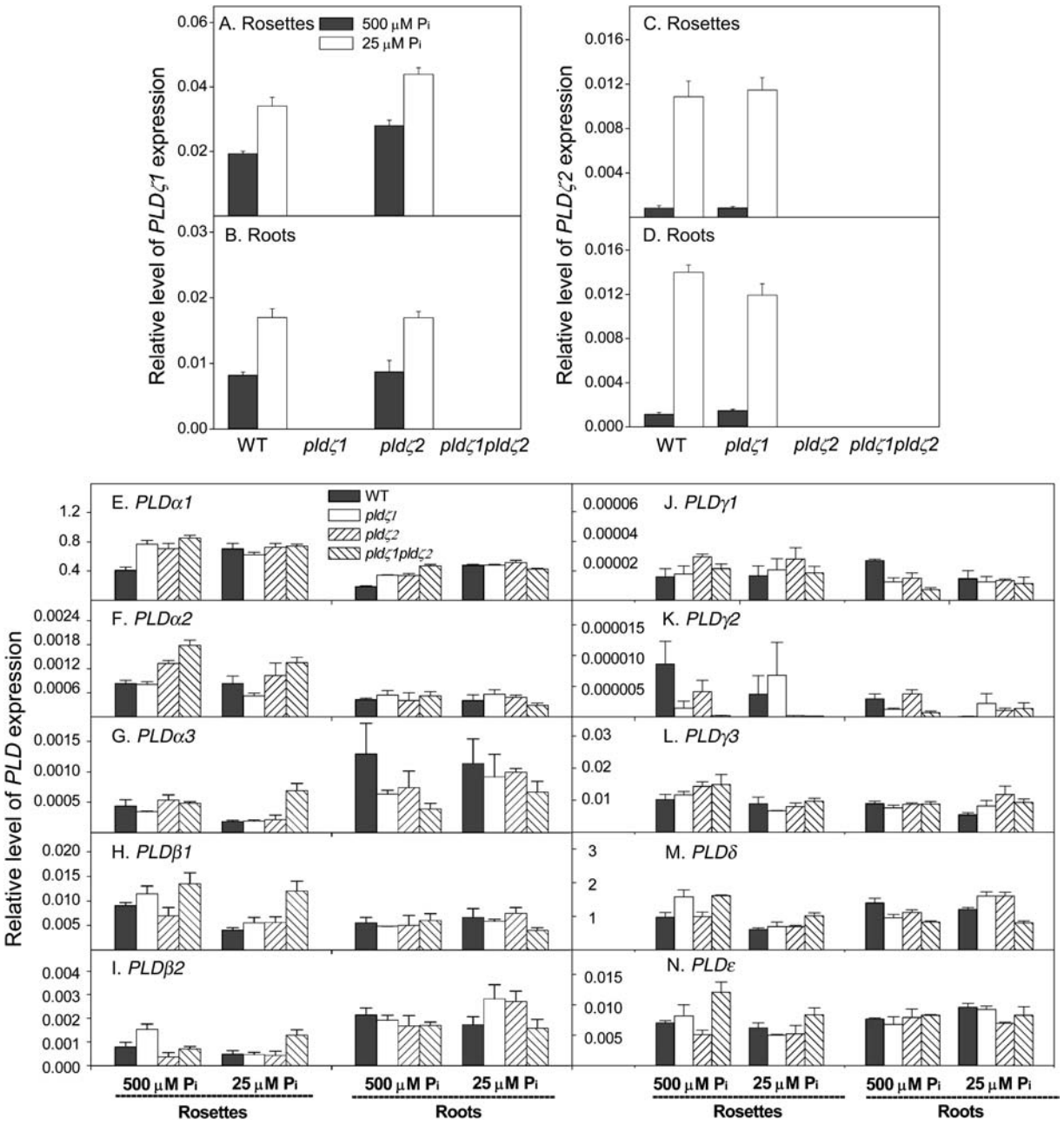

Figure 5. Effect of phosphate levels on the expression of 12 *PLD* genes in Arabidopsis. Primers specific to each *PLD* gene (Table II) were used for real-time PCR to determine the expression levels in wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under the two phosphate conditions. Three-day-old seedlings were transferred to fresh 500 or 25 μM phosphate agar media, and, after 7 d, total RNA was isolated from rosettes and roots. The levels of expression are expressed relative to the expression of *UBQ10*. Values are the mean of three replicates ± SE from one of the two independent experiments that gave similar results.

levels in rosettes, but not in roots (Fig. 5, F–I). However, the other five *PLD*s, *γ1*, *γ2*, *γ3*, *δ*, and *ε*, were expressed in both rosettes and roots, at levels similar to wild type in low-phosphate medium (Fig. 5, J–N).

