Phospholipase Dα1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling

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Phospholipase D (PLD) and protein phosphatase 2C (PP2C) both play a role in mediating plant responses to abscisic acid (ABA). In this article, we show that PLDα1 and its product, phosphatidic acid (PA), regulate a PP2C, ABI1, which is a negative regulator of ABA responses in Arabidopsis. Leaves from a T-DNA insertional mutant of PLDα1 and PLDα1-antisense plants lose more water than do wild-type plants. The stomatal closure of PLDα1-null leaves is insensitive to ABA but is promoted by PA. ABA treatment promotes an increase in PA from phosphatidylcholine in wild type but not in PLDα1-null cells. PLDα1-derived PA binds to ABI1; the PA–ABI1 binding is demonstrated by coprecipitating PA with ABI1 from plant cells, measuring binding of PA from vesicles to ABI1, and assaying ABI1 bound to PA immobilized on a filter. Deletion and site-specific mutational analyses show that arginine 73 in ABI1 is essential for PA–ABI1 binding. PA binding decreases the phosphatase activity of ABI1. The lack of ABA-induced production of PA in PLDα1-null cells results in a decrease in the association of ABI1 with the plasma membrane in response to ABA. These results indicate that PA produced by PLDα1 inhibits the function of the negative regulator ABI1, thus promoting ABA signaling. The identification of ABI1 as a direct target of the lipid messenger PA provides a functional link between the two families of important signaling enzymes, PLD and PP2C.

Abscisic acid (ABA) plays an important role in plant growth, development, and responses to environmental stresses, such as drought, salinity, and low temperature (1). Reversible protein phosphorylation is involved in the early events of ABA signal transduction (2, 3). Specific protein kinases are activated in response to ABA and have been proposed to play a positive role in ABA signaling (4, 5, 6). PLD activity has been implicated in ABA responses in Arabidopsis (7). Moreover, ABA-regulated stomatal movement (8) and ABA-induced gene expression (9) have been reported. In recent years, specific PLDs have been shown to mediate many plant functions, including cell patterning, programmed cell death, and stress tolerance (10, 11). However, the direct target of PLD action is unknown. In this study, we present evidence for a direct link between PLDα1 and PP2C in the ABA signaling response.

Materials and Methods

Plant Materials and Growth. A T-DNA-insertional mutant of PLDα1 (PLDα1-KO) was identified from SALK-053785 seeds obtained from the Arabidopsis Biological Resource Center (Columbus, OH). The site of T-DNA insertion was confirmed by DNA sequencing. The generation of PLDα1-AS plants was described in ref. 2. Seeds of PLDα1-KO, PLDα1-AS, and wild type of Arabidopsis thaliana (ecotype Columbia) were sown in soil and kept at 4°C for 2 days. Plants were grown in a growth chamber with cool white fluorescent light of 100 μmol m⁻²s⁻¹ under 14-h light/10-h dark and 23°C/18°C cycles.

Water Loss and Stomatal Aperture Measurements. Detached leaves from 6-week-old plants were exposed to cool white light (125 μmol m⁻²s⁻¹) at 23°C. Leaves were weighed at various time intervals, and the loss of fresh weight (%) was used to indicate water loss. Stomatal aperture was measured according to a published procedure (23) with minor modifications. Epidermal peels were stripped from fully opened leaves of 6-week-old plants and floated in a solution of 10 mM KCl, 0.2 mM CaCl₂, 0.1 mM EGTA, and 10 mM Mes-KOH (pH 6.15). After incubation for 2 h under cool white light (150 μmol m⁻²s⁻¹) at 23°C to induce stomatal opening, 50 μM ABA and/or phosphatidic acid (PA) was added. For PA treatment, dioleoyl PA in chloroform was dried under N₂ and suspended at 500 μM in the above solution by sonication. PA was diluted to the final concentration with the same solution. Stomatal aperture was recorded under a microscope with a digital camera and analyzed by using IMAGEPRO software (Media Cybernetics, Silver Spring, MD).

