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, PLD\(\alpha\)1 and PLD\(\delta\), in ABA signaling in guard cells, we analyzed ABA responses in guard cells using Arabidopsis wild type, pld\(\alpha\)1 and pld\(\delta\) single mutants, and a pld\(\alpha\)1 pld\(\delta\) double mutant. ABA-induced stomatal closure was suppressed in the pld\(\alpha\)1 pld\(\delta\) double mutant but not in the pld single mutants. The pld\(\alpha\)1 and pld\(\delta\) mutations reduced ABA-induced phosphatidic acid production in epidermal tissues. Expression of either PLD\(\alpha\)1 or PLD\(\delta\) complemented the double mutant stomatal phenotype. ABA-induced stomatal closure in both pld\(\alpha\)1 and pld\(\delta\) single mutants was inhibited by a PLD inhibitor (1-butanol), suggesting that both PLD\(\alpha\)1 and PLD\(\delta\) 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 alkalinization, but these changes are reduced in guard cells of the pld\(\alpha\)1 pld\(\delta\) double mutant. Inward-rectifying K\(^+\) channel currents of guard cells were inhibited by ABA in the wild type but not in the pld\(\alpha\)1 pld\(\delta\) double mutant. ABA inhibited stomatal opening in the wild type and the pld\(\delta\) mutant but not in the pld\(\alpha\)1 mutant. In wild-type rosette leaves, ABA significantly increased PLD\(\delta\) transcript levels but did not change PLD\(\alpha\)1 transcript levels. Furthermore, the pld\(\alpha\)1 and pld\(\delta\) mutations mitigated ABA inhibition of seed germination. These results suggest that PLD\(\alpha\)1 and PLD\(\delta\) 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 phosphatidic 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, \(\alpha, \beta, \gamma, \delta, e,\) and \(\zeta\) (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 PLD\(\alpha\)1 positively regulates ABA-induced stomatal closure (Zhang et al., 2004) and the inhibition of stomatal opening by ABA (Mishra et al., 2006). Furthermore, PLD\(\alpha\)1 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 pld\(\alpha\)1 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.

PLD\(\delta\) is involved in responses to drought and salinity stress (Katagiri et al., 2001) and cold stress (Li et al., 2004). PLD\(\alpha\)1 and PLD\(\delta\) are required for tolerance to high salinity and hyperosmotic stress (Bargmann et al., 2009). Therefore, we hypothesized that PLD\(\delta\) functions cooperatively with PLD\(\alpha\)1 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), Ca\(^{2+}\), 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 ([Ca\(^{2+}\)]\(_{cyt}\)) oscillation/elevation.
and cytosolic alkalization (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 Ca\(^{2+}\) elevation, and (3) ABA inhibition of inward-rectifying K\(^{+}\) (K\(_{\text{in}}\)) 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, pldα1 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 pldα1 and pldα1 pldδ mutants, and the transcripts of PLDδ were not detected in the pldδ and pldα1 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 pldα1, P < 10\(^{-3}\) for pldδ)

![Figure 1](image-url)
Complementation of the Stomatal Phenotype of the \( plda1 \) \( pldδ \) Double Mutant with PLDa1 or PLDδ

To test whether the expression of PLDa1 or PLDδ complements the stomatal phenotype of the double mutant, we generated the \( plda1 \) \( pldδ \) mutants transformed with PLDa1 or PLDδ. PLDa1 and PLDδ 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 PLDδ are responsible for the ABA-insensitive phenotype observed in the \( plda1 \) \( pldδ \) mutants.

PA-Induced Stomata Closure in \( pld \) Mutants

To confirm the function of PLDs in ABA signaling in guard cells, we examined the effects of a PLD inhibitor, 1-BuOH, on ABA-induced stomatal closure. ABA at 1 \( \mu \)M closed stomata of \( plda1 \) and \( pldδ \) 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 \( plda1 \) and \( pldδ \) mutants. PA at 10 and 50 \( \mu \)M induced stomatal closure in the wild type and the \( pld \) mutants (Fig. 3B), suggesting that downstream of PA production in the ABA signal cascade is intact in the \( pld \) mutants.

