Phospholipase DZ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in *Arabidopsis* roots

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Low phosphate (Pi) availability is one of the major constraints for plant productivity in natural and agricultural ecosystems. Plants have evolved a myriad of developmental and biochemical mechanisms to increase internal Pi uptake and utilization efficiency. One important biochemical pathway leading to an increase in internal Pi availability is the hydrolysis of phospholipids. Hydrolyzed phospholipids are replaced by nonphosphorus lipids such as galactolipids and sulfolipids, which help to maintain the functionality and structure of membrane systems. Here we report that a member of the *Arabidopsis* phospholipase D gene family (*PLDZ2*) is gradually induced upon Pi starvation in both shoots and roots. From lipid content analysis we show that an *Arabidopsis pldz2* mutant is defective in the hydrolysis of phospholipids and has a reduced capacity to accumulate galactolipids under limiting Pi conditions. Morphological analysis of the *pldz2* root system shows a premature change in root architecture in response to Pi starvation. These results show that PLDZ2 is involved in the eukaryotic galactolipid biosynthesis pathway, specifically in hydrolyzing phosphatidylcholine and phosphatidylethanolamine to produce diacylglycerol for digalactosyldiacylglycerol synthesis and free Pi to sustain other Pi-requiring processes.

phosphate starvation | phospholipids | root architecture | sulfolipids

Phosphate (Pi) influences virtually all developmental and biochemical processes in plants. Pi is not only a constituent of key cell molecules such as ATP, nucleic acids, and phospholipids, but it is also a pivotal metabolic regulator of many processes including energy transfer, protein activation, and carbon and nitrogen metabolism. However, Pi availability can be one of the major constraints for plant growth in both natural and agricultural ecosystems because of its low mobility and high absorption capacity in the soil. As a response to this limitation, plants have evolved a range of developmental, biochemical, and symbiotic adaptive strategies to cope with low Pi availability (1, 2). In *Arabidopsis*, a general, 3-fold strategy to cope with low Pi availability has been described. (*i*) The release and uptake of Pi from external sources that are not readily available for plant uptake. This mechanism includes the transcriptional activation of high-affinity Pi transporters and the excretion of RNases, acid phosphatases, and organic acids (3–5). (*ii*) Changes in the architecture of the root system that reflect alterations in cell length, root meristem activity, root hair elongation, and an increased number of lateral roots (6, 7). These changes presumably increase the exploratory capacity of the root and the absorptive surface area. (*iii*) Optimization of Pi utilization due to a wide range of metabolic alterations, and the mobilization of Pi from internal reserves by the hydrolysis of nucleic acids, proteins, and the recycling of Pi from membrane phospholipids.

During Pi deprivation, the total content of diverse phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol, which constitute ≈30% of total Pi storage molecules in the plant, decrease. A concomitant increase in the synthesis of nonphosphorus lipids such as the galactolipid digalactosyldiacylglycerol (DGDG) and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG) occurs presumably to preserve membrane integrity (8–10). Although DGDG is typically found in plastid membranes, during Pi deprivation it has been proposed that DGDG replaces phospholipids in the plasma membrane. Therefore, when Pi availability is limited, the hydrolysis of phospholipids can be considered both a key biochemical pathway to liberate Pi from an important storage source and the substrate for galactolipid biosynthesis.

Galactolipids are synthesized by transfer of galactose from UDP-galactose to diacylglycerol (DAG) by monogalactosyldiacylglycerol synthase (MGD) and DGDG synthase (11). Under Pi-limiting conditions it has been shown that the expression of genes encoding MGDG synthase and DGDG synthase significantly increases and correlates with the accumulation of DGDG (12, 13). It has been proposed that part of the DAG needed for the increased biosynthesis of nonphosphorus lipids is obtained by hydrolysis of phospholipids, although this has not been demonstrated experimentally. Two alternative pathways for the production of DAG from phospholipids have been proposed: direct hydrolysis by phospholipase C and an indirect two-step reaction involving phospholipase D (PLD) that yields phosphatidic acid (PA) and PA phosphatase (PAP), which releases DAG and Pi. The involvement of these two pathways suggests the existence of transcriptionally induced phospholipases that may degrade phospholipids in both leaves and roots. Recently, Nakamura *et al*. (14) reported an increase in PC-hydrolyzing activity under Pi-deprivation conditions and identified a novel phospholipase C (NPC4), encoded at locus *At3g03530*, which shows significantly increased transcription under Pi deprivation. However, lipid composition analysis of an *Arabidopsis npc4* T-DNA insertion mutant did not show a significant change in total DGDG content or accumulation of PC when compared with a WT control. The authors propose an alternative Pi-recycling pathway that produces DAG for DGDG biosynthesis to replace hydrolyzed membrane phospholipids (14).

In *Arabidopsis* the *PLD* gene family is composed of 12 functional members (15, 16). Global gene expression analysis using microarray technology identified an *Arabidopsis* PLD gene as a candidate to encode a PLD that participates in the hydrolysis of phospholipids to provide DAG for galactolipid synthesis under Pi stress; the array showed that *PLDZ2* (locus *At3g05630*) is highly induced in both shoots and roots under Pi starvation.

In this study we show that the expression of *PLDZ2* is specifically regulated by Pi availability and that PLDZ2 actively participates in

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; GUS, β-glucuronidase; Pi, phosphate; DAG, diacylglycerol; dag, day after germination; PAP, PA phosphatase; PLD, phospholipase D; MGD, monogalactosyldiacylglycerol synthase.

