Cooperative Function of PLDδ and PLDα1 in Abscisic Acid-Induced Stomatal Closure in Arabidopsis

Misugi Uraji, Takeshi Katagiri, Eiji Okuma, Wenxiu Ye, Mohammad Anowar Hossain, Choji Masuda, Aya Miura, Yoshimasa Nakamura, Izumi C. Mori, Kazuo Shinozaki, and Yoshiyuki Murata*

Graduate School of Natural Science and Technology, Okayama University, Kita-ku, Okayama 700–8530, Japan (M.U., E.O., W.Y., M.A.H., C.M., A.M., Y.N., Y.M.); Plant Molecular Biology Laboratory, RIKEN Tsukuba Institute, Tsukuba 305–0074, Japan (T.K., K.S.); and Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama 710–0046, Japan (I.C.M.)

Phospholipase D (PLD) is involved in responses to abiotic stress and abscisic acid (ABA) signaling. To investigate the roles of two Arabidopsis (Arabidopsis thaliana) PLDs, PLDα1 and PLDδ, in ABA signaling in guard cells, we analyzed ABA responses in guard cells using Arabidopsis wild type, pldα1 and pldδ single mutants, and a pldα1 pldδ double mutant. ABA-induced stomatal closure was suppressed in the pldα1 pldδ double mutant but not in the pld single mutants. The pldα1 and pldδ mutations reduced ABA-induced phosphatic acid production in epidermal tissues. Expression of either PLDα1 or PLDδ complemented the double mutant stomatal phenotype. ABA-induced stomatal closure in both pldα1 and pldδ single mutants was inhibited by a PLD inhibitor (1-butanol), suggesting that both PLDα1 and PLDδ function in ABA-induced stomatal closure. During ABA-induced stomatal closure, wild-type guard cells accumulate reactive oxygen species and nitric oxide and undergo cytosolic alkalization, but these changes are reduced in guard cells of the pldα1 pldδ double mutant. Inward-rectifying K+ channel currents of guard cells were inhibited by ABA in the wild type but not in the pldα1 pldδ double mutant. ABA inhibited stomatal opening in the wild type and the pldδ mutant but not in the pldα1 mutant. In wild-type rosette leaves, ABA significantly increased PLDδ transcript levels but did not change PLDα1 transcript levels. Furthermore, the pldα1 and pldδ mutations mitigated ABA inhibition of seed germination. These results suggest that PLDα1 and PLDδ cooperate in ABA signaling in guard cells but that their functions do not completely overlap.

Stomatal pores are formed by pairs of guard cells and mediate transpiration and carbon dioxide uptake. Abscisic acid (ABA) synthesized in plants subjected to drought stress induces stomatal closure in order to suppress water loss in plants (Assmann and Shimazaki, 1999; Schroeder et al., 2001). Phospholipase D (PLD) activity increases in response to hyperosmotic stress and dehydration in Craterostigma plantagineum (Frank et al., 2000; Munnik et al., 2000) and Arabidopsis (Arabidopsis thaliana; Katagiri et al., 2001). PLDs hydrolyze phospholipids, phosphatidylcholine (PC), and phosphatidylethanolamine (PE), releasing phosphatic acid (PA) and their head group in the plasma membrane. The released PA is thought to function as a signal molecule in cellular signaling. The Arabidopsis genome has 12 PLD genes that are classified into six subfamilies, α, β, γ, δ, ε, and ζ (Wang, 2005). Pharmacological studies examining the effect of a PLD inhibitor (1-butanol [1-BuOH]) and exogenous application of PA in Vicia faba have suggested that ABA signaling is mediated by PLDs (Jacob et al., 1999). Previous studies have reported that PLDα1 positively regulates ABA-induced stomatal closure (Zhang et al., 2004) and the inhibition of stomatal opening by ABA (Mishra et al., 2006). Furthermore, PLDα1 activity is reported to be modulated by a G protein, GPA1, in the process of inhibition of stomatal opening by ABA (Zhao and Wang, 2004; Mishra et al., 2006). However, a pldα1 loss-of-function mutation alone did not inhibit ABA-induced stomata closure (Siegel et al., 2009), which suggests that other PLDs are involved in ABA signaling in guard cells.

PLDδ is involved in responses to drought and salinity stress (Katagiri et al., 2001) and cold stress (Li et al., 2004). PLDα1 and PLDδ are required for tolerance to high salinity and hyperosmotic stress (Bargmann et al., 2009). Therefore, we hypothesized that PLDδ functions cooperatively with PLDα1 in the ABA signal pathway in guard cells.