Abolishment of ABA-Induced Production of ROS and NO and Cytosolic Alkalization in \( plda1 \) \( pldδ \) Guard Cells

We measured ABA-induced ROS accumulation in guard cells using a hydrogen peroxide (H2O2)-sensitive fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-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 \( plda1, P < 0.02\) for \( pldδ \)), 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 H$_2$O$_2$ in the pld mutants was examined. Application of 100 $\mu$M H$_2$O$_2$ 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 ($P < 0.03$), pld$d$ ($P < 10^{-3}$), and plda1 pld$d$ ($P < 0.03$) mutants (Supplemental Fig. S1). These results indicate that PLDa1 and PLD$d$ 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 pld$d$ 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 pld$d$ 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 pld$d$ 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 Ca$^{2+}$ Oscillations in plda1 pld$d$ Guard Cells

We monitored [Ca$^{2+}$]$_{cyt}$ in guard cells using a Ca$^{2+}$-sensing fluorescent protein, Yellow Cameleon 3.6 (YC3.6). When the wild-type guard cells were treated with 10 $\mu$M ABA, 83% of the guard cells showed [Ca$^{2+}$]$_{cyt}$ transient elevation(s), hereafter [Ca$^{2+}$]$_{cyt}$ oscillation ($n = 30$; Fig. 5, A and C). When the double mutant guard cells were treated with 10 $\mu$M ABA, 87% of the guard cells showed [Ca$^{2+}$]$_{cyt}$ oscillations ($n = 32$; Fig. 5, B and C). The frequency of [Ca$^{2+}$]$_{cyt}$ oscillations was not significantly different between the wild type and the double mutant, suggesting that neither PLDa1 nor PLD$d$ is involved in [Ca$^{2+}$]$_{cyt}$ oscillation in guard cells in response to ABA.

ABA Inhibition of K$^{+}$in Channel Currents in Guard Cells

In the absence of ABA, K$^{+}$in 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$^{+}$in 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 PLD$d$ are involved in the inhibition of the K$^{+}$in 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 $\mu$M inhibited stomatal opening in the wild type ($P < 0.02$) and the pld$d$ single mutant ($P < 0.01$) but did not inhibit it in the plda1 single mutant ($P = 0.76$) or the plda1 pld$d$ double mutant ($P = 0.15$; Fig. 7). On the other hand, 10 $\mu$M ABA slightly inhibited stomatal opening in the plda1 single mutant and the plda1 pld$d$ double mutant. These results suggest that PLD$d$ 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 PLD$d$ 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 PLD$d$ 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 PLDδa1, PLDδd, 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 PLDδa1 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 PLDδa1 is constitutive and that transcription of PLDδδ is ABA inducible. They also suggest that PLDδ functions in ABA signaling by regulating gene transcription.

DISCUSSION

Cooperative Function of PLDδa1 and PLDδδ 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),
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 PLDa1 but also PLDδ positively regulate ABA-induced stomatal closure. The disruption by the double mutation was complemented by the expression of PLDa1 or PLDδ by the 35S promoter, suggesting that PLDa1 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.

PLDa1 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; Garcia-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, B and C), 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 H2O2 (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 ab1 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.

[Ca2+]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 Ca2+, since these PLDs contain a conserved C2 domain that participates in Ca2+/phospholipid binding (Li et al., 2009), suggesting that PLD activities are affected by [Ca2+]cyt elevation in guard cells. PA production and [Ca2+]cyt oscillation/elevation may occur not only in tandem but also in parallel in ABA signaling in guard cells.

In this study, ABA induced [Ca2+]cyt oscillation but not ROS production in the pldα1 pldδ double mutant (Fig. 5). These results contradict the current ABA-signaling model, in which Ca2+-permeable cation channels in the plasma membrane are activated by H2O2 (Pei et al., 2000; Kwak et al., 2003). However, long-term Ca2+ programmed stomatal closure requires stimulus-specific calcium oscillations; that is, certain specific Ca2+ signatures inhibit stomatal reopening after Ca2+ (reactive) stomatal closure (Allen et al., 2000, 2001). Therefore, the H2O2-independent [Ca2+]cyt oscillations in the pldα1 pldδ double mutant may have failed to induce stomatal closure and may be attributed to a malfunction of [Ca2+]cyt homeostasis due to the double mutation. Furthermore, spatiotemporal modulation of ROS production and/or differences of pattern of [Ca2+]cyt elevation are important in guard cell ABA signaling (Allen et al., 2001; Jannat et al., 2011a, 2011b). Spatiotemporal analysis of ROS production and [Ca2+]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\textsuperscript{+} Channel Currents by ABA in GCPs

Three second messengers, H\textsubscript{2}O\textsubscript{2}, NO, and sphingosine-1-phosphate, have been reported to inhibit K\textsuperscript{+} 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\textsuperscript{+} channel currents of V. faba GCPs (Jacob et al., 1999). Our findings that exogenous PA inhibited K\textsuperscript{+} channel currents in Arabidopsis GCPs (Supplemental Fig. S3) and that ABA strongly inhibited K\textsuperscript{+} channel currents of wild-type GCPs but not of the plda1 pld\textdelta GCPs (Fig. 6) suggest that, in ABA signaling, PLD\textalpha and PLD\textdelta are also involved in the inhibition of K\textsuperscript{+} channel currents.