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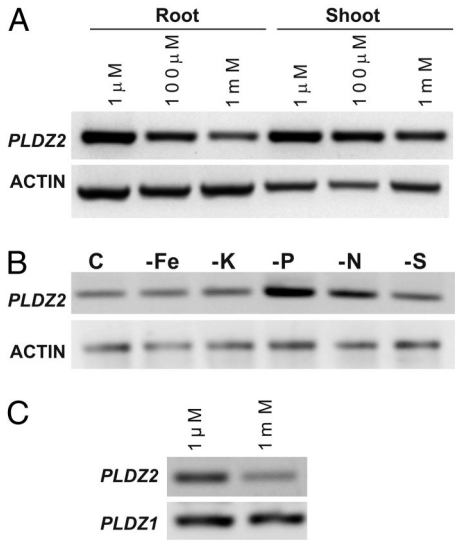

Fig. 1. Molecular analysis of *PLDZ2* expression by RT-PCR. (*A*) Shoot and root expression patterns under diverse Pi stress assays. (*B*) Specificity of transcriptional induction from diverse nutrient conditions assayed. (*C*) Induced and constitutive expression of *PLDZ2* and *PLDZ1*, respectively, as a response to Pi stress.

the hydrolysis of PC and PE to release Pi from phospholipids and provide DAG for the biosynthesis of DGDG.

Results

Data from microarray analysis showed that *PLDZ2* is induced in *Arabidopsis* after exposure to Pi-limiting conditions (17). To confirm these results we carried out semiquantitative RT-PCR analysis of RNA extracted from roots and shoots of seedlings grown in 0.1× MS media containing 1, 0.1, and 0.01 mM Pi. A basal level of *PLDZ2* mRNA was observed in seedlings grown in media containing 1 mM Pi, which increased in both roots and shoots of plants exposed to lower Pi concentrations (Fig. 1*A*). The level of *PLDZ2* transcripts was ≈1.5- and 4-fold higher, respectively, in 0.1 and 0.01 mM Pi than in 1 mM Pi. The induction of *PLDZ2* steady-state mRNA by Pi deprivation was confirmed by Northern blot analysis (see Fig. 7 and *Supporting Text*, which are published as supporting information on the PNAS web site). To determine whether the expression of *PLDZ2* is regulated by nutritional stress in general or specifically by Pi starvation, RT-PCR analysis of RNA extracted from seedlings grown in media lacking Fe, K, S, N, or P was carried out. As can be seen in Fig. 1*B*, the level of *PLDZ2* transcripts of plants grown in media lacking Fe, K, and S was comparable to that present in control seedlings grown in complete media. However, a reproducible increase in *PLDZ2* transcript levels was observed for seedlings grown in media deprived of Pi and N, suggesting that PLDZ2 is specifically regulated by starvation for specific nutrients rather than as a general response to nutritional stress.

PLDZ2 is a member of a subclass of the PLD gene family composed of two closely related genes (*PLDZ1* and *PLDZ2*) that are characterized by the presence of PX (phox homology) and PH (pleckstrin homology) domains and are thereby more closely related to the two mammalian PLDs than to the other *Arabidopsis* PLD gene family members (15, 16). To determine whether both genes or only *PLDZ2* are induced by Pi starvation, we carried out RT-PCR analysis of the expression of *PLDZ1* and *PLDZ2* under adequate (1 mM) or limiting (1 μM) Pi conditions. It was observed that, whereas the transcript level of *PLDZ2* clearly increases upon Pi deprivation, no significant changes were detected for *PLDZ1* (Fig. 1*C*).

To explore in more detail the tissue-specific pattern of expression of the *PLDZ2* gene, a transcriptional gene fusion between the

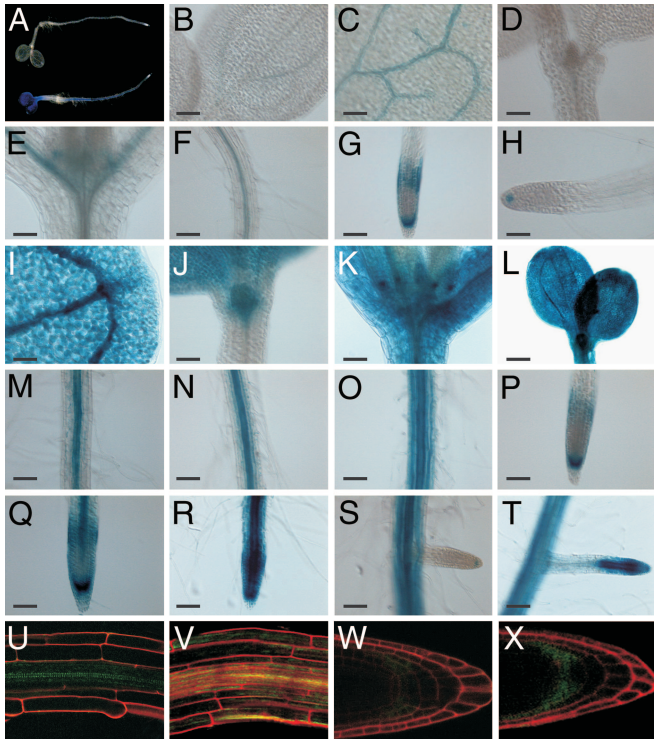

Fig. 2. The effect of Pi availability on the temporal and spatial expression patterns of *PLDZ2*. Transgenic plants with the transcriptional fusion *pPLDZ2:GFP:UidA* were analyzed by Nomarsky optics (*A–T*) and confocal microscopy (*U–X*). (*A*) The differential expression pattern in 2-dag transgenic seedlings grown under high (1 mM) and low (1 μM) Pi availability conditions. The expression pattern under adequate Pi growth conditions is shown for cotyledons of 2-dag (*B*) and 10-dag (*C*) seedlings, shoot apical meristem of 2-dag (*D*) and 10-dag (*E*) seedlings, and differentiation zone (*F*), primary root meristem (*G*), and lateral root (*H*) of a 10-dag seedling. By contrast, the effect of Pi starvation on the expression pattern is observed in cotyledons of 2-dag (*I*) and 10-dag (*L*) seedlings; shoot apical meristem of 2-dag (*J*) and 10-dag (*K*) seedlings; differentiation zone of 2-dag (*M*), 4-dag (*N*) and 10-dag (*O*) seedlings; primary root meristem of 2-dag (*P*) and 10-dag (*Q*) seedlings; and emerging (*S*) and mature (*T*) lateral roots of 10-dag seedlings. (*U–X*) Differential expression pattern of the GFP reporter gene in the differentiation zone of 10-dag seedlings (*U* and *V*) and root meristem of 2-dag seedlings (*W* and *X*).