ABA signal transduction in guard cells is mediated by various signaling molecules, including reactive oxygen species (ROS), nitric oxide (NO), Ca2+, sphingosine-1-phosphate, and inositol 1,4,5-triphosphate (Zhang et al., 2001; Desikan et al., 2002; Coursol et al., 2003; Sokolovski et al., 2005; Lee et al., 2007; Roelfsema and Hedrich, 2010). In addition, ABA-induced stomatal closure is accompanied by various events, including cytosolic free calcium concentration ([Ca2+]cyt) oscillation/elevation.
and cytosolic alkalinization (Pei et al., 2000; Murata et al., 2001; García-Mata et al., 2003; Sokolovski and Blatt, 2004; Suhita et al., 2004; Islam et al., 2010a).

In this study, we investigated the roles of PLDα1 and PLDδ in (1) ABA-induced stomatal closure, (2) ABA-induced production of ROS and NO, cytosolic pH alkalization, and cytosolic free Ca2+ elevation, and (3) ABA inhibition of inward-rectifying K+ (K+in) channel currents and stomatal opening. We report that PLDα1 and PLDδ have overlapping functions in ABA-induced stomatal closure and different functions in the ABA inhibition of light-induced stomatal opening.

RESULTS

Expression of PLDα1 and PLDδ in Guard Cells

We examined the accumulation of transcripts of PLDα1 (At3g15370) and PLDδ (At4g35790) in isolated guard cell protoplasts (GCPs) using reverse transcription (RT)-PCR. POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA1 (KAT1) was used as a specific GCP marker (Leonhardt et al., 2004). As shown in Figure 1A, the transcripts of PLDα1 and PLDδ were detected in both GCPs and mesophyll cell protoplasts (MCPs). The PLDα1 transcription level in GCPs appeared to be lower than that in MCPs, whereas the PLDδ transcription level in GCPs was slightly higher than that in MCPs (Fig. 1A).

ABA-Induced Stomatal Closure in pld Mutants

We isolated mutants with T-DNA inserted in PLDα1 (SALK_063785) and PLDδ (KAZUSA T-DNA tag line) loci (Fig. 1B). A double mutant, plda1 pldδ, was generated by crossing the single mutants. Disruption of the genes was assessed by RT-PCR with total RNA isolated from whole leaves. The transcripts of PLDα1 were not detected in the plda1 and plda1 pldδ mutants, and the transcripts of PLDδ were not detected in the pldδ and plda1 pldδ mutants (Fig. 1C).

Utilizing these loss-of-function mutants, we examined the involvement of PLDα1 and PLDδ in ABA-induced stomatal closure. Application of 1 μM ABA induced stomatal closure in the wild type (P < 10⁻⁴) and both pld single mutants (P < 10⁻³ for plda1, P < 10⁻³ for pldδ).
compared with the solvent control (Fig. 1D) but not in the double mutant ($P = 0.096$). On the other hand, the application of 10 $\mu$M ABA slightly induced stomatal closure in the double mutant (Fig. 1D), indicating greatly reduced ABA sensitivity of double mutant stomata.

Drought induced an accumulation of PA in wild-type rosette leaves (Fig. 1E). The drought-induced PA accumulation in rosette leaves was suppressed in the 

$$\text{pld}a1 \text{ pld}d\theta$$

double mutant but not in either single mutant (Fig. 1E). ABA-induced PA accumulation in wild-type rosette leaves was not detected (data not shown), in agreement with previous results (Katagiri et al., 2001). On the other hand, in epidermal tissues, 50 $\mu$M ABA induced an accumulation of PA in the wild type ($P < 0.05$; Fig. 1F). ABA also induced a similar accumulation of PA in the 

$$\text{pld}d\theta$$

single mutant ($P < 0.03$), which was not significantly different from the accumulation induced in the wild type ($P = 0.593$; Fig. 1F). ABA did not induce PA accumulation in either the 

$$\text{pld}a1$$

single mutant ($P = 0.222$) or the 

$$\text{pld}a1 \text{ pld}d\theta$$

double mutant ($P = 0.163$; Fig. 1F). Moreover, suppression of PA production by the double mutation was stronger than that of the 

$$\text{pld}a1$$

single mutation ($P < 0.04$). These results suggest that PLDa1 and PLD6 are involved in PA production induced by ABA signaling in guard cells.

Complementation of the Stomatal Phenotype of the 

$$\text{pld}a1 \text{ pld}d\theta$$

Double Mutant with PLDa1 or PLD6

To test whether the expression of PLDa1 or PLD6 complements the stomatal phenotype of the double mutant, we generated the 

$$\text{pld}a1 \text{ pld}d\theta$$

mutants transformed with PLDa1 or PLD6. PLDa1 and PLD6 transcripts were detected in the respective complement lines (Fig. 2A). The transformed plants showed a restored stomatal response to ABA (Fig. 2B), suggesting that mutations of PLDa1 and PLD6 are responsible for the ABA-insensitive phenotype observed in the 

$$\text{pld}a1 \text{ pld}d\theta$$

mutants.

