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ORIGINAL ARTICLE

# Phospholipase Dδ is involved in nitric oxide-induced stomatal closure

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**Abstract** Nitric oxide (NO) has recently emerged as a second messenger involved in the complex network of signaling events that regulate stomatal closure. Little is known about the signaling events occurring downstream of NO. Previously, we demonstrated the involvement of phospholipase D (PLD) in NO signaling during stomatal closure. PLDδ, one of the 12 *Arabidopsis* PLDs, is involved in dehydration stress responses. To investigate the role of PLDδ in NO signaling in guard cells, we analyzed guard cells responses using *Arabidopsis* wild type and two independent *pldδ* single mutants. In this work, we show that *pldδ* mutants failed to close the stomata in response to NO. Treatments with phosphatidic acid, the product of PLD activity, induced stomatal closure in *pldδ* mutants. Abscisic acid (ABA) signaling in guard cells involved $H_2O_2$ and NO production, both required for ABA-induced stomatal closure. *pldδ* guard cells produced similar NO and $H_2O_2$ levels as the wild type in response to ABA. However, ABA- or $H_2O_2$-induced stomatal closure was impaired in *pldδ* plants. These data indicate that PLDδ is downstream of NO and $H_2O_2$ in ABA-induced stomatal closure.

**Keywords** Abscisic acid · Hydrogen peroxide · Nitric oxide · Phospholipase Dδ · Stomatal closure

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| AU | Arbitrary unit |
| DAF-2-DA | 4,5-Diaminofluorescein diacetate |
| GSNO | S-nitrosoglutathione |
| $H_2$DCFDA | 2′,7′,-Dichlorofluorescein diacetate |
| NO | Nitric oxide |
| PA | Phosphatidic acid |
| PE | Phosphatidylethanolamine |
| PLD | Phospholipase D |
| ROS | Reactive oxygen species |

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## Introduction

Plants regulate gas exchange through stomata, which are pores located in the epidermis of the aerial part of the plant. The stomatal pore is bounded by a pair of specialized cells, called guard cells, which regulate the pore size through reversible volume changes. To regulate stomatal movements, guard cells sense and rapidly respond to several signals such as light, $CO_2$ and humidity, and to the hormones abscisic acid (ABA), auxin, methyl jasmonate and ethylene (Kim et al. 2010). ABA plays a crucial role in several plant stress responses, including cold, drought and salinity (Mahajan and Tuteja 2005). Under drought stress, ABA promotes stomatal closing and inhibits stomatal opening, thereby reducing transpiration and water loss. Stomatal closure is, therefore, an essential event for plant survival upon drought stress conditions. The signal transduction network triggered by ABA is one of the best characterized signaling processes in guard cells (Fan et al. 2004). Among the second messengers characterized to participate in ABA-induced stomatal closure are nitric oxide (NO), $H_2O_2$ and phospholipase D (PLD)-derived phosphatidic acid (PA) (Jacob et al. 1999; Pei et al. 2000; Garcia-Mata and Lamattina 2002).

NO is a key signaling molecule involved in plant responses to biotic and abiotic stresses (Beligni and

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Lamattina 2001; Floryszak-Wieczorek et al. 2007). Evidences from different groups indicate that NO donors promote stomatal closure (Garcia-Mata and Lamattina 2001; Neill et al. 2002) and increase plant tolerance to drought stress (Garcia-Mata and Lamattina 2001). Endogenous NO synthesis is triggered during the induction of stomatal closure by ABA, methyl jasmonate, UV-B light, and bicarbonate (Neill et al. 2002; Garcia-Mata and Lamattina 2002; Suhita et al. 2004; He et al. 2005; Kolla and Raghavendra 2007). Using pharmacological and genetic approaches, it has been demonstrated that ABA-induced NO production requires $H_2O_2$ generation (Bright et al. 2006). Once produced, NO induces an increase in cytosolic $Ca^{2+}$ concentration and inhibition of inward-rectifying $K^+$ ($K^+_{in}$) channels through cGMP/cADPR-dependent processes (Garcia-Mata et al. 2003).