PLDZ2 promoter and coding sequences of the β-glucuronidase (GUS) and GFP reporter genes was generated and used to produce transgenic *Arabidopsis* plants. Histochemical GUS analysis and confocal GFP analysis of seedlings grown under adequate Pi conditions showed that up to 4 days after germination (dag) *PLDZ2* expression was detected only in the meristematic region of the primary root. At later stages (10 dag), low levels of expression were detected in the vascular tissues of the cotyledons and leaves (Fig. 2*C*), the vascular tissues near the shoot meristem (Fig. 2*E*), and the central cylinder of the primary root (Fig. 2 *F* and *U*). In emerging lateral roots, expression of *PLDZ2* was observed in a well defined zone of the root tip that at later stages (Fig. 2*H*) became quite similar to that observed in the primary root (data not shown). In seedlings germinated in Pi-limiting conditions, a high level of expression was detected as early as 1 dag (Fig. 2 *I* and *L*) in all tissues of cotyledons and leaves and was maintained until the end of the experiment (14 dag; data not shown); in the shoot meristematic region, *PLDZ2* expression was detected in the shoot meristematic dome at 2–4 dag (Fig. 2*J*), and at later stages all tissues, including the hypocotyls (Fig. 2*K*), showed high levels of expression including. In primary roots, GUS activity was clearly detected in the central cylinder 2 dag (Fig. 2*M*) and became increasingly more intense until all tissues of the root showed high levels of *PLDZ2* expression (Fig.

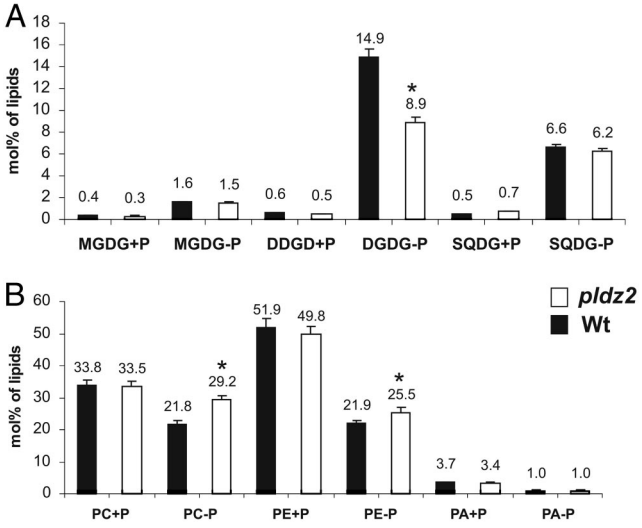

Fig. 3. Total amounts of diverse glycerolipids in WT and *pldz2* roots as a function of Pi availability. Shown is a comparison of relative amounts of lipids between WT (black bars) and *pldz2* (white bars) roots of 20-dag seedlings. (*A*) Major nonphosphorus lipids DGDG, MGDG, and SQDG. (*B*) Relative amounts of PC, PE, and PA. Values are means ± SE ($n = 3$). Asterisks indicate major changes between WT and *pldz2*. Values were analyzed by Duncan's method for statistical validation.

2 *N*, *O*, and *V*). The primary root of Pi-starved seedlings showed a pattern of expression similar to that observed in plants grown under standard Pi conditions up to 2 dag (Fig. 2*P*); levels of expression later increased until all tissues of the root tip showed high levels of expression (Fig. 2 *Q* and *R*). In emerging lateral roots of Pi-deprived plants, a pattern of expression similar to that observed in plants under normal Pi was initially observed (see Fig. 8, which is published as supporting information on the PNAS web site); however, 2 days after emergence the entire lateral root showed *PLDZ2* expression (Fig. 2*T*). Confocal microscopy of the root tip 2 dag of both Pi-sufficient and Pi-deficient plants showed that *PLDZ2* is specifically expressed in a region below the quiescent center, comprising most of the initial cells and the first layer of columella cells (Fig. 2 *W* and *X*). The expression pattern of *PLDZ2*, determined by using reporter genes, closely resembled that observed for the endogenous *PLDZ2* transcript using whole-mount *in situ* hybridization (Fig. 8).

Galactolipids and Sulfolipids Accumulate in the Roots of Pi-Deprived *Arabidopsis*. Several studies have demonstrated that Pi availability alters the lipid composition in diverse plant tissues (12, 13). In *Arabidopsis* leaves it has been reported that major changes include a decrease in phospholipids and a concomitant accumulation of the galactolipid DGDG (8). A detailed analysis of the effect of Pi starvation on lipid composition in roots has not been reported to our knowledge. To determine whether Pi-limiting conditions promote lipid turnover from phospholipids to nonphosphorus lipids in roots, we quantified the lipid composition in WT Col-0 seedlings grown in either sufficient (1 mM) or limiting (1 μM) Pi conditions (Fig. 3, black bars). A remarkable increase in the relative amount of DGDG from 0.6 to 14.9 mol% was observed in the roots of seedlings grown under low-Pi growth conditions. Similarly, the relative amount of sulfolipids (SQDG), another class of nonphosphorus lipids, increases from 0.5 to 6.6 mol% (Fig. 3*A*). In contrast, a significant reduction of the major membrane phospholipids is observed. Relative amounts of PC and PE decrease from 33.8 to 21.8 mol% and from 51.9% to 21.9%, respectively (Fig. 3*B*). Lipid composition of green tissues was also analyzed (see Table 1, which is published as supporting information on the PNAS web site), confirming the changes in lipid levels reported by other groups (8). These results suggest the existence of metabolic pathways that are activated under Pi starvation in *Arabidopsis* roots to recycle Pi from phospholipids (PC and PE) and to replace them with DGDG and SQDG.