When the expression of all 12 *PLD*s was compared under standard and phosphate-limited conditions, the expression of *PLDζ2* showed the most prominent increase in low phosphate. The levels of *PLDζ2* transcript in roots and rosettes were more than 10-fold higher under phosphate-limited than under standard conditions (Fig. 5, C and D). The expression of *PLDζ1* increased about 2-fold (Fig. 5, A and B), as did *PLDβ2*, but the expression of other *PLD*s exhibited no major changes in response to low-phosphate conditions (Fig. 5, E–N). The *PLD* expression patterns are consistent with the observation that *PLDζ*s are involved in root elongation during phosphate limitation. The results also suggest that *PLDζ2* plays a more important role than *PLDζ1* in the ability of a plant to cope with phosphate deficiency.

PLDζ2 Is Involved in PA Production in Roots under Phosphate Limitation

To investigate the metabolic consequences resulting from the loss of the single or double *PLDζ*s, we analyzed the level and composition of PA, the direct lipid product of *PLD* activity. Under standard phosphate conditions, there was no significant difference in PA levels in roots in single *pldζ1* or *pldζ2* mutants, but the PA level tended to be lower in the *pldζ1pldζ2* double mutant than in roots of wild-type plants (Fig. 6A). In low-phosphate medium, however, *pldζ2* had only 70% as much PA as in wild-type plants, but *pldζ1* had a similar level of PA compared to wild type (Fig. 6A). The PA level of *pldζ1pldζ2* was the same as that of *pldζ2*, implying that the decrease in the double mutant resulted from the loss of *pldζ2*. This difference was also observed when the data were expressed as mol% of total phospholipids. This was because the absolute amounts of total phospholipids were not significantly different between wild type and mutants in roots under growth conditions, and also because the amount of PA accounted for only a small fraction of total phospholipids. Thus, the decrease in PA in the double mutant was not due to an alteration in content of other phospholipids. These results show that *PLDζ2*, but not *PLDζ1*, plays a major role in PA production during phosphate deprivation, and that PLDζ1 does not compensate for PLDζ2 function in *pldζ2*. This metabolic activity of PLDζ2 is consistent with gene expression data showing that transcript levels of *PLDζ2* were induced most by phosphate deficiency (Fig. 5, C and D). All PA molecular species were lower in roots of *pldζ2* and the double mutant than in wild type or *pldζ1* (Fig. 6B), suggesting that *PLDζ2* produces a variety of PA molecular species.

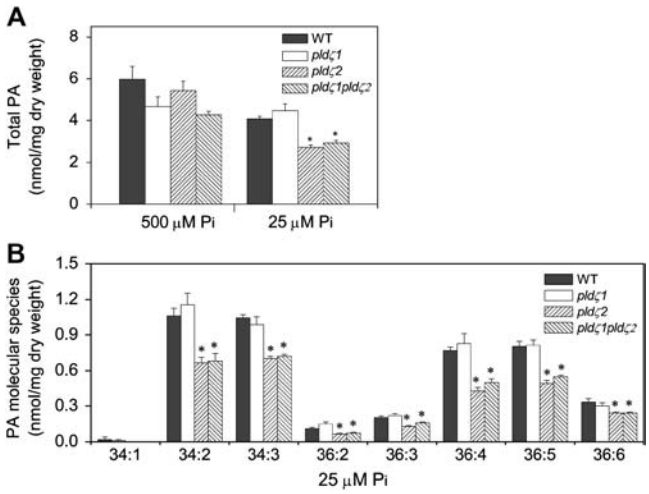

Figure 6. Total PA and PA molecular species in wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2*. PA was analyzed in roots of wild type and *PLDζ* mutant lines. The content of total PA and PA molecular species was determined by electrospray ionization/tandem mass spectrometry. A, Total PA content in roots in standard and low-phosphate conditions is shown. B, PA molecular species in roots of wild type, *pldζ1*, *pldζ2*, and *pldζ1pldζ2* under low-phosphate conditions. Values are means ± SE ($n = 5$). The stars indicate that mutant and wild-type values are significantly different ($P < 0.05$).