PA-Induced Stomata Closure in 

$$\text{pld}$$

Mutants

To confirm the function of PLDs in ABA signaling in guard cells, we examined the effects of a PLD inhibitor, 

$$1\text{-BuOH}$$

on ABA-induced stomatal closure. ABA at 10 $\mu$M closed stomata of 

$$\text{pld}a1$$

and 

$$\text{pld}d\theta$$

at a level comparable to the level in the wild type in the absence of 1-BuOH, whereas 1-BuOH at 50 $\mu$M suppressed the ABA-induced stomatal closure (Fig. 3A), which suggests that activation of PLDs is involved in ABA-induced stomatal closure.

PLDs hydrolyze phospholipids, releasing PA. The released PA mediates ABA signaling, leading to stomatal closure (Jacob et al., 1999; Mishra et al., 2006; Zhang et al., 2009). We examined exogenous PA-induced stomatal closure in the 

$$\text{pld}a1$$

and 

$$\text{pld}d\theta$$

mutants. PA at 10 and 50 $\mu$M induced stomatal closure in the wild type and the 

$$\text{pld}$$

mutants (Fig. 3B), suggesting that downstream of PA production in the ABA signal cascade is intact in the 

$$\text{pld}$$

mutants.

Abolishment of ABA-Induced Production of ROS and NO and Cytosolic Alkalization in 

$$\text{pld}a1 \text{ pld}d\theta$$

Guard Cells

We measured ABA-induced ROS accumulation in guard cells using a hydrogen peroxide ($\text{H}_2\text{O}_2$)-sensitive fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$). ABA induced ROS accumulation in guard cells in the wild type ($P < 0.003$; Fig. 4A), in agreement with previous results (Pei et al., 2000; Murata et al., 2001; Munemasa et al., 2007). ABA significantly induced ROS production in guard cells of both single mutants ($P < 0.05$ for 

$$\text{pld}a1$$

, $P < 0.02$ for 

$$\text{pld}d\theta$$

), while the amounts of accumulated ROS were decreased. However, ROS production was nearly abolished in the double mutant ($P = 0.619$; Fig. 4A).
Stomatal closure in response to H₂O₂ in the pld mutants was examined. Application of 100 μM H₂O₂ induced stomatal closure in the wild type (P < 10⁻⁴; Supplemental Fig. S1), as reported previously (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003; Zhang et al., 2009, 2011), and also in the plda1 (P < 0.03), pldδ (P < 10⁻⁷), and plda1 pldδ (P < 0.03) mutants (Supplemental Fig. S1). These results indicate that PLDa1 and PLDδ function upstream of ROS production in ABA signaling.

Production of NO in guard cells was examined using 4,5-diaminofluorescein-2 diacetate (DAF-2DA). ABA induced NO accumulation in wild-type guard cells (P < 0.05) but not in the plda1 pldδ double mutant (P = 0.79; Fig. 4B). Cytosolic alkalization was investigated using 2',7'-bis-(2-carboxyethyl)-5,(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM). ABA elicited cytosolic alkalization in the wild-type guard cells (P < 0.002) but not in the plda1 pldδ double mutant (P = 0.95; Fig. 4C).

Exogenous PA induced ROS accumulation and cytosolic alkalization in guard cells in the wild type and the plda1 pldδ mutant (Supplemental Fig. S2, A and C) but did not affect NO accumulation in guard cells in either the wild type or the double mutant (Supplemental Fig. S2B).

ABA-Induced Cytosolic Ca²⁺ Oscillations in plda1 pldδ Guard Cells

We monitored [Ca²⁺]cyt in guard cells using a Ca²⁺-sensing fluorescent protein, Yellow Cameleon 3.6 (YC3.6). When the wild-type guard cells were treated with 10 μM ABA, 83% of the guard cells showed [Ca²⁺]cyt transient elevation(s), hereafter [Ca²⁺]cyt oscillation (n = 30; Fig. 5, A and C). When the double mutant guard cells were treated with 10 μM ABA, 87% of the guard cells showed [Ca²⁺]cyt oscillations (n = 32; Fig. 5, B and C). The frequency of [Ca²⁺]cyt oscillations was not significantly different between the wild type and the double mutant, suggesting that neither PLDa1 nor PLDδ is involved in [Ca²⁺]cyt oscillation in guard cells in response to ABA.