The Role of PLDZ2 in Lipid Turnover Under Pi Stress. Because we observed that the expression of *PLDZ2* significantly increases during Pi starvation, it is possible that this enzyme participates in the hydrolysis of phospholipids to recycle Pi and to produce DAG for the synthesis of galactolipids. To investigate the possible role of PLDZ2 in this process, we quantified the lipid composition in tissues from the homozygous insertion mutant SALK_094369 (denominated hereafter as *pldz2*) and compared them with those of the WT. The T-DNA insertion in *pldz2* is located in the ninth exon, 2,416 nucleotides downstream of the ATG codon (see Fig. 9*A*, which is published as supporting information on the PNAS web site). The presence and location of the T-DNA insert were verified by PCR-based genotyping, and homozygous lines were also identified by PCR (Fig. 9*B*). The F_2 progeny of *pldz2* segregated 3:1 for resistance to kanamycin, indicating a single T-DNA insertion. RT-PCR analysis of RNA from WT and *pldz2* showed the absence of *PLDZ2* transcripts in the mutant line, corroborating the disruption of *PLDZ2* (Fig. 9*C*). The presence of a single T-DNA insertion in *pldz2* was further demonstrated by DNA gel blot analysis (data not shown).

Under Pi-sufficient conditions, the content of phospholipids, galactolipids, and sulfolipids in *pldz2* roots did not show any significant change in comparison with those of the WT (Fig. 3 *A* and *B*). In roots of Pi-deprived WT plants a 4-, 20-, and 11-fold increase in MGDG, DGDG, and SQDG was detected when compared with Pi-sufficient plants. Interestingly, the accumulation of DGDG in the roots of Pi-deprived *pldz2* plants was reduced 40% when compared with the accumulation observed in the WT. No significant difference between the accumulation of MGDG and SQDG was detected between *pldz2* and WT plants subjected to Pi starvation (Fig. 3*A*). Moreover, an accumulation of PC and PE in Pi-starved *pldz2* roots was observed when compared with the roots of WT plants grown under the same conditions (Fig. 3*B*). Surprisingly, no changes in the content of PA, the direct product of PLDZ2 action, were observed in the mutant under any growth condition (Fig. 3*B*). The lack of observed changes in PA composition between the control and the mutant plants indicates that PA is probably a transitory molecule that may be rapidly degraded to DAG and Pi by a PAP. No changes in other potential substrates of PLD such as phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol were detected between *pldz2* and WT in either sufficient or limiting conditions (see Fig. 10, which is published as supporting information on the PNAS web site). Similar results in terms of a reduction in DGDG accumulation and an increase in PC and PE content were observed for *PLDZ2* silenced lines using interferent RNA technology (see Fig. 11, which is published as supporting information on the PNAS web site).

To determine a more detailed specificity for the PLDZ2 substrates, we analyzed the relative amounts of the different molecular species of DGDG, PC, and PE in *pldz2* and WT roots under Pi starvation (Fig. 4 *A–C*). The results show a reduction of 34:2, 34:3, 36:4, 36:5, and 36:6 DGDG molecular species in *pldz2* roots when compared with the WT (Fig. 4*A*) and an accumulation of 34:2, 34:3, 36:5, and 36:6 for PC molecular species (Fig. 4*B*) and of 34:2, 34:3, and 36:6 for PE molecular species when compared with those of the WT in each case (Fig. 4*C*). The finding that the same type of molecular species of DGDG that is reduced in *pldz2* correspond to those of PC and PE that accumulate in the mutant suggest a role for PLDZ2 in hydrolyzing phospholipids to provide DAG for DGDG synthesis under Pi stress.

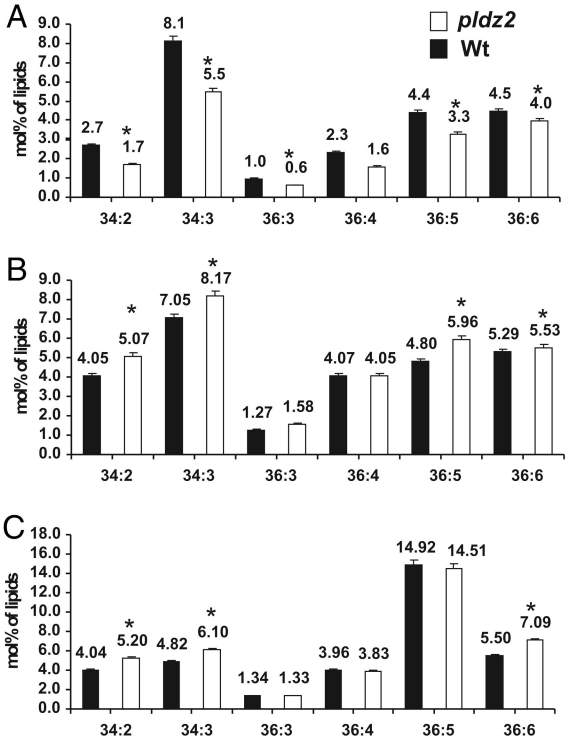

Fig. 4. Alterations in DGDG, PC, and PE molecular species *pldz2* roots under Pi-deprivation conditions. Shown is a comparison between WT (black bars) and *pldz2* (white bars) amounts of the diverse molecular species of DGDG (*A*), PC (*B*), and PE (*C*). Values are means ± SE ($n = 3$). Asterisks indicate major changes between WT and *pldz2* and/or treatments. Values were analyzed by Duncan's method for statistical validation.