Construction of ABI1 Mutants. cDNA fragments for the full-length and four C-terminal deletion mutants of ABI1 were amplified by PCR using ABI1 cDNA as template. A common forward primer, 5'-TAGGATCCATGGAGGAAGTATCTCCG-3', was paired with the following five reverse primers: 5'-AAGGCTGT-TCAAGGGTTTCTCCTCTGTA-3' for the full-length ABI1 (1–423); 5'-TAAGGCTTACTGAGCGGTGATCTC-3' for ABI1a (1–104); 5'-TAAGGCTTTTCTCCTTAGGCATCTCC-3' for ABI1b (1–204); 5'-TAAGGCTTAC-GAGCTCATTTCAGCTGAAT-3' for ABI1c (1–304); and 5'-GAGCTCATTTCAGCTGAAT-3'.

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Abbreviations: PLD, phospholipase D; PP2C, protein phosphatase 2C; ABA, abscisic acid; PA, phosphatidic acid; AS, antisense; HA, hemagglutinin; NBD, 12[(7-nitro-2-1,3-benzoxadiazol-4-yl)-amino]dodecanoyl; PtdDlt, phosphatidylbutanol; PC, phosphatidylcholine; DAG, diacylglycerol.

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Protoplast Isolation, Phospholipid Labeling, and PLD Activity Assays. Protoplasts were prepared from fully expanded leaves of 4-to-6-week-old Arabidopsis plants. The procedures for protoplast isolation and subsequent labeling of protoplasts with fluorescent dye (359) were described in ref. 20. Protoplasts (3 × 10^6 ml^-1), labeled with NBD-PC, were incubated with 50 μM ABA at 22°C. To determine in vivo PLD activity based on the production of phosphatidylbutanol (PtdBut), 0.1% 1-butanol (vol/vol) was added with ABA. At the end of a treatment, the mixture was centrifuged at 10,000 g for 10 min. The cellular extract was immunoprecipitated, and associated NBD-PA was quantified as described in ref. 25. Vesicles were incubated with ABI1 with agitation. ABI1 was immunoprecipitated, and associated NBD-PA was quantified (20).

Protein Extraction, Immunoblotting, and Immunolocalization of ABI1. Proteins were extracted from leaves or protoplasts (20, 22). The extracts were separated by SDS/10% PAGE and blotted onto nitrocellulose. The expression of ABI1 was verified by Western blotting using a monoclonal HA antibody (1:100) for 8 h at 23°C. The nuclei were stained with 2 μg/ml propidium iodide and 5 μg/ml antipain. ABI1 bound to the filters was visualized by staining alkaline phosphatase activity. To detect PA binding in vesicles, lipid vesicles composed of 50 μm NBD-PA and 25 μm PC were prepared by sonication in a buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM NaHPO₄, and 1.8 mM KH₂PO₄ (25). Vesicles were incubated with ABI1 with agitation. ABI1 was immunoprecipitated, and associated NBD-PA was quantified (20).

Results

Ablation of PLD1 Increases Leaf Water Loss and Decreases ABA-Induced Stomatal Movement. The expression of PLD1 was abolished in Arabidopsis by AS suppression (AS) and T-DNA inser- tional mutagenesis (KO; Fig. 1A). The loss of PLD1 was confirmed by the lack of PLD1 protein in PLD1-AS and PLD1-KO leaves (Fig. 1B). Compared with wild-type leaves, PLD1-AS leaves had ~5% PLD1 activity, whereas the knockout plants had no such activity. PLD1-KO resulted from an insertion of T-DNA at nucleotide 1027 downstream of the initiation codon of PLD1. The PLD1-KO allele cosegregated with kanamycin resistance and susceptibility in a 3:1 ratio, suggesting that the knockout mutant contains a single T-DNA in the genome. Introducing a wild-type PLD1 into the knockout plants genetically complemented the expression and function of PLD1 (data not shown).