ABA Inhibition of K⁺ Channel Currents in Guard Cells

In the absence of ABA, K⁺ channel currents in GCPs were not significantly different between the wild type and the double mutant (P = 0.70). However, in the presence of ABA, K⁺ channel currents were reduced in the wild type (P < 10⁻³; Fig. 6, A and B) but not in the double mutant (P = 0.24; Fig. 6, C and D). These results indicate that PLDa1 and PLDδ are involved in the inhibition of the K⁺ channel by ABA signaling.

Inhibition of Stomatal Opening by ABA

One of ABA’s many roles is to inhibit light-induced stomatal opening (Shimazaki et al., 2007), and PLDa1 is reported to be involved in the ABA inhibition of light-induced stomata opening (Mishra et al., 2006). ABA at 1 μM inhibited stomatal opening in the wild type (P < 0.02) and the pldδ single mutant (P < 0.01) but did not inhibit it in the plda1 single mutant (P = 0.76) or the plda1 pldδ double mutant (P = 0.15; Fig. 7). On the other hand, 10 μM ABA slightly inhibited stomatal opening in the plda1 single mutant and the plda1 pldδ double mutant. These results suggest that PLDδ functions differently from PLDa1 in the inhibition of light-induced stomatal opening.

Inhibition of Seed Germination by ABA

The inhibitory effect of ABA on seed germination was slightly reduced in both of the single mutants and strongly reduced in the double mutant (Fig. 8). These results suggest that PLDa1 and PLDδ cooperatively function not only in stomatal closure but also in seed germination. In contrast, root growth was inhibited by ABA in a dose-dependent manner in the wild type and the pld mutants (data not shown). Hence, PLDa1 and PLDδ do not appear to be involved in all ABA signaling in Arabidopsis.
Effects of ABA on Transcription of PLDδ

Rosette leaves were placed in stomatal assay solution with and without 50 μM ABA under the light condition for 2 h. Total RNA was isolated from the leaves, and transcript levels of PLDa1, PLDδ, and RESPONSIVE TO DEHYDRATION B (RD29B), a positive control for the ABA response (Yamaguchi-Shinozaki and Shinozaki, 1993), were measured by quantitative real-time PCR. The amounts of PLDa1 transcripts in the untreated and treated leaves were not significantly different (P = 0.13; Fig. 9, left panel), but the amount of PLDδ transcript in the ABA-treated leaves was three times higher than that in the untreated leaves (P < 0.002; Fig. 9, middle panel). RD29B transcripts were remarkably increased by ABA (Fig. 9, right panel), as expected. These results indicate that transcription of PLDa1 is constitutive and that transcription of PLDδ is ABA inducible. They also suggest that PLDδ functions in ABA signaling by regulating gene transcription.

**DISCUSSION**

Cooperative Function of PLDa1 and PLDδ in ABA Signaling in Arabidopsis Guard Cells

In the plda1 single mutant, most studies have reported that ABA-induced stomatal closure is impaired (Zhang et al., 2004, 2009; Mishra et al., 2006), (Fig. 9, right panel), as expected. These results indicate that transcription of PLDa1 is constitutive and that transcription of PLDδ is ABA inducible. They also suggest that PLDδ functions in ABA signaling by regulating gene transcription.
although one study found no strong ABA-insensitive phenotype (Siegel et al., 2009), in agreement with our research here.

Our results show that the plda1 pldδ double mutation disrupted ABA-induced PA production and ABA-induced stomatal closure but that the single mutations did not, suggesting that not only PLDα1 but also PLDδ positively regulate ABA-induced stomatal closure. The disruption by the double mutation was complemented by the expression of PLDα1 or PLDδ by the 35S promoter, suggesting that PLDα1 and PLDδ cooperatively function in ABA signaling in guard cells.

A PLD inhibitor, 1-BuOH, inhibited ABA-induced stomatal closure in the plda1 and pldδ single mutants as it did in the wild type (Fig. 3A), suggesting that other PLDs are involved in ABA-induced stomatal closure in each pld mutant. Moreover, ABA-induced stomatal closure in the plda1 pldδ double mutant was not completely inhibited (Fig. 1D), but it was completely inhibited by the application of 1-BuOH (Fig. 3A). This implies that other PLDs are involved in ABA signaling in guard cells.