DISCUSSION

Phosphorus is an essential element for plant growth, development, and reproduction. It plays an important role not only in regulation of various enzymes but also as a constituent of components such as membrane phospholipids and nucleic acids (Schachtman et al., 1998). Plants have developed distinct systems to cope with phosphate deficiency. When a plant grows under low-phosphate conditions, highly integrated systems in plants are activated (Lynch, 1995; Vance et al., 2003) to increase the assimilation of free inorganic phosphate. One alteration is a morphological modification of root architecture upon phosphate limitation, which presumably facilitates phosphate uptake by enlargement of absorptive root surface areas (Lynch, 1995; Vance et al., 2003). We show here that *PLDζ1* and *PLDζ2* are involved in the root growth modification and that these two *PLD* gene products have both overlapping and unique functions.

These data show that the loss of *PLDζ1* and *PLDζ2* decreases primary root growth and that loss of *PLDζ2* results in lower PA content under a low-phosphate condition. Root elongation is regulated by many factors. A recent study indicates that PA interacts with AtPDK1, stimulates a protein kinase cascade, and promotes root apical growth and initiation (Anthony et al., 2004). However, the in vivo source of PA is unclear. This is because, in addition to PLD, signaling PA can be generated from other routes, such as diacylglycerol kinase-mediated phosphorylation of diacylglycerol. The decrease in PA formation in the *PLDζ2* mutant raises the possibility that PA generated by PLDζs may promote root elongation and that AtPDK1 might be the target for PA under low-phosphate conditions. However, there is a discrepancy between the metabolic alteration and morphological phenotype: Decreased apical growth in primary roots occurred only in plants lacking both *PLDζ*s, indicating that either *PLDζ1* or *PLDζ2* can function to promote apical growth. However, *PLDζ2* is sufficient to produce PA in the absence of *PLDζ1*, while *PLDζ1* does not seem to produce PA in the absence of *PLDζ2*. The production of PA by the *PLDζ2* gene product occurs in concert with the induction of *PLDζ2* during phosphate deprivation. These results suggest that PLDζ2-derived PA is not the only mediator of root elongation and that *PLDζ1* plays a significant role, uncorrelated with the production of measurable levels of PA.

Interestingly, the consequence of loss of *PLDζ*s is different in the elongation of primary roots from that of lateral roots. Recently, it was reported that phosphate transport double mutants, *plt1;1* and *plt1;4*, exhibited lower plant growth, including that of primary roots (Shin et al., 2004). However, the *plt1;1plt1;4*

Table I. *Primers used for identification and verification of T-DNA insertions in Figure 2*

Gene	Primer Names and Sequences
PLDζ1	1a 5′-CGCTACTTTCAGATGCAGCCTGAGCAATT-3′ 1b 5′-ACTGATATGGACTGCTGTCTTCCCA-3′ 1c 5′-CGGCGATATTAGCCCTGTACTCTT-3′ 1d 5′-GCCATTCTTTAACCTGTTCCTGCT-3′ 1e 5′-CAAGATGAAGGCTGAGAAGGCTAGAGAGA-3′ 1f 5′-ATCAGAGAAATGGCATCTGAGCAGTTGAT-3′
PLDζ2	2a 5′-CAGGTGAAGGAACACAAC-3′ 2b 5′-CAGACCATTGGCTAACAC-3′ 2c 5′-AGACAGGAGAAAGTACCCGCGAAT-3′ 2d 5′-TGTGGTGGTGAGGCATCAACAATG-3′ 2e 5′-GGATTTTTGATATGCAAGGATCAGTTGTG-3′ 2f 5′-GTCTCGTGAACCTAGTAAGCTCCTATCGT-3′
T-DNA left border	JL202 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′ LB 5′-GCGTGGACCGCTTGCTGCAACT-3′

double mutant showed faster lateral root growth than wild type when high levels of phosphate were supplied to phosphate-starved roots (Shin et al., 2004). These results support the notion that the response to low phosphate could be different in primary as compared to lateral roots. Our data indicate that *PLDζ*s are involved in these different responses to low phosphate between primary roots and lateral roots; they promote primary root elongation but inhibit lateral root elongation under low-phosphate conditions (Fig. 3, B and D).