Both PLD1-AS and PLD1-KO leaves lost more water than wild-type leaves did, with PLD1-KO displaying the highest rate of water loss (Fig. 1C). In terrestrial plants, >95% of water loss occurs through transpiration from stomata (5). Thus, we measured the stomatal aperture in response to ABA. Stomatal aperture of wild-type leaves decreased with an increase in ABA concentrations, but the stomatal aperture in PLD1-KO was insensitive to added ABA (Fig. 1D). However, the stomatal aperture of both wild-type and PLD1-AS leaves responded to the PLD lipid product PA (Fig. 1E). After treatment of epidermal peels with 50 μM PA, there was no difference in stomatal aperture between the two genotypes (Fig. 1E). These results show that PLD1 is involved in the ABA response and also suggest that PLD1-derived PA mediates the ABA-induced stomatal movement.

ABA Stimulates PLD1 Activity and PA Production. To establish that PLD1 is activated in ABA responses, we determined the PLD activity in vivo after cells were exposed to ABA. To facilitate a...
Fig. 1. Effect of PLDα1 on leaf water loss and ABA-induced stomatal closure. (A) Gene structure of PLDα1 and the site of the T-DNA insertion. Boxes represent exons. (B) PLDα1 activity in wild-type (WT), PLDα1-AS (AS), and PLDα1-KO (KO) leaves. (Inset) Immunoblotting of PLDα1 in wild-type (WT), PLDα1-AS (AS), and knockout (KO) leaves. (C) Water loss from detached leaves. Water loss is expressed as the percentage of initial fresh weight. Values are means ± SD of three replicates (n = 12). (D) Stomatal apertures in wild-type and PLDα1-KO leaves affected by increasing concentrations of ABA. After a 2 h treatment at each ABA concentration, 50–75 stomata were measured. Values are means ± SD of five experiments. (E) Stomatal apertures of wild-type and PLDα1-KO epidermal peels after treatments of ABA or PA (n = 50–75).

uniform labeling of membrane phospholipids and a synchronized response to ABA, leaf protoplasts were isolated and prelabeled with fluorescent NBD-PC. PC was used because it is the preferred substrate of PLDα1 (26). The level of PA increased in wild-type protoplasts and reached a plateau 30 min after the ABA treatment, but no apparent change in PA level occurred in PLDα1-KO cells (Fig. 2A). To further verify that the increase in PA came from PLD activity, we compared the formation of PtdBut in PLDα1-KO and wild-type protoplasts. Although the production of PA could result from other activities besides that of PLD [e.g., diacylglycerol (DAG) kinase or de novo synthesis], the transphosphatidylation activity is unique to PLD (15). ABA treatment increased the levels of PtdBut in wild-type but not in PLDα1-null cells (Fig. 2B). These results show that PLD is activated in response to ABA and that PLDα1 is responsible for the ABA-induced production of PA from PC in the leaf cells.

Fig. 2. Role of PLDα1 in ABA-promoted PLD activity in Arabidopsis. (A) ABA-induced production of PA in wild-type (WT) and PLDα1-KO (KO) protoplasts. (B) ABA-promoted production of PtdBut in protoplasts. NBD-PC-labeled protoplasts were treated with 50 μM ABA. PA or PtdBut were expressed as percentage of NBD-PA or NBD-PtdBut fluorescence over the total fluorescence of lipids. Values are means ± SD of three experiments.

PLD-Derived PA Interacts with ABI1 PP2C. To determine how PLDα1 mediates plant response to ABA, we investigated the possibility that its product, PA, might interact with proteins involved in the ABA signaling cascade. PP2C-like enzymes such as ABI1 have been shown to be mediators of ABA responses (3, 5, 6, 27). We hypothesized that its product, PA, might interact with proteins involved in the ABA signaling cascade. PP2C-like enzymes such as ABI1 have been shown to be mediators of ABA responses (3, 5, 6, 27). We hypothesized that the transphosphatidylation activity of PLDα1 was the reason for its ABA responsiveness (15). ABA treatment increased the levels of PtdBut in wild-type but not in PLDα1-null cells (Fig. 2B). These results show that PLD is activated in response to ABA and that PLDα1 is responsible for the ABA-induced production of PA from PC in the leaf cells.