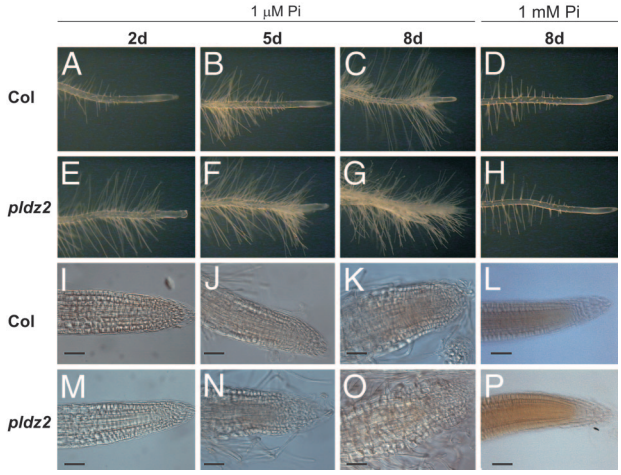

Fig. 5. Morphological phenotype of *pldz2* revealed by Pi stress. Morphological alterations in the roots of WT Col-0 and *pldz2* seedlings grown under Pi starvation (1 μM) conditions are shown, with the exception of *D*, *H*, *L*, and *P*, which are 8-dag seedlings grown in sufficient (1 mM) Pi conditions. (*A–C* and *E–G*) changes in primary root zones of 2-, 5-, and 8-dag seedlings (*A–C*) compared with the altered growth pattern of mutant seedlings of the same age (*E–G*) in three major zones of the primary root. (*I*, *J*, and *M–O*) Alterations caused by Pi starvation on primary root meristem organization and proliferation of 2-, 5-, and 8-dag WT seedlings (*I* and *J*) compared with the precocious meristem exhaustion process in mutant seedlings of 2-, 5-, and 8-dag (*M–O*).

Mutations in *PLDZ2* Accelerate Primary Root Meristem Exhaustion During Pi Deprivation. One of the most conspicuous responses of the *Arabidopsis* root system to Pi deprivation is the formation of an abundance of long root hairs and the arrest of primary root growth caused by the exhaustion of the root meristem (1, 2, 6, 7). Our results show that mutations in *PLDZ2* decrease phospholipid hydrolysis and DGDG accumulation. This observation can be explained if we assume that PA produced by PLDZ2 is processed by a PAP to yield DAG, which is used by MGDG synthase as the first step to produce DGDG and Pi. To determine whether the release of Pi from an important storage source such as phospholipids affects the *Arabidopsis* root architecture response to Pi-limiting conditions, we examined the root tip of seedlings grown under Pi-sufficient and Pi-limiting conditions. No differences in primary root length, root hair elongation, or root meristem structure were observed between the WT and the *pldz2* seedlings when grown in media containing 1 mM Pi (Fig. 5 *D*, *H*, *L*, and *P*). However, under Pi-limiting conditions, WT *Arabidopsis* seedlings showed a typical increase in root hair length starting at 5 dag, with long root hairs forming closer to the root meristem at 8 dag (Fig. 5 *B* and *C*). In contrast, in *pldz2* long root hairs appeared close to the primary root tip as early as 2 dag and completely covered the root meristematic zone 8 dag (Fig. 5 *E–G*). Closer examination of the meristematic zone showed that 2 dag in both Pi-sufficient and Pi-deficient treatments mutant and WT seedlings maintained a normal root meristem morphology (Fig. 5 *I* and *M*); however, 5 dag the root tip of mutant Pi-deprived seedlings showed clear morphological alterations and by 8 dag showed complete disorganization that was not matched in the WT even 8 dag (Fig. 5 *J*, *K*, *N*, and *O*). This early loss of meristem structure was reflected in the primary root growth of *pldz2* that, under Pi-limiting conditions, attained a maximal length of 1.44 ± 0.26 cm 10 dag whereas the WT reached a length of 1.75 ± 0.28 cm in the same period (data not shown).

Discussion

Plants as sessile organisms have evolved adaptive mechanisms to cope with adverse environmental conditions. One of the most common environmental constraints that plants encounter in natural and agricultural ecosystems is low Pi availability. To cope with limiting Pi conditions plants activate diverse mechanisms that allow them to increase Pi availability, uptake, and utilization (1–7). One of the biochemical mechanisms that plants activate to recycle Pi from storage compounds during Pi deprivation is the hydrolysis of phospholipids (8–10).

The replacement of phospholipids by nonphosphorus lipids was first reported in bacteria. Cyanobacteria mutants with impaired SQDG synthesis are severely affected in growth only under Pi-limiting conditions, suggesting that sulfolipids can replace phospholipids to maintain membrane functionality and integrity (18). In plants it has been shown that not only sulfolipids but also galactolipids increase after Pi deprivation (8, 19). Previous analysis of Pi-deprived WT *Arabidopsis* seedlings showed an increase in the relative amount of SQDG and a concomitant decrease in phosphatidylglycerol (19). Also, it has been shown that Pi stress increases the relative amounts of galactolipids, mainly DGDG, whereas the relative amounts of PC and PE greatly decrease (8). These studies suggest the existence of metabolic pathways that are activated under Pi starvation, which perform two major reactions: (*i*) the hydrolysis of phospholipids to generate DAG, which necessarily involves the enzymatic action of a phospholipase, and (*ii*) the use of DAG for the synthesis of galactolipids and sulfolipids. Although the participation and induction of genes involved in SQDG (*SQD1* and *SQD2*), DGDG (*DGD1* and *DGD2*), and MGDG (*MGD2* and *MGD3*) biosynthesis in both roots and leaves as a response to Pi starvation have been experimentally demonstrated (12, 13), no direct evidence has been reported to our knowledge on the existence of specific phospholipases that hydrolyze phospholipids to provide DAG for the synthesis of nonphosphorus lipids.