The lack of dramatic differences in root hair growth and patterning in the single and double *PLDζ* mutants is perplexing because a previous study showed that *PLDζ1* was involved in root hair initiation and patterning. GL2 is a key component of a regulatory circuit mediating root hair patterning in Arabidopsis (Masucci et al., 1996). *PLDζ1* is a direct target of GL2 as GL2 binds to its promoter region of *PLDζ1* (Ohashi et al., 2003). Inducible expression of *PLDζ1* promoted ectopic root hair initiation, whereas inducible suppression inhibited root hair initiation (Ohashi et al., 2003). These results suggest that GL2 regulates root hair development through modulation of *PLDζ1* and lead to the prediction that loss of *PLDζ1* would affect root hair patterning. However, our data show that knockout of

Table II. *Primers used for real-time PCR in Figure 1, A and B, and Figure 5*

Gene		Primer Sequences	Product Size
PLDα1	At3g15730	Forward 5′-TCTCTGCTTTGCTGCTGTTGTAGC-3′ Reverse 5′-CACAAAGCTACATTCTCTCACCACGTC-3′	150 bp
PLDα2	At1g52570	Forward 5′-GAGGATCCGCGAGATTATCTGACA-3′ Reverse 5′-TGTGCTCGGATATAGTCAGTGTCC-3′	116 bp
PLDα3	At5g25370	Forward 5′-ATGGTTAATGCAACGGCAGACGAG-3′ Reverse 5′-CCCGGTAAATCGTCATTTCGAGGA-3′	77 bp
PLDβ1	At2g42010	Forward 5′-AGGAAGGATCCAGTGGCACACTTT-3′ Reverse 5′-TCTCCCACCCTCTTATTATCAACTCTCA-3′	82 bp
PLDβ2	At4g00240	Forward 5′-CTGCTAGATGATTGCTTTGTGGAGC-3′ Reverse 5′-CTCCGACACTTCCTCACTCCT-3′	108 bp
PLDγ1	At4g11850	Forward 5′-ACTTTTTCTGTCTTGGAACCAGAG-3′ Reverse 5′-GCATTTGCATTTGCGTTTGGCTGA-3′	97 bp
PLDγ2	At4g11830	Forward 5′-TGCTCCCTTTGCGTCTAGGTTTCT-3′ Reverse 5′-TCTATTCCAGCAGCAACACCACGA-3′	78 bp
PLDγ3	At4g11840	Forward 5′-GGTTTCCATAACACCCTTGTTGGT-3′ Reverse 5′-TTCTCACCATCCACTTTATGATTCCT-3′	73 bp
PLDδ	At4g35790	Forward 5′-TGGGCGCATACCAACCTAATCA-3′ Reverse 5′-TGGCTCCACAAACTCATCTCCA-3′	128 bp
PLDε	At1g55180	Forward 5′-GGGACACCGAGATTGCAATAGGTT-3′ Reverse 5′-AGCGATAACCGGTAAGCTTGGA-3′	79 bp
PLDζ1	At3g16785	Forward 5′-TGGATGGCAACCGCAAAGACAA-3′ Reverse 5′-ATCGTTGTGTGTCCCAGCTTCT-3′	137 bp
PLDζ2	At3g05630	Forward 5′-TTTGAGGACGGTCCAATTGCCA-3′ Reverse 5′-ACAACACCGATCTCAGAGTCTCGT-3′	143 bp
UBQ10	At4g05320	Forward 5′-CACACTCCACTTGGTCTTGCGT-3′ Reverse 5′-TGGTCTTTCCGGTGAGAGTCTTCA-3′	71 bp

PLDζ1 and *PLDζ2* in both the Col-0 and Ws backgrounds had no effect on root hair patterning and only a very subtle phenotype of slightly globular root tips under low-phosphate conditions. One potential cause of the apparent discrepancy is the difference in experimental approaches: The previous studies were done with an inducible *PLDζ1* suppression/overexpression system, not gene knockouts. It might be possible that the antisense suppression was not specific to *PLDζ1*, and other PLDs might also be suppressed. Another potential explanation is that the threshold of *PLDζ1* expression and protein levels might be important in root hair initiation and growth because the antisense suppression might not completely ablate the proteins and expression of PLDζ1, and the alterations on root hairs might result from the suboptimal PLDζ1 levels in the cell. Taken together, our results indicate that more than one PLD is involved in root hair initiation and patterning, that *PLDζ1* and *PLDζ2* may have overlapping functions, and that *PLDζ*s promote primary root growth when plants are challenged with low-phosphate levels.