PLDα1 and PLDδ Are Involved in ROS and NO Production and Cytosolic Alkalization But Not in Calcium Oscillation in ABA-Induced Stomatal Closure

ABA induces ROS production in guard cells, resulting in stomatal closure (Pei et al., 2000; Murata et al., 2001). The ROS production is mediated by NADPH oxidases, encoded by RESPIRATORY BURST OXIDASE HOMOLOG D (AtrbohD) and AtrbohF genes (Kwak et al., 2003). OPEN STOMATA1 kinase has been reported to activate AtrbohF via phosphorylation in ABA signaling (Sirichandra et al., 2009), and PA has also been reported to activate AtrbohF via binding (Zhang et al., 2009), suggesting that ABA-induced ROS production occurs downstream of PA production in ABA signaling in guard cells.

In this study, ABA-induced ROS production in guard cells was partially suppressed in the single mutants and completely suppressed in the double mutant (Fig. 4A),
suggesting that both PLDα1 and PLDδ are involved in ABA-induced ROS production and that PLDα1 and PLDδ cooperatively function upstream of ABA-induced ROS production in guard cells. Like ROS production, NO production and cytosolic alkalization are also accompanied by ABA-induced stomatal closure (Irving et al., 1992; Desikan et al., 2002; Suhita et al., 2004; García-Mata and Lamattina, 2007; Gonugunta et al., 2008; Islam et al., 2010a). ABA-induced NO production and cytosolic alkalization were also impaired in the \( pldα1 pldδ \) double mutant (Fig. 4, BandC), suggesting that PLDα1 and PLDδ positively regulate NO production and cytosolic alkalization in ABA signaling.

PLDs hydrolyze phospholipids, releasing PA as a second messenger. Our findings that PA induces ROS production (Supplemental Fig. S2A) and cytosolic alkalization (Supplemental Fig. S2C) confirm that PLDα1 and PLDδ function upstream of ROS production and cytosolic alkalization in ABA signaling.

In our study, PA treatment did not evoke NO production in guard cells (Supplemental Fig. S2B). Previous reports have shown that NO production in guard cells is dependent on ABA-induced \( H_2O_2 \) (Bright et al., 2006), suggesting that NO production is downstream of ROS production. However, another report has shown that NO induces PA production (Distéfano et al., 2008). The protein phosphatase 2C \( abi1 \) mutation impaired ABA-induced ROS production (Murata et al., 2001) but not ABA-induced NO production (Desikan et al., 2002). Moreover, Lozano-Juste and León (2010) have proposed a NO-independent regulatory mechanism of ABA-induced stomatal closure. Taken together, these results indicate that PA is closely involved in ROS production and cytosolic alkalization in ABA signaling and that the roles of NO production in ABA signaling remain to be investigated.

[Ca\(^{2+}\)]\(_{cyt}\) oscillation/elevation is known to occur during ABA-induced stomatal closure (Allen et al., 2000, 2001) and is closely related with ROS production in guard cells (Pei et al., 2000; Islam et al., 2010a, 2010b). Activation of PLDα1 and PLDδ requires \( Ca^{2+} \), since these PLDs contain a conserved C2 domain that participates in \( Ca^{2+}/phospholipid \) binding (Li et al., 2009), suggesting that PLD activities are affected by \[Ca^{2+}\]\(_{cyt}\) elevation in guard cells. PA production and \[Ca^{2+}\]\(_{cyt}\) oscillation/elevation may occur not only in tandem but also in parallel in ABA signaling in guard cells.

In this study, ABA induced \[Ca^{2+}\]\(_{cyt}\) oscillation but not ROS production in the \( pldα1 pldδ \) double mutant (Fig. 5). These results contradict the current ABA-signaling model, in which \( Ca^{2+}\)-permeable cation channels in the plasma membrane are activated by \( H_2O_2 \) (Pei et al., 2000; Kwak et al., 2003). However, long-term \( Ca^{2+} \) programmed stomatal closure requires stimulus-specific calcium oscillations; that is, certain specific \( Ca^{2+} \) signatures inhibit stomatal reopening after \( Ca^{2+} \) (reactive) stomatal closure (Allen et al., 2000, 2001). Therefore, the \( H_2O_2\)-independent \[Ca^{2+}\]\(_{cyt}\) oscillations in the \( pldα1 pldδ \) double mutant may have failed to induce stomatal closure and may be attributed to a malfunction of \[Ca^{2+}\]\(_{cyt}\) homeostasis due to the double mutation. Furthermore, spatiotemporal modulation of ROS production and/or differences of pattern of \[Ca^{2+}\]\(_{cyt}\) elevation are important in guard cell ABA signaling (Allen et al., 2001; Jannat et al., 2011a, 2011b). Spatiotemporal analysis of ROS production and \[Ca^{2+}\]\(_{cyt}\) elevation should resolve this discrepancy.