Two potential pathways for the hydrolysis of phospholipids to release Pi from its storage source and to produce DAG, the substrate for galactolipid synthesis, have been proposed: a direct pathway involving phospholipase C and an indirect pathway involving PLD and PAP activities (14, 19, 20). Microarray analysis showed that two genes encoding phospholipases are strongly induced during Pi deprivation in *Arabidopsis*: *NPC4*, a member of the *PLC* family, and *PLDZ2*. Our results show that *PLDZ2* is indeed induced >4-fold when grown under Pi-limiting conditions (17). Analysis of the tissue-specific expression of a gene fusion of the *PLDZ2* promoter to the GUS and GFP reporter genes showed that the expression of the *PLDZ2* gene is activated gradually in all tissues of *Arabidopsis* seedlings in response to Pi deprivation. Although PLD has been shown to participate in signaling pathways that involve the production of PA (21–23), the pattern of expression (strong induction in all tissues) observed for *PLDZ2* during Pi deprivation is more suggestive of a metabolic role for PLDZ2 rather than participation in a signaling pathway. The low levels of expression of *PLDZ2* in the vascular tissues and the root tip of *Arabidopsis* plants grown under Pi-sufficient conditions could suggest an additional signaling role for PLDZ2. However, the lack of a visible phenotype for the *pldz2* mutant in Pi-sufficient conditions, even in adult plants (data not shown), makes this possibility unlikely unless there is redundancy with *PLDZ1*, the other member of the *PLDZ* clad.

To determine the potential role of PLDZ2 in the hydrolysis of phospholipids during Pi starvation, we analyzed the phospholipids and nonphosphorus lipids in WT and *pldz2* plants. As previously reported, we found that in green tissues of the *Arabidopsis* WT plants there is a 2.3- and 4.8-fold increase in the accumulation of DGDG and SQDG, respectively, after Pi starvation, concomitant with a 50% reduction in PC and PE. A similar accumulation of DGDG and SQDG was observed in green tissues of *pldz2*, strongly suggesting that PLDZ2 does not participate in the synthesis and accumulation of galactolipids in photosynthetic tissues. In contrast, the dramatic increase in DGDG observed in the WT roots was reduced by ≈40% in the roots of *pldz2*, whereas the reduction in the content of PC and PE in WT roots was not observed in this mutant. These results show that PLDZ2 plays an important role in the hydrolysis of phospholipids to generate DAG, which in turn may be used by the plant as substrate of DGDG synthase in roots but not in green tissues.

These phenomena may be explained if we consider that DGDG in leaves and roots is produced via different pathways. Härtel *et al.* (24) demonstrated that in *Arabidopsis* leaves, independent of Pi status, most DGDG synthesis takes place in plastids and depends on the activity of the DGDG synthase DGD1. However, the induction of DGDG synthesis by Pi starvation in the roots of *dgd1* is identical to that of WT, suggesting that an alternative pathway produces the newly formed galactolipids. Moreover, it has been shown that enrichment of DGDG in Pi-deprived *Arabidopsis* roots occurs in nonphotosynthetic and extraplastidic membranes by the action of DGDG synthase DGD2 (24–26).

In many plant species, such as *Arabidopsis*, two different pathways of galactolipid biosynthesis have been described: the prokaryotic pathway localized in plastids and the eukaryotic pathway localized in the endoplasmic reticle. Most of the accumulated MGDG in *Arabidopsis* is synthesized via the prokaryotic pathway, whereas the predominant fraction of DGDG is derived from the eukaryotic pathway (26). This phenomenon correlates with the presence of the MGDG synthase MGD1, which is responsible for the synthesis of the bulk of MGDG that accumulates in the inner envelope of the chloroplast and the localization of MGD2 and MGD3 (with preferences for eukaryotic lipid precursors) and DGD1 and DGD2 in the outer chloroplast membranes. Genes involved in DGDG synthesis (MGD2, MGD3, and DGD2) are strongly induced after Pi deprivation, whereas the content of MGDG and MDG1 remain unchanged. In plant cells SQDG synthase is also located in the inner membrane of the plastids, and

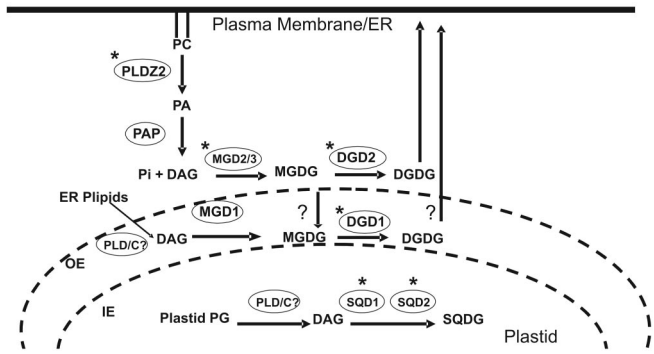

Fig. 6. Schematic pathway of lipid turnover induced by Pi stress. PLDZ2 degrades plasma membrane and/or endoplasmic reticle phospholipids PC and PE to produce PA, which in turn may be converted to DAG and Pi by a proposed PAP. DAG generated via PLDZ2 is the precursor for the synthesis of DGDG in a DGD1-independent pathway that is highly regulated by Pi availability. Newly produced DGDG is transported to extraplastidic root cell membranes.