PLD\textalpha and PLD\textdelta Differentially Function in ABA Signaling in Guard Cells

In this study, we observed several differences of phenotype between the plda1 and pld\textdelta single mutants. In the case of ABA-induced stomatal closure, the plda1 single mutation partially impaired stomatal closure induced by 1 \mu M ABA but the pld\textdelta single mutation did not (Fig. 1D). ROS production by ABA in the plda1 single mutant was smaller than that in the pld\textdelta single mutant, even though the difference was not significant (Fig. 4A). Similarly, ABA-induced PA production in the plda1 single mutant was less than that in the pld\textdelta single mutant (Fig. 1F). Moreover, the plda1 single mutation weakened the ABA inhibition of light-induced stomatal opening but the pld\textdelta single mutation did not (Fig. 7). Together, these results suggest that PLD\textalpha and PLD\textdelta have somewhat different roles in ABA signaling.

PLD\textalpha is located in the cytosol and plasma membrane and prefers PC to PE as a substrate (Fan et al., 2001; Qin et al., 2002; Li et al., 2009). These differences in phenotype between the plda1 and pld\textdelta mutants thus may be due to differences in the location of PA production and the molecular species of the produced PA between PLD\textalpha and PLD\textdelta.

Moreover, PLD\textalpha activity is regulated by the binding of PLD\textalpha with the G protein \alpha-subunit GPA1 at a DRY motif (Zhao and Wang, 2004), while PLD\textdelta 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 plda1 and pld\textdelta mutants.

The PLD\textalpha gene is constitutively expressed even under drought and saline conditions, whereas the PLD\textdelta gene is inductively expressed by dehydration and salinity (Katagiri et al., 2001). Antisense suppression of PLD\textalpha increases PLD\textdelta gene expression (Mane et al., 2007). Our study shows that expression of the PLD\textalpha gene was constitutive regardless of ABA treatment and that expression of the PLD\textdelta gene was ABA inducible (Fig. 9). This suggests that PLD\textdelta activity is regulated at the transcriptional level in the stomatal response to ABA. Thus, the plda1 single mutant phenotype is susceptible to a change in PLD\textdelta expression that is influenced by growth conditions. As a result, we could see both ABA-sensitive and ABA-hyporesponsive phenotypes in the plda1 single mutant. By contrast, PLD\textalpha activity may be posttranslationally regulated by other factors, such as GPA1. In other words, PLD\textalpha may mainly function under moderate environmental stress conditions and PLD\textdelta may cooperatively work with PLD\textalpha under severe environmental stress conditions.

Other Physiological Functions of PLD\textalpha and PLD\textdelta 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\textbeta1 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\textalpha mutants (data not shown), while a reduction of PA production due to the plda1 pld\textdelta double mutation increased the sensitivity to hyperosmotic and salt stress in Arabidopsis roots (Bargmann et al., 2009). In roots, PLD\textalpha and PLD\textdelta appear to be involved in the responses to salinity and hyperosmolarity but not in the response to ABA.

CONCLUSION

Our results show that PLD\textalpha and PLD\textdelta 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 plda1, pld\textalpha, and plda1 pld\textdelta mutants were grown in a growth chamber at 22°C and 60% humidity with a 16-h light period with 80 \mu mol m\textsuperscript{-2} s\textsuperscript{-1} photon flux density and 8 h of dark. Water containing 0.1% Hypoxone was applied two to three times in 1 week on the plant growth tray. [Ca\textsuperscript{2+}]\text{cyt} in guard cells was measured using a Ca\textsuperscript{2+}-sensing fluorescent protein, YC3.6 (Nagai et al., 2004; Mori et al., 2006). To obtain YC3.6-expressing plants, wild-type and plda1 pld\textdelta 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 using BIOTaq DNA polymerase (Bioline) and primer sets as follows: PLD458 (At4g35790), 5'-GCAACGGCTTTGCATATTGATCTAG-3'; 5'-TTGCCACCGTCACTGACCTC-3'; PLD6 (At4g35790), 5'-CTGGCCCTGCAAGAAAAG-3' and 5'-TTGCTAACAACATACATCATCTGC-3'; and RD29B (At5g52300), 5'-AGAAGAAGGCTGCTGGGGAAAG-3' and 5'-CAACTCTAATCCGACCAGAAT-3'. Amplified DNA sequence was confirmed by sequencing with an ABI310 sequencer (ABI). The PCR products were modified to allow insertion in Gateway binary vectors. Amplified DNA was used for the measurement of guard cell [Ca2+]i oscillations as described previously (Islam et al., 2010a, 2010b; Hossain et al., 2011). The abaxial side of an excised leaf was gently mounted on a glass slide with a medical adhesive (stock no. 7730; Hollister) followed by removal of the adaxial epidermis and the mesophyll tissue with a razor blade in order to keep the lower epidermis intact on the slide. The remaining abaxial epidermis was incubated in a solution containing 5 mM KCl, 50 μM CaCl2, and 10 mM MES-Tris (pH 6.15) under light for 2 h at 22°C to promote stomatal opening. Turgid guard cells were used to measure [Ca2+]i oscillations. Guard cells were treated with 10 μM ABA using a peristaltic pump at 5 min after monitoring. For dual-emission ratio imaging of YC3.6, we used a 440AF21 excitation filter, a 445DRLP dichroic mirror, a 480DF30 emission filter for cyan fluorescent protein (CFP), and a 535DF25 emission filter for yellow fluorescent protein (YFP). The CFP and YFP fluorescence intensities of guard cells were imaged and analyzed using the W-View system and AQUA COSMOS software (Hamamatsu Photonics). CFP and YFP fluorescence were simultaneously monitored following simultaneous excitation of CFP and YFP.