Figure 8. Effects of 3 μM ABA on the germination of wild-type (WT) and \( pld \) mutant seeds. Averages from three independent experiments (100 seeds per replication) are shown. Error bars represent se.

Figure 9. Real-time PCR analysis of PLDα1, PLDδ, and RD29B gene expression in the wild type. Total RNA was isolated from leaves treated with 50 μM ABA and from untreated leaves, as was done in the stomatal assay procedure. Transcript levels were normalized to the expression of ACTIN2 in the control. Three independent experiments were done. Error bars represent se.
Inhibition of $K^+_{in}$ Channel Currents by ABA in GCPs

Three second messengers, H$_2$O$_2$, NO, and sphingosine-1-phosphate, have been reported to inhibit $K^+_{in}$ channel currents of GCPs in V. faba (Zhang et al., 2001; Sokolovski et al., 2005) and of Arabidopsis GCPs (Coursol et al., 2003). Exogenous PA was also reported to inhibit $K^+_{in}$ channel currents of V. faba GCPs (Jacob et al., 1999). Our findings that exogenous PA inhibited $K^+_{in}$ channel currents in Arabidopsis GCPs (Supplemental Fig. S3) and that ABA strongly inhibited $K^+_{in}$ channel currents of wild-type GCPs but not of the pld1 pld6 GCPs (Fig. 6) suggest that, in ABA signaling, PLD$\alpha$1 and PLD$\delta$ are also involved in the inhibition of $K^+_{in}$ channel currents.

PLD$\alpha$1 and PLD$\delta$ Differentially Function in ABA Signaling in Guard Cells

In this study, we observed several differences of phenotype between the pld$\alpha$1 and pld$\delta$ single mutants. In the case of ABA-induced stomatal closure, the pld$\alpha$1 single mutation partially impaired stomatal closure induced by 1 $\mu$M ABA but the pld$\delta$ single mutation did not (Fig. 1D). ROS production by ABA in the pld$\alpha$1 single mutant was smaller than that in the pld$\delta$ single mutant, even though the difference was not significant (Fig. 4A). Similarly, ABA-induced PA production in the pld$\alpha$1 single mutant was less than that in the pld$\delta$ single mutant (Fig. 1F). Moreover, the pld$\alpha$1 single mutation weakened the ABA inhibition of light-induced stomatal opening but the pld$\delta$ single mutation did not (Fig. 7). Together, these results suggest that PLD$\alpha$1 and PLD$\delta$ have somewhat different roles in ABA signaling.

PLD$\alpha$1 is located in the cytosol and plasma membrane and prefers PC to PE as a substrate (Fan et al., 1999; Li et al., 2009), whereas PLD$\delta$ is mainly located at the plasma membrane and prefers PE to PC (Gardiner et al., 2001; Qin et al., 2002; Li et al., 2009). These differences in phenotype between the pld$\alpha$1 and pld$\delta$ mutants thus may be due to differences in the location of PA production and the molecular species of the produced PA between PLD$\alpha$1 and PLD$\delta$.

Moreover, PLD$\alpha$1 activity is regulated by the binding of PLD$\alpha$1 with the G protein $\alpha$-subunit GPA1 at a DRY motif (Zhao and Wang, 2004), while PLD$\delta$ potentially interacts with G protein because it contains a DRY motif and a hydrophobic motif, which are highly conserved in G protein-binding proteins. Hence, the difference in affinity for G protein may also be responsible for the phenotype differences between the pld$\alpha$1 and pld$\delta$ mutants.

The PLD$\alpha$1 gene is constitutively expressed even under drought and saline conditions, whereas the PLD$\delta$ gene is inductively expressed by dehydration and salinity (Katagiri et al., 2001). Antisense suppression of PLD$\alpha$1 increases PLD$\delta$ gene expression (Mane et al., 2007). Our study shows that expression of the PLD$\alpha$1 gene was constitutive regardless of ABA treatment and that expression of the PLD$\delta$ gene was ABA inducible (Fig. 9). This suggests that PLD$\delta$ activity is regulated at the transcriptional level in the stomatal response to ABA. Thus, the pld$\alpha$1 single mutant phenotype is susceptible to a change in PLD$\delta$ expression that is influenced by growth conditions. As a result, we could see both ABA-sensitive and ABA-hyporesponsive phenotypes in the pld$\alpha$1 single mutant. By contrast, PLD$\alpha$1 activity may be posttranslationally regulated by other factors, such as GPA1. In other words, PLD$\alpha$1 may mainly function under moderate environmental stress conditions and PLD$\delta$ may cooperatively work with PLD$\alpha$1 under severe environmental stress conditions.