MATERIALS AND METHODS

Mutant Isolation

Two Arabidopsis (*Arabidopsis thaliana*) ecotypes, Col-0 and Ws, were employed. Potential *pldζ1* (Salk_083090) and *pldζ2* (Salk_094369) mutant lines, which are in Col-0, were identified from the Salk T-DNA lines (Alonso et al., 2003) through the analysis of the SiGnAL database (http://www.signal.salk.edu/cgi-bin/tdnaexpress), and 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: Primers 1a and 1b were used to identify *pldζ1* and primers 2a and 2b to identify *pldζ2* (Table I). *pldζ1-ws* and *pldζ2-ws* insertion mutants are in the Ws background, and mutants were identified by screening pools of T-DNA insertion lines according to the protocol of the Arabidopsis Gene Knockout Research Facility at the University of Wisconsin (Sussman et al., 2000). PCR was carried out on pooled genomic DNA with a T-DNA left-border primer, JL202, and gene-specific primers, 1e and 1f for the *PLDζ1* gene and 2e and 2f for the *PLDζ2* gene. Pools were deconvoluted and individual lines identified by PCR. The number of single T-DNA inserts in *pldζ1-ws* and *pldζ2-ws* knockout lines was assessed by Southern-blot analysis.

To generate *pldζ1pldζ2* double mutants in both ecotypes, homozygous single mutants in *PLDζ1* were crossed with mutants in *PLDζ2*. F_1 plants were self-pollinated and individual F_2 plants were screened for homozygous double mutants. Because *PLDζ1* and *PLDζ2* genes are both located on chromosome III, a large population of plants was screened to obtain homozygous double mutants. Of 200 individual F_2 plants screened by PCR, eight plants were found to be homozygous for *PLDζ1* and heterozygous for *PLDζ2*. These plants were self-pollinated and double homozygous *pldζ1pldζ2* plants were obtained in the F_3 generation.

Plant Growth and Phosphate Treatments

For mutant isolation and routine plant growth, seeds were sown in soil and treated at 4°C for 2 d. Plants were grown in growth chambers under a 12-h-day/12-h-night at 23°C/18°C cycle. For phosphate treatments, seeds were surface sterilized and germinated on modified Murashige and Skoog medium under a 12-h-day/12-h-night at 22°C cycle. The modified 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 $(NH_4)_6Mo7O24$. Three-day-old seedlings on standard 500 μM phosphate medium were transferred onto either 500 or 25 μM phosphate medium and grown for an additional 7 d. The seedlings grown on both media were used to determine dry weight, lipid composition, and RNA isolation.

Measurements of Roots and Root Hairs

Seedlings were grown for 3 d on agar plates containing 500 μM phosphate and then transferred to agar plates containing either 500 or 25 μM phosphate agar plates. The replacement of the root tip was measured between the fourth and seventh days after transfer, and the data are based on 20 seedlings per genotype. The lateral root length was measured at the seventh day after transferring, and data are based on four lateral roots per seedling and 20 seedlings per genotype. Forty-eight hours after transfer, images of root hair patterns in the region 3 to 4 mm above the root tips were captured using a MagnaFire (Optronics) system. Root hair length was determined by measuring 100 root hairs located 10 mm from the root tip in 12 individual plants from each line.

Lipid Profiling

The processes of lipid extraction, analysis, and quantification were performed as described (Wanjie et al., 2005). Briefly, rosettes or roots from 25 seedlings were collected at the sampling time and immersed immediately into 3 mL hot isopropanol with 0.01% butylated hydroxytoluene at 75°C to inhibit lipolytic activities. The tissues were extracted with chloroform-methanol five times with 30 min agitation each time. The remaining plant tissues were dried in an oven at 105°C 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). The molecular species of PA were quantified in comparison to the two internal standards using a correction curve determined between standards. Five replicates of each treatment for each phenotype were processed and analyzed. The Q-test for discordant data was done on the replicates of the total lipid. Paired values were subjected to Student's *t* test to determine statistical significance.

Real-Time PCR

Total RNA was isolated using a rapid cetyl-trimethyl-ammonium bromide method (Stewart and Via, 1993), and RNA was precipitated using 2 M LiCl overnight under 4°C. RNA integrity was checked by 1% (w/v) agarose gel electrophoresis 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 RT. For RT, first-strand cDNA was synthesized from 1 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad) in a total reaction volume of 20 μL according to the manufacturer's instructions. The efficiency of the cDNA synthesis was assessed by real-time PCR amplification of a control gene encoding UBQ10 (At4g05320), and the *UBQ10* gene threshold cycle (C_t) value was 20 ± 0.5. Only cDNA preparations that yielded similar C_t values for the control genes were used for determination of *PLD* gene expression. The level of *PLD* expression was normalized to that of *UBQ10* by subtracting the C_t value of *UBQ10* from the C_t value of *PLD* genes. PCR was performed with a MyiQ sequence detection system (Bio-Rad) using SYBR Green to monitor double-stranded DNA synthesis. Each reaction contained 7.5 μL 2× 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.

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