Whole-Cell Patch-Clamp Recording of K+ Channel Currents

Arabidopsis GCPs were enzymatically isolated from rosette leaves of 4- to 6-week-old plants as described previously (Nagamasa et al., 2007). Whole-cell currents were measured using a patch-clamp amplifier (model CEZ-2200; Nihon Kohden). Data were analyzed with pCLAMP 8.2 software (Molecular Devices). The pipette solution contained 30 mM KCl, 70 mM K-Glu, 2 mM MgCl2, 3.35 mM CaCl2, 6.7 mM EGTA, and 10 mM HEPES adjusted to pH 7.1 with Tris, and the bath solution contained 30 mM KCl, 2 mM MgCl2, 40 mM CaCl2, and 10 mM MES titrated to pH 5.5 with Tris (Saito et al., 2008). Osmolarity of the pipette solution and the bath solution was adjusted with d-sorbitol to 500 and 485 mmol kg−1, respectively. In order to examine the effect of ABA, GCPs were treated with 10 μM ABA for 2 h before recordings.

32P Labeling of Phospholipids of Arabidopsis Leaf Discs and Epidermis

Phospholipids were labeled with 32P as described previously (Katagiri et al., 2001). Leaf discs with a diameter of 3 mm and epidermal tissues were prepared from 3- to 4-week-old Arabidopsis plants. The discs and epidermal peels were incubated in MES-KOH buffer (pH 5.6) supplemented with 3.7 MBq mL−1 [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 Eppendort 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 incubated for 30 min at room temperature, and then the excess dye was washed out with the assay solution. Collected tissues were again incubated with solution and 50 μM ABA or 50 μM PA for 20 min in the dark condition. The image was captured using a fluorescence microscope (Bio Zero BZ-8000; KEYENCE), and the pixel intensity of the fluorescence in guard cells was measured using ImageJ 1.42q (National Institutes of Health). For ABA- and PA-induced NO detection in guard cells, 10 μM 4,5-diaminofluorescein-2 diacetate was added instead of 50 μM H2DCF-DA (Munemasa et al., 2011).
Lipid Extraction and Analysis

Lipids were extracted from Arabidopsis leaves and epidermal tissues using a modified Bligh-Dyer procedure. The tissues were homogenized in methanol and chloroform, and the lipids were then extracted with chloroform:methanol:water (13:3:1, v/v) and separated on a silica-gel 60 thin-layer chromatography plate (Merck) in an ethyl acetate:hexane:formic acid:water (13:2:3:10, v/v). The lipids were dried by vacuum centrifugation, dissolved in chloroform, and separated on a silica-gel 60 thin-layer chromatography plate (Merck) in an ethyl acetate:hexane:formic acid:water (13:2:3:10, v/v).

Statistical Analysis

The significance of differences between mean values of stomatal aperture and root growth was assessed by Student’s t-test and two-factor factorial ANOVA. The frequency of [Ca^{2+}]-related oscillations was assessed by χ² test. Differences were considered significant at P < 0.05.

Supplemental Data

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

Supplemental Figure S1. Effects of exogenous H2O2 (100 μM) on stomatal aperture in the wild type (WT) and pld 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 pld 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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Involvement of PLDδ in ABA Signaling in Guard Cells

50 μM ABA for 2 h. After each treatment, an equal volume of MES-KOH buffer supplemented with 0.75% (v/v) 1-BuOH 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).