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contrast, no PA was precipitated with ABI1 in PDLα1-null cells with or without ABA treatment (Fig. 3 B and C). These results indicate that (i) PA is associated with ABI1, (ii) the formation of PA–ABI1 complexes is promoted by ABA, and (iii) the activation of PDLα1 produces PA that binds to ABI1.

To augment and characterize the PA–ABI1 interaction, filter-binding and vesicle-binding assays were performed. ABI1 bound to PA but not to other acidic phospholipids [phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidylinositol (PI)], phosphatidylethanolamine (PE), or DAG (Fig. 3D). Some binding to PC occurred (Fig. 3D), and PC binding to ABI1 immunoprecipitated from protoplasts also was detectable (Fig. 3B). The amount of PC binding was ~25% of the amount of PA bound to ABI1 (Fig. 3B and data not shown), although the total amount of NBD-PC in the starting cell lysate was at least 10-fold greater than the amount of NBD-PA. This finding could indicate that the affinity of ABI1 for PA is 40-fold higher than that for PC. In addition, the amount of PC bound to ABI1 was not affected by ABA. Fatty acid species of PA affect the binding of PA to ABI1; dioleoyl PA was bound more than other PA species tested (Fig. 3E). The binding of dioleoyl PA increased with increasing amounts of PA (Fig. 3F). The binding of PA in lipid vesicles composed of PC and NBD-PA to ABI1 also was demonstrated (Fig. 4D).

**Arg-73 Is Required for ABI1 Binding to PA.** To identify the protein region involved in PA binding, serial deletion mutants of ABI1 were constructed and expressed in protoplasts (Fig. 4A and B). C-terminal deletions up to 104 aa residues did not significantly affect PA binding (Fig. 4C), suggesting that the PA-binding domain resides within the first 104 aa residues. The first 104 aa sequence of ABI1 showed no sequence stretches that were highly similar to the PA-interaction regions identified in animals. A short sequence fragment from residues 67 to 97 displayed a low level of sequence similarity to the PA-binding motif identified in the protein kinase Raf1 (Fig. S4; ref. 28). In the putative PA-binding regions in animal proteins, there is not high homology among the PA-binding motifs, but basic residues always are involved (29). Therefore, we mutated the three basic residues (ABI1RKK67-68GA and ABI1R73A) of ABI1 and expressed these proteins in protoplasts (Fig. 4C). The two mutated proteins had PP2C activity similar to the full-length ABI1 (Fig. 5B). However, ABI1R73A had no ability to bind PA, whereas ABI1RKK67-68GA bound PA like wild-type ABI1 (Fig. 5D).

To corroborate the binding results in the cell, we introduced ABI1R73A and wild-type ABI1 into protoplasts, followed by ABA treatment and immunoprecipitation (Fig. 5E). No PA was associated with ABI1R73A, whereas PA was coprecipitated with the wild-type ABI1 as demonstrated earlier (Fig. 5E and F). In-solution binding with lipid vesicles also showed the lack of PA binding by ABI1R73A (data not shown).

**Effects of PA on ABI1 Activity and Membrane Association.** To investigate the function of PA–ABI1 interaction, we measured the effect of PA on the PP2C activity of the immunoaffinity-purified ABI1. PA decreased ABI1 phosphatase activity; ~50% of phosphatase activity was lost at 100 μM dioleoyl PA (Fig. 6A). At 100 μM, other acidic lipids, phosphatidylinositol, phosphatidylserine, and phosphatidylglycerol, had no effect on ABI1 enzymatic activity (Fig. 6B). Neither PC, the substrate lipid of PDLα1, nor DAG, a dephosphorylated PA derivative, inhibited ABI1 activity. PA had no inhibitory effect on the phosphatase activity of the non-PA-binding ABI1 mutation R73A (Fig. 6A). In addition, we tested PAs with different acyl species shown in Fig. 3E, and, consistent with the binding results, dioleoyl PA gave the strongest inhibition (data not shown). These data indicate that lipid inhibition of ABI1 activity is specific to PA and that PA–ABI1 binding is required for the inhibition.
ABA-induced production of PA, the subcellular association of membrane became clearer in wild-type than in we also expressed the non-PA-binding ABI1 R73A in wild-type and Cc