Other Physiological Functions of PLD$\alpha$1 and PLD$\delta$ in Response to ABA

In this study, the inhibitory effect of ABA on seed germination was slightly reduced in both of the single mutants and strongly reduced in the double mutant (Fig. 8), in agreement with the result of Katagiri et al. (2005) that an accumulation of PA facilitates the inhibition of seed germination by ABA in Arabidopsis. However, in Oryza sativa, PLD$\beta$1 mutation mitigates the inhibition of germination by ABA (Li et al., 2007). It is unknown whether the mutation also reduces PA production.

In this study, root growth was inhibited by ABA in a dose-dependent manner in the wild type and the pld mutants (data not shown), while a reduction of PA production due to the pld$\alpha$1 pld$\delta$ double mutation increased the sensitivity to hyperosmotic and salt stress in Arabidopsis roots (Bargmann et al., 2009). In roots, PLD$\alpha$1 and PLD$\delta$ appear to be involved in the responses to salinity and hyperosmolarity but not in the response to ABA.

CONCLUSION

Our results show that PLD$\alpha$1 and PLD$\delta$ cooperatively function upstream of the production of ROS and NO and the cytosolic alkalization in ABA signaling of Arabidopsis guard cells.

MATERIALS AND METHODS

Plant Materials, Growth, and Transformation

Arabidopsis (Arabidopsis thaliana) wild type (Columbia-0) as well as pld$\alpha$1, pld$\delta$, and pld$\alpha$1 pld$\delta$ mutants were grown in a growth chamber at 22°C and 60% humidity with a 16-h light period with 80 µmol m$^{-2}$ s$^{-1}$ photon flux density and 8 h of dark. Water containing 0.1% Hypoxen was applied two times in 1 week on the plant growth tray. [Ca$^{2+}$]$_{cyt}$ in guard cells was measured using a Ca$^{2+}$-sensing fluorescent protein, YC3.6 (Nagai et al., 2004; Mori et al., 2006). To obtain YC3.6-expressing plants, wild-type and pld$\alpha$1 pld$\delta$ double mutant plants were crossed with a Columbia-0 plant that had previously been transformed with YC3.6.

For a germination test, 100 seeds of the same age were sown on germination medium agar plates (Katagiri et al., 2001) supplemented with 1% (w/v) Suc. Germination was defined as the emergence of the radicle.
Measurement of Stomatal Aperture

Stomatal apertures were measured as described previously (Hossain et al., 2011). Briefly, excised rosette leaves were floated on an assay solution containing 5 mM KCl, 50 µM CaCl₂, and 10 mM MES-Tris, pH 6.15, for 2 h in the light to induce stomatal opening followed by the addition of ABA or PA or H₂O₂. After a 2-h incubation, the leaves were shredded in a commercial blender for 30 s, and the remaining epidermal tissues were collected using nylon mesh. For stomatal opening, excised rosette leaves were floated on the assay solution for 2 h in the dark to induce stomatal closure. These leaves were transferred in the light for 3 h with ABA. The leaves were shredded for 30 s, and the remaining epidermis was collected. For each sample, 20 stomatal apertures were measured.

Isolation of MCPs and GCPs

MCPs and GCPs were enzymatically isolated from 4-week-old Arabidopsis plants as described previously (Leonhardt et al., 2004).

RNA Extraction and Real-Time PCR

Total RNA was isolated from whole leaves, isolated MCPs, and GCPs using Trizol reagent (Invitrogen). cDNA was prepared from 1 µg of RNA using Moloney murine leukemia virus reverse transcriptase (Takara) and oligo(dT) primers according to the manufacturer’s instructions. PCR was performed with 1 µL of RT reaction mixture containing BIOTAQ DNA polymerase (Biotien) and primer sets as follows: PLDα1 (At5g13570), 5′-GGCCGGCTGTTTGATGATTGCCTC-3′ and 5′-TTCGACCCGTTCACTGCTACTCT-3′; PLDβ1 (At4g35790), 5′-CTGGCCCTGTCAGAGAAA-3′ and 5′-TTGCTTAACATACATACATCATC-TGC-3′; and RD29B (At5g52300), 5′-AGAAGGAATGGTGCCGAAGAAG-3′ and 5′-CACTACTTCCTACCCGGAAAT-3′. Arabidopsis ACTIN2 (At5g18708) was amplified as an internal control using primer set 5′-CTCGAGA-GATTCAGATGCCCA-3′ and 5′-AAGCATGGGATGGGAAGTGG-3′, which were based on GenBank accession number At4g35790, 5′-AAAGGTTGTAA-3′ and 5′-CACCATGGCG-3′. Arabidopsis ACTIN2 (At5g18708) was amplified as an internal control using primer set 5′-AACATGCGTGAGGAATGG-3′ and 5′-CCATAGACGTGCGAAGTT-3′. Primers were constructed on exons of the gene that interfere with an intron. Amplified DNA products were separated using an agarose gel followed by image analysis with LAS-3000 (GE Healthcare).