PA has been implicated as a second messenger during responses to stress (Li et al. 2009). PLD, which hydrolyses structural phospholipids to PA and free head groups, is a family of widely studied phospholipases in plants. *Arabidopsis* has 12 PLD genes grouped into 6 types, PLDα(3), *β*(2), *γ*(3), *δ*, *ε* and *ζ*(2), based on structural and biological properties (Elias et al. 2002). PLD has been involved in cold, drought and salinity stress responses (Li et al. 2009). Also, it was shown that PLD-derived PA mediates ABA responses (Jacob et al. 1999; Zhang et al. 2004). In addition, we recently showed that PLD activation is required for NO-induced stomatal closure in *Vicia faba* guard cells (Distéfano et al. 2008). Only two AtPLDs isoenzymes, PLDα1 and PLD*δ*, have been related to stomatal closure and dehydration stress (Sang et al. 2001; Katagiri et al. 2001; Zhang et al. 2009; Uraji et al. 2012).

Previous reports showed that *pldα1* mutant plants (*pldα1*) are (i) insensitive to ABA-induced stomatal closure (Sang et al. 2001; Zhang et al. 2004), (ii) impaired in $H_2O_2$ and NO production in ABA-treated guard cells (Zhang et al. 2009), and (iii) show less PA levels in response to dehydration treatments (Sang et al. 2001; Zhang et al. 2004, 2009; Bargmann et al. 2009b). Recent evidences indicate that PLDα1 is upstream of NO production (Zhang et al. 2009). Interestingly, our recent report showed that NO induces PA formation via PLD activation (Distéfano et al. 2008). Since AtPLDα1 is upstream of NO-induced stomatal closure (Zhang et al. 2009), but PLD activation is downstream of NO (Distéfano et al. 2008), we hypothesized that another PLD isoenzyme could be activated by NO.

PLD*δ* is activated in response to $H_2O_2$ (Zhang et al. 2003), dehydration (Katagiri et al. 2001), cold (Li et al. 2004), and salt stress (Bargmann et al. 2009b). It is associated with the plasma membrane (Wang and Wang 2001) and has been suggested to be the microtubule-binding PLD in *Arabidopsis* (Gardiner et al. 2003; Andreeva et al. 2009; Ho et al. 2009). *PLDδ* mRNA levels increase in response to severe dehydration, high salts concentration (Katagiri et al. 2001) and in ABA-treated guard cell protoplasts (Wang et al. 2011). In the present work, we study the involvement of PLD*δ* in NO-induced stomatal closure.

## Materials and methods

### Plant material and growth conditions

The knock-out PLD mutants were isolated from *Arabidopsis thaliana* Columbia-0 (wild type) ecotype. *pldδ* knock-out T-DNA insertion line SALK_023247 (*pldδ-1*), *pldα1* knock-out T-DNA insertion line SALK_067533 (*pldα1*), and the double-mutant *pldα1/pldδ-1* were kindly provided by Dr. Munnik. An independent Col-0 T-DNA insertion line for *pldδ*, SALK_092469 (*pldδ-3*), was obtained from the Ohio State University Arabidopsis Biological Resources Center (ABRC). Homozygous line T-DNA insertion was verified by PCR, and knock-out null mutants was confirmed by RT-PCR (Fig. 1). Seeds were germinated in soil:vermiculite:perlite, 1:1:1, by weight and kept at 4 °C for 2 days. Then, they were grown at 25 °C using a 16-h photoperiod at 200 μmol $s^{-1}$ $m^{-2}$. Four-week-old plants were used to do the experiments.

The following primers were used to verify *pldδ* and *pldα1* knock-out plants:

| | |
|---|---|
| fw1 | 5′-TGTACTCGGTGCTTCGGGAAA-3′ |
| fw2 | 5′-CCGCTTTTATTGGAGGTCTGGATCTTT GTGATGGC-3′ |
| fw3 | 5′-CCGTTCTATCGACTCAGGGTCCGTGA AAGGA-3′ |
| rv1 | 5′-GCCATCACAAAGATCCAGACCTCCAA TAAAAGCGG-3′ |
| rv2 | 5′-TCCTTTCACGGACCCTGAGTCGATA GAACGG-3′ |
| rv3 | 5′-TCACCTGCAGTGGTTAAAGTGTCAGG-3′ |
| fw4 | 5′-GACGATGAATACATTATCATTG-3′ |
| rv4 | 5′-GTCTGAGCTGCAGTTGTAAGGATTGGAG GC-3′ |
| rv5 | 5′-TGAGTCCAAAGGTACATAACAAC-3′ |
| LB | 5′-TGGTTCACGTAGTGGGCCATCG-3′ |