type and subcellular distribution of ABI1 was compared between wild-
in response to ABA. Therefore, the effect of ABA on the

activation of ATHB6, a transcriptional factor that negatively

nuclei is believed to be required for ABI1 binding to and

indicate that more ABI1 is associated with the plasma membrane (Fig.

besides the direct effect on the catalytic activity, PA could have a membrane- tethering function for proteins and thus regulate the intracellular distribution and assembly of signaling proteins.

We reasoned that because PLDα1-null cells diminished the ABA-induced production of PA, the subcellular association of ABI1 might be different between wild-type and PLDα1-null cells in response to ABA. Therefore, the effect of ABA on the subcellular distribution of ABI1 was compared between wild-type and PLDα1-null cells. Before adding ABA, ABI1 was localized predominantly in the cytoplasm in both genotypes (Fig.

6 Cb vs. Cc, marked by a white arrow). On the other hand, after adding ABA, more fluorescent labeling was visible in the perinuclear region in PLDα1-KO cells than in wild-type ones. We also expressed the non-PA-binding ABI1R73A in wild-type and PLDα1-KO. The distribution of ABI1R73A in the two genotypes was not affected by ABA treatments (data not shown).

These results suggest that in response to ABA, more ABI1 is associated with the plasma membrane in wild-type than in PLDα1-null cells, whereas more ABI1 is translocated to nuclei in PLDα1-null cells.

Discussion

Phospholipases and intracellular lipid messengers have been implicated in many plant processes (15, 16), but there has been no evidence for the direct link of a phospholipase → a lipid messenger → a target protein in a specific signaling pathway in plants. The paucity of such information has been an impediment to the understanding of lipid signaling in plants. Results of this study have filled the gap by providing a link between PLD and PP2C through the lipid mediator PA.

The identification of PP2C as a direct target of PLD-derived PA opens a door to investigating and understanding the regulation and function of this large and complex family of protein phosphatases. Arabidopsis contains 69 PLP2C-like genes (30). Most of plant PP2Cs, including ABI1, ABI2, and MP2C, have a common structure: a highly conserved catalytic domain but a unique N-terminal region (31). The N-terminal region of PP2Cs has been proposed to mediate the interactions with cellular substrates, regulatory proteins, and secondary messengers (32). ABI1 lacking the N-terminal 105 aa blocks the ABA signaling response more obviously than the full-length ABI1 does, suggesting that the N-terminal domain has an inhibitory role in PP2C function and a stimulatory role in ABA response (3). We show here that a mutation in the N-terminal region (R73A) abolishes ABI1 binding to PA and blocks PA-conferred inhibition of ABI1 PP2C activity (Fig.

6). The results may explain, at least in part, why deletion of the N-terminal region stimulates ABI1 phosphatase activity and suppresses ABA signaling. We also tested the possibility that the dominant mutation of abi1 (G180D) would affect the binding of ABI1 to PA. The abi1 protein showed PA binding that was similar to wild-type ABI1 binding (data not shown), indicating that the abi1 mutation does not affect PA binding to ABI1.

It is worth noting that the levels of added PA in the study are close to the physiological range. The PA concentration in Arabidopsis leaves is estimated to be 50–100 μM based on the PA content in leaves (20, 21, 26). The levels of PA from fluorescence PC increased 2-fold in protoplasts of Arabidopsis leaves (Fig.

2A) and of Vicia faba guard cells (14). When 50 μM ABA was applied to Arabidopsis leaves, a 50% increase in PA occurred within 10 min (data not shown). However, the actual concentration of signaling PA is difficult to determine because PA resides primarily in membranes and also because metabolic compartmentalization of PA is expected to occur in the cell.