Quantitative real-time PCR was performed with an MX3000P QPCR System (Agilent Technologies) using SYBR Green Brilliant II QPCR Master Mix; Stratagene) to monitor double-stranded DNA synthesis. A standard curve was constructed for each gene using gene fragments that had been previously amplified and quantified. The levels of gene transcript were normalized to that of ACTIN2 and expressed relative to the amounts observed under control conditions.

Generation of Complement Transgenic Plants in plda pldβ

PLDA1 was amplified by PCR from Arabidopsis leaf cDNA using primers 5′-CACCATGCGCAGACATCTGTCACCGG-3′ and 5′-AAAGGTGTTAAGGATGAGGCAAGTG-3′, which were based on GenBank accession At5g13570. Similarly, PLD8 was amplified using primers 5′-CACCATGGCGCAGAAAGATCGGAGGACCC-3′ and 5′-AAACCTGTTATTAGGATCGGTAACG-3′, which were based on At4g35790. These primer sequences were modified to allow insertion in Gateway binary vectors. Amplified DNA fragments were cloned into pENTR/D-TOPO vector (Invitrogen). The final sequence was confirmed by sequencing with an ABI310 sequencer (ABI). The resulting entry clones were introduced into Gateway binary vector pGWB2 (Nakagawa et al., 2007) following the manufacturer’s instructions. The plda1 pldβ double mutant was transformed using a floral dip procedure (Clough and Bent, 1998), and transformed plants were confirmed to be carrying the transgene by growth on kanamycin- and hygromycin-containing Murashige and Skoog agar medium.

Measurement of ROS and NO Production

ROS production in guard cells was analyzed using H₂DCF-DA (Munemasa et al., 2011). The discs and epidermal tissues were incubated in MES-KOH buffer without [³²P]orthophosphoric acid for 12 h. For the control, ³²P-labeled leaf discs were transferred into the MES-KOH buffer without [³²P]orthophosphoric acid. For dehydration treatment, dry ³²P-labeled leaf discs were incubated in an Essendorf tube for 2 h. For ABA treatment, ³²P-labeled leaf discs were transferred into MES-KOH buffer supplemented with 50 µM ABA and then incubated for 2 h. The ³²P-labeled epidermal tissues were collected by centrifugation and washed with MES-KOH buffer. The corrected epidermal tissues were incubated in MES-KOH buffer in the absence or presence of...
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50 μM ABA for 2 h. After each treatment, an equal volume of MES-KOH buffer supplemented with 0.75% (v/v) 1BuOH was added to each sample. After a 10-min incubation, the reaction was stopped by the addition of 10 μL of 60% (w/v) HClO₄. The mixture was incubated in liquid nitrogen for 1 min, and then lipids were extracted from leaf discs and epidermal tissues (Katagiri et al., 2001).

Lipid Extraction and Analysis

Lipids were extracted with the addition of 3.75 volumes of CHCl₃:methanol: HCl (50:001:0, v/v) and then with an equal volume of 0.9% (w/v) NaCl and 3.75 volumes of CHCl₃, as described previously (Katagiri et al., 2001). The lower organic phase was washed with 3.75 volumes of methanol:water: HCl (50:0:1, v/v). Lipids were dried by vacuum centrifugation, resolved in CHCl₃, and separated on a silica-gel 60 thin-layer chromatography plate (Merck) in an ethyl acetate solvent system with an organic upper phase of ethyl acetate:2,4-trimethylpentane:formic acid:water (13:2:3:10, v/v).

Statistical Analysis

The significance of differences between mean values of stomatal aperture and root growth were assessed by Student’s t test and two-factor factorial ANOVA. The frequency of [Ca²⁺]ᵢₓ oscillations was assessed by χ² test. Differences were considered significant at P < 0.05.

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: PLDα1, At4g15730; PLDδ, At4g35790; KAT1, At5g46240; RD29B, At5g52300; ACTIN2, At1g18780.

Supplemental Data

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

Supplemental Figure S1. Effects of exogenous H₂O₂ (100 μM) on stomatal aperture in the wild type (WT) and plδ mutants.

Supplemental Figure S2. Effects of exogenous PA (50 μM) on the production of ROS and NO and cytosolic alkalization in guard cells of the wild type (WT) and plδ mutants.

Supplemental Figure S3. Effects of PA (50 μM) on K⁺ in channel currents of guard cells of the wild type.

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