LB was used in combination with fw1 to analyze the presence of the T-DNA insertion in *pldδ-1*. LB was used in combination with rv2 to analyze the presence of the T-DNA insertion in *pldδ-3*. LB was used in combination with rv4 to analyze the presence of the T-DNA insertion in *pldα1*. fw1 was used in combination with rv1 to verify the homozygosis of the *pldδ-1* line, the combination fw2/rv2 to verify the homozygosis of the *pldδ-3* line, and fw4 was used in combination with rv4 to verify the homozygosis of

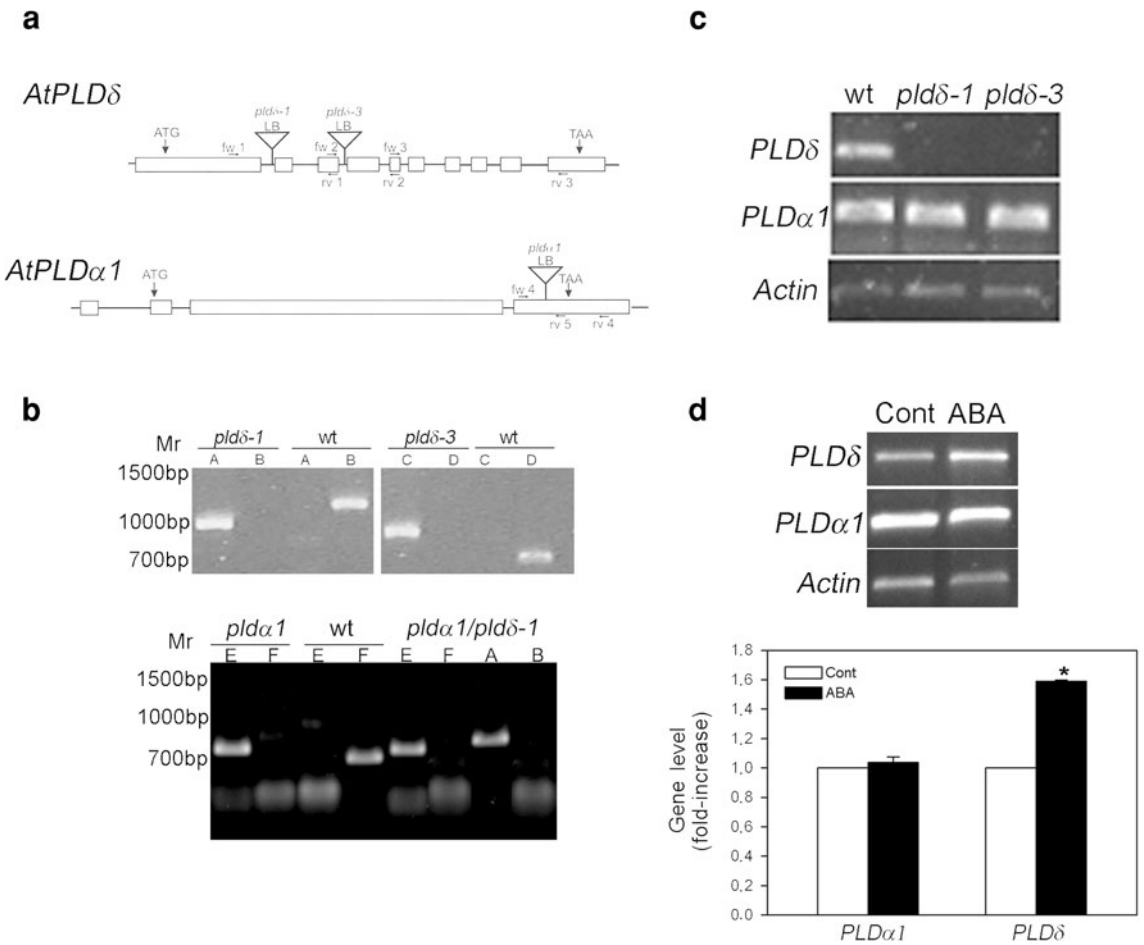


**Fig. 1** *PLDδ* and *PLDα1* expression and characterization of PLD knock-out lines. **a** Gene structure of *AtPLDδ* and At*PLDα1*. *Boxes* represents exons, *triangle* represents the T-DNA insertion lines *pldδ-1* (SALK_023247), *pldδ-3* (SALK_092469) and *pldα1* (SALK_067533). *Arrows* indicate primers used to check mutants. Drawing is approximately to scale. **b** Genomic DNA was extracted from wild-type (*wt*; Col-0), *pldδ-1, pldδ-3*, *pldα1* and the double mutant *pldα1/pldδ-1* plants. The presence of the T-DNA insertion was verified by PCR using primer combinations fw1/LB (**a**), LB/rv2 (**c**), LB/rv4 (**e**) (see Table 1). Homozygosis was verified by PCR using *PLDδ* or *PLDα1*-specific primer combinations fw1/rv1 (**b**), fw2/rv2 (**d**) and fw4/rv4 (**f**) (see Table 1). Products were separated by gel electrophoresis. **c** *PLDδ* and *PLDα1* expression level were analyzed by RT-PCR in leaves from *wt* or *pldδ* plants, as indicated. The primer combination fw3/rv3 (*PLDδ*) and fw4/rv5 (*PLDα1*) were used (see "Materials and methods") with *actin* as a loading control. **d** Wild-type epidermal peels were incubated in opening buffer under light for 3 h. The peels were treated with buffer (control) or 50 μM ABA for 90 min. Total RNA was extracted from peels and the mRNA was analyzed by RT-PCR. *Actin* levels were used as loading control. The amplification products were observed on a 1 % agarose gel stained with Syber safe. The products were scanned and quantified by ImageQuant program, levels of *PLDα1* or *PLDδ* were normalized against *actin* and