SQDG biosynthesis takes place exclusively in the plastids (8). The finding that *pldz2* has altered DGDG content in roots, but not in green tissues, and accumulates normal levels of MGDG and SQDG strongly suggests that PLDZ2 provides DAG for DGDG synthesis from hydrolysis of phospholipids from extraplastidial membranes through the eukaryotic pathway. Because the plastidic pathway yields only 34:3 MGDG with one 18- and one 16-carbon fatty acid (10, 19), the accumulation of both 34:3 and 36:3 DGDG in *pldz2* (with two 18-carbon fatty acids) supports the notion that PLDZ2 most probably only participates in the eukaryotic DGDG biosynthesis pathway. Therefore, we propose that PLDZ2 forms part of a Pi starvation-induced DGDG pathway, in which PLDZ2 hydrolyses endoplasmic reticle or plasma membrane phospholipids to produce DAG that in turn is used by the MGDs and DGDs located in the outer membrane of the plastid (Fig. 6). In agreement with this hypothesis, it has been recently demonstrated that DGDG accumulation is highly up-regulated by Pi starvation in the plasma membrane fraction of oat roots and that PLD-type and PAP activity are the major lipase activities induced by Pi starvation in nonplastidic root membranes (20, 27).

It has been reported that some of the characteristics of the root system response to Pi deprivation are an increase in the length of root hairs, an arrest in the growth of the primary root due to a loss of meristematic activity, and an abundant formation of lateral roots close to the root tip (6, 7). The finding that root hair elongation, meristem degradation, and the formation of lateral roots close the root tip occur at least 2 days earlier in *pldz2* than in the WT under Pi-limiting conditions suggests that PLDZ2, in addition to providing DAG for the synthesis of galactolipids, plays a role in providing Pi from phospholipids to sustain other Pi-requiring cellular processes.

Materials and Methods

Plant Material and Growth Conditions. WT and *pldz2* are *Arabidopsis* ecotype Col-0. Mutant and WT seeds were surface-sterilized with 95% (vol/vol) ethanol for 5 min and 20% (vol/vol) bleach for 7 min. Seeds were germinated and grown on agar plates with limiting (1 μM) or sufficient (1 mM) NaH_2PO_4 in a 0.1× MS medium as described (28).

Expression Analysis. For RT-PCR analysis, total RNA from different tissues and treatments of 11-day-old WT and mutant plants was extracted by using the Concert Plant RNA Reagent from Invitrogen according to the manufacturer's instructions. For reverse-transcriptase analysis, 100 ng of each RNA was used in a 12.5-μl reaction mixture of the SuperScript One-Step RT-PCR with Platinum Taq Kit from Invitrogen. For *PLDZ2* transcript detection we

used the following specific primers: *PLDZ2*-RTF (5′-CGACGACGGTTTGGGGAG-3′) and *PLDZ2*-RTR (5′-CCCAAGCTGTGTAGCCAC-3′). For *PLDZ1* transcript detection we used the following specific primers: *PLDZ1*-RTF (5′-GGATCAACGCGACGGGAG-3′) and *PLDZ1*-RTR (5′-CCTGCAAACCTCCCGTG-3′). The amplification reactions were performed under the following conditions: 52°C for 30 min, 94°C for 2 min, 35 cycles of 94°C for 20 sec, 55°C for 30 sec, and a final extension step at 72°C for 1.0 min. We followed the same protocol and conditions for control reactions using specific primers for the *Arabidopsis ACTIN2* gene AC-RTF (5′-GTACAACCGGTATTGTGCTGGAT-3′) and AC-RTR (5′-GCTTGGTGCAAGTGCTGTGATTTC-3′). For Northern blot analysis of *PLDZ2*, the 361-bp PCR fragment was used as specific probe. Northern blot analysis was performed as described (29).

Expression Pattern Analysis by GUS/GFP Promoter Fusion. For the *PLDZ2* promoter fusion, a 1.3-kb region upstream of the ORF of *PLDZ2* was amplified from genomic *Arabidopsis* DNA by PCR using the following primers*:* FWDAttB1p*PLDZ2* (5′-GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-TAA-gga-gta-cca-ttc-aca-aac-3′) and REVAttB2p *PLDZ2* (5′-GGGG-AC-CAC-TTT-GTA-CAA-GAA-AGC-TGG-GTA-ccg-tcg-aca-tcg-att-aac-3′). The PCR product was cloned in the vector pDONR221 and transferred to pKGWFS7 (30) by recombination using Gateway BP and LR Clonase enzyme mixes, respectively, to produce the *pPLDZ2:GUS-GFP* gene fusion, which was then introduced into the *Arabidopsis* genome via the *Agrobacterium*-mediated transformation system using the protocol reported by Martinez-Trujillo *et al.* (31). GUS histochemical analysis was carried out as reported (6). The stained seedlings were cleared by the method described by Malamy and Benfey (32), and a representative plant, from 12 independent transformation events, was chosen and photographed by using Nomarski optics in a Leica DMR microscope. For GFP visualization, plants were transferred to a solution of 10 μg/ml propidium iodide for 3 min. Imaging was monitored in a LSM510 Meta Zeiss confocal microscope, with argon blue laser at 488 nm and helium-neon green laser for GFP and propidium iodide excitation. Images obtained from the same root zone were merged.

Mutant Genotyping and Molecular Characterization. The Salk_094369 mutant line was identified by using the SiGnAL Database from The Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress), and seeds were kindly provided by the Ohio State University *Arabidopsis* Biological Resources Center. The mutant population was genotyped, and homozygous mutant plants were isolated by using the T-DNA left-border primer and gene-specific primers as recommended on the SiGnAL web site.

Lipid Extraction and Analysis. Lipids from leaf and root tissues were isolated (details are available on request). Lipid extracts were observed and isolated by using the TLC technique as described (24). For quantitative analysis, individual lipids were isolated from TLC plates and used to prepare fatty acid methyl esters. The methyl esters were quantified by GC-MS by using myristic acid as an internal standard. Duplicates of each experiment were also analyzed by electrospray ionization-tandem MS technology in the Kansas Lipidomics Research Center (Manhattan, KS) as described (33). Duncan's method was used to determine whether the differences described in each experiment, between *pldz2-pldz2*, *pldz2*-WT, *Silz2- Silz2*, *Silz2*-WT, and WT-WT plant tissues and treatments were statistically significant with a 95% confidence coefficient.