PA has been shown to be a potent inhibitor of human protein phosphatase-1 (33), but PA inhibition of ABI1 in vitro is incomplete. It is most likely that PA functions as part of the ABI1 regulatory complex that involves the function of several mediators, such as H2O2 and free fatty acids. H2O2 has been reported to inactivate ABI1 and ABI2 (32). ABA triggers H2O2 accumulation in guard cells, which in turn activate Ca2+ channels and promote stomatal closure (34). In addition, linoleic acid inhibits ABI2 (32) and MP2C (35). PLDα1 and PA are involved in H2O2 production in Arabidopsis leaves by activation of NADPH oxidase activity (36). Activation of PLDα1 also leads to an increase in free linoleic and linolenic acid, which are involved in oxylipin synthesis and defense signaling (37, 38). Thus, besides the direct effect of PA on ABI1 phosphatase activity, PLDα1 and PA also may indirectly regulate ABI1 activity by affecting the production of H2O2 and linolenic acid.

Another role for PA–protein interaction is to tether effector proteins to a specific region of cell membranes. The present data indicate that more ABI1 is associated with the plasma membrane in wild-type than in PLDα1-null cells after ABA treatment. This difference is correlated with the results that the PA level increases more in wild-type than in PLDα1-null cells in response to ABA. However, more ABI1 is associated with the nuclear region in PLDα1-null cells. The translocation of ABI1 from cytosol to nuclei is believed to be required for ABI1 binding to and activation of ATHB6, a transcriptional factor that negatively regulates ABA responses (8, 9). Knockout of PLDα1 does not alter ABA-induced expression of ABI1, but the mRNA level of ATHB6, relative to that of ABA in PLDα1-null cells is 2-fold higher than that in wild-type cells (Fig. 8, which is published as supporting information on the PNAS web site). The result is
consistent with the hypothesis that, compared with wild-type cells, more ABI1 is translocated into nuclei in PLDΔ1-null cells because of a decrease in the PA anchorage of ABI1 to membranes. The membrane-tethering role of PA might precede its activity inhibition. One intriguing observation is that almost no PA was precipitated with ABI1 in PLDΔ1-null cells, whereas PA was pulled down from wild-type cells without added ABA. The higher basal level of PA (Fig. 2A) in wild-type cells might account for, in part, the difference, but the results seem to argue for the importance of close association between PLDΔ1 and PP2C on membranes. The localization results suggest that some ABI1 is associated with the plasma membrane even without added ABA (Fig. 6C). Earlier results indicate that some PLDΔ1 was associated with the plasma membrane and its membrane association increased upon stress perturbation (38). Thus, colocalization of PLDΔ1 and ABI1 to the plasma membrane is probable and might be crucial to the PA–ABI1 interaction. Based on these results, we propose a model of interaction between PLDΔ1/PA and ABI1 in the ABA signaling process (Fig. 7). PLDΔ1 is activated in response to ABA, which produces PA. PA binds to ABI1 protein, resulting in the anchorage of ABI1 to the plasma membrane and a decrease in ABI1 PP2C activity. This membrane tethering reduces the movement of ABI1 from the cytosol into the nucleus, which decreases the ABI1-mediated activation of ATHB6. The negative effect of ABI1 in ABA signaling is suppressed. Therefore, PLDΔ1 and PA positively mediate ABI1 signaling processes; abrogation of PLDΔ1 renders plants less sensitive to ABA. In addition, PLDΔ1 has been shown to interact directly with the alpha subunit (Gαs) of heterotrimeric G proteins (39), and Gαs also plays a role in mediating ABA response (40). These results show that PLD and the lipid messenger PA are intermediary links between important cellular regulators in plant cells. Recent studies also indicate that PA increases the activity of protein kinases (41, 42). These findings raise an intriguing possibility that PA may regulate the homeostasis of protein phosphorylation by concerted regulation of the function of protein phosphatases and kinases.

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