expressed as a fold-increase with respect to control samples. The quantification of three independent experiments is shown. *Error bars* indicate SE of the means. *Asterisk* denotes statistical difference respective control (Student's *t* test, $P < 0.05$)

the *pldα1* line. fw3 was used in combination with rv3 to confirm that PLDδ transcript extending downstream of the insertion could not be detected in *pldδ* mutant plants. The expected product sizes are listed in Table 1. See Fig. 1 for more details.

PCR conditions were as follows: 94 °C for 5 min and then 38 cycles at 94 °C for 30 s, 56 °C for 50 s, and 72 °C for 1 m 30 s. Amplicons were observed on a 1 % agarose gel stained with Syber safe (Invitrogen, Gaithersburg, MD, USA).

## Epidermal peel preparation and stomatal aperture measurement

Epidermal peels were peeled from the abaxial surface of fully expanded leaves. The peels were pre-incubated in opening buffer [10 mM MES, pH 6.1 (MES titrated to its pKa with KOH) with 10 mM KCl] under white light at 25 °C, to promote stomatal opening. After 3 h pre-incubation, S-nitrosoglutathione (GSNO) prepared according to Gordge et al. (1996), ABA (Sigma, St Louis, MO, USA),

**Table 1** Primer combinations used to check *pldδ* and *pldα1* knock-out plants

| Name | Combination used | Expected product size (bp) |
|---|---|---|
| A | fw1–LB | 900 |
| B | fw1–rv1 | 1,179 |
| C | LB–rv2 | 800 |
| D | fw2–rv2 | 637 |
| E | LB–rv4 | 600 |
| F | fw4–rv5 | 432 |
| *PLDδ* | fw3–rv3 | 1,005 |
| *PLDα1* | fw4–rv5 | 432 |
| *Actin* | ActinR–ActinF | 651 |

The name of the products and the expected product size corresponds to those indicated in Fig. 1

$H_2O_2$, PA or phosphatidylethanolamine (PE) were added to the opening buffer and subsequently incubated for 1 h. For the addition of PA (16:0–18:1; 18:1–18:1) or PE (16:0–18:1) (Avanti Polar Lipids, Birmingham, AL, USA) to epidermal cells, lipids were prepared as follows: lipids dissolved in $CHCl_3$ were dried, opening buffer was added to the lipid to reach the final concentration, then allowed to hydrate for at least 30 min and sonicated three times for 5 s before treatment.

Stomatal apertures were measured from digital pictures taken with a Nikon Coolpix 990 (Nikon, Tokyo, Japan) camera coupled to an optical microscope (Nikon Eclipse 2000). Then, the pore width was digitally calculated using the image analysis software Matrox Inspector 2.2 (Matrox Electronic System, Dorval, Canada). Aperture values are the mean of 90–120 stomata measured from at least three independent experiments. Values are expressed as mean ± standard error of means (SE). Differences between means were statistically analyzed using SigmaStat software (Systat Software Inc., Chicago, IL, USA).

### *PLDα1* and *PLDδ* expression in epidermal peels and leaves

*Arabidopsis* epidermal peels were floated in opening buffer (10 mM MES, pH 6.1, 10 mM KCl) for 3 h and then treated in the same buffer with or without 50 μM ABA for 90 min. Total RNA from epidermal peels or expanded leaves of 4-week-old *Arabidopsis* plants was extracted with Trizol reagent (Invitrogen). Two μg of total RNA was used for RT reaction with an oligo dT primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA).

The *PLDα1* transcript was amplified using the gene-specific primers fw4 and rv5 designed to amplify 432 bp. The *PLDδ* transcript was amplified using the gene-specific primers fw3 and rv3 designed to amplify 1,005 bp. The *Actin* transcript was amplified using the gene-specific primers Actin-F (5′-AAACCCTCGTAGATTGGCACA-3′) and Actin-R (5′-AAACCCTCGTAGATTGGCACA-3′) designed to amplify 651 bp from actin2 transcripts. Numbers of cycles were setup in order to evaluate transcripts levels in a lineal phase of the PCR experiment. Thus, PCR conditions were as follows: 94 °C for 5 min and then 25 cycles for actin and *PLDδ*, or 29 cycles for *PLDα1,* at 94 °C for 30 s, 56 °C for 50 s, and 72 °C for 75 s. Amplicons were observed on a 1 % agarose gel stained with Syber safe (Invitrogen), scanned with STORM (Molecular Dynamics, Sunnyvale, CA, USA) and quantified by ImageQuant program. The transcript level was normalized against the transcript level of actin for every sample. Transcript levels are expressed as a fold-increase with respect to non-treated wild type.

### $H_2O_2$ and NO detection in guard cells

For NO or $H_2O_2$ detection, epidermal peels were floated in opening buffer (10 mM MES, pH 6.1 with 10 mM KCl) for 3 h under light at 25 °C to induce stomatal opening and then loaded with 10 μM of the NO-sensitive dye, 4,5-diaminofluorescein diacetate (DAF-2DA) (Sigma-Aldrich) or 10 μM of the $H_2O_2$-sensitive dye, 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCFDA; Molecular Probes, Eugene, OR, USA) for 10 min and washed for 20 min in opening buffer. Then, 20 μM ABA was added for 10 min for NO detection or 5 min for $H_2O_2$ detection. Fluorescence was observed using an epifluorescence microscope (Eclipse E200, Nikon with excitation at 488 nm and emission at 505–530 nm). Images acquired from microscope were analyzed using Image J software (NIH, Bethesda, MD, USA). Pixel intensities are presented as the mean ± SE.

### Data analysis and statistics

All data were taken from at least three independent experiments. Different treatments were tested using Student's *t* test as indicated in the legends to figures using SIGMAPLOT 11 (Systat Software).

## Results

### PLDδ is required for NO-induced stomatal closure

To analyze the role of PLDδ in stomatal closure, we first analyzed whether it is expressed in guard cells. With that aim we extracted RNA from epidermal peels, where guard cells are the only intact cell type (Distéfano et al. 2008). We measured *PLDδ* expression in wild-type *Arabidopsis*