Analysis of Mutant and WT Root Architecture. The root system of WT and *pldz2* seedlings was analyzed with a SZH10 stereomicroscope (Olympus). All root zones were clearly visible by using different objectives. Primary root length was determined for each root by using a plastic ruler. For primary root meristem analysis, roots from mutant and WT seedlings were cleared as described and analyzed by Nomarski optics microscopy.

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1. Lynch, J. (1995) *Plant Physiol.* **109,** 7–13.
2. Raghothama, K. G. (1999) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 665–693.
3. Baldwin, J. C., Karthikeyan, A. S. & Raghothama, K. G. (2002) *Plant Physiol.* **125,** 728–737.
4. Bariola, P. A., Howard, C. J., Taylor, C. B., Verburg, M. T., Jaglan, V. D. & Green, P. J. (1994) *Plant J.* **6,** 673–685.
5. Rubio, V., Francisco, L., Roberto, S., Ana, C., Martin, J. I., Antonio, L. & Paz-Ares, J. (2001) *Gene Dev.* **15,** 2122–2133.
6. Sánchez-Calderón, L., López-Bucio, J., Chacón-López, A., Cruz-Ramírez, A., Nieto-Jacobo, F., Dubrovsky, J. G. & Herrera-Estrella, L. (2005) *Plant Cell Physiol*. **6,** 174–184.
7. López-Bucio, J., Cruz-Ramírez, A. & Herrera-Estrella, L. (2003) *Curr. Opin. Plant Biol*. **6,** 280–287.
8. Essigmann, B., Güller, S., Narang, R.-A., Linke, D. & Benning, C. (1988) *Proc. Natl. Acad. Sci. USA* **95,** 1950–1955.
9. Andersson, M. X., Stridh, M. H., Larson, K. E., Liljenberg, C. & Sandelius, A. S. (2003) *FEBS Lett.* **537,** 128–132.
10. Kelly, A. A., Froehlich, J. E. & Dörmann, P. (2003) *Plant Cell* **15,** 2694–2706.
11. Dörmann, P. & Benning, C. (2002) *Trends Plant Sci.* **7,** 112–118.
12. Kelly, A. A. & Dörmann, P. (2002) *J. Biol. Chem.* **277,** 1166–1173.
13. Awai, K., Marechal, E., Block, M. A., Brun, D., Masuda, T., Shimada, H., Takamiya, K., Ohta, H. & Joyard, J. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 10960–10965.
14. Nakamura, Y., Awai, K., Masuda, T., Yoshioka, Y., Takamiya, K. & Ohta, H. (2005) *J. Biol. Chem.* **280,** 7469–7476.
15. Qin, C. & Wang, X. (2002) *Plant Physiol.* **128,** 1057–1068.
16. Eliáš, M., Potocký, M., Cvrčková1, F. & Žárskýl, V. (2002) *BMC Genomics* **3**, 2.
17. Misson, J., Raghothama, K. G., Jain, A., Jouhet, J., Block, M. A., Bligny, R., Ortet, P., Creff, A., Somerville, S., Rolland, N., *et al.* (2005) *Proc. Natl. Acad. Sci. USA* **102,** 11934–11939.
18. Güler, S., Seeliger, A., Härtel, H., Renger, G. & Benning, C. (1996) *J. Biol. Chem.* **271,**7501–7507.
19. Frentzen, M. (2004) *Curr. Opin. Plant Biol.* **7,** 270–276.
20. Andersson, M. X., Larsson, K. E, Tjellström, H., Liljenberg, C. & Sandelius, A. S. (2005) *J. Biol. Chem.* **280,** 27578–27586.
21. Ohashi, Y., Oka, A., Rodrigues-Pousada, R., Possenti, M., Ruberti, I., Morelli, G. & Aoyama, T. (2003) *Science* **300,** 1427–1430.
22. Anthony, R. G., Henriques, R., Helfer, A., Meszaros, T., Rios, G., Testerink, C., Munnik, T., Deak, M., Koncz, C. & Bogre, L. (2004) *EMBO J.* **23,** 572–581.
23. Van Leeuwen, W., Okrësz, L., Bögre, L. & Munnik, T. (2004) *Trends Plant Sci.* **9,** 378–384.
24. Härtel, H., Dörmann, P. & Benning, C. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 10649–10654.
25. Benning, C. & Otha, H. (2005) *J. Biol. Chem.* **280,** 2397–2400.
26. Kelly, A. & Dörmann, P. (2004) *Curr. Opin. Plant Biol.* **7,** 262–269.
27. Mats, X., Andersson, J., Kjellberg, M. & Sandelius, A.-S. (2004) *Biochim. Biophys. Acta* **1684,** 46–53.
28. López-Bucio, J., Hernández-Abreu, E., Sánchez-Calderón, L., Nieto-Jacobo, M. F., Simpson, J. & Herrera-Estrella, L. (2002) *Plant Physiol.* **129,** 244–256.
29. Karthikeyan, A. S., Varadarajan, D. K., Mukatira, U. T., D'Urzo, M. P., Damsz, B. & Raghothama, K. G. (2002) *Plant Physiol.* **130,** 221–233.
30. Karimi, M., Inzé, D. & Depicker, A. (2002) *Trends Plant Sci.* **7,** 193–195.
31. Martinez-Trujillo, M., Limones-Briones, V., Cabrera-Ponce, J. L. & Herrera-Estrella, L. (2004) *Plant Mol. Biol. Rep.* **22,** 63–70.
32. Malamy, J. E. & Benfey, P. N. (1997) *Development (Cambridge, U.K.)* **124,** 33–44.
33. Wanjie, S. W., Welti, R., Moreau, R. A. & Chapman, K. D. (2005) *Lipids* **40,** 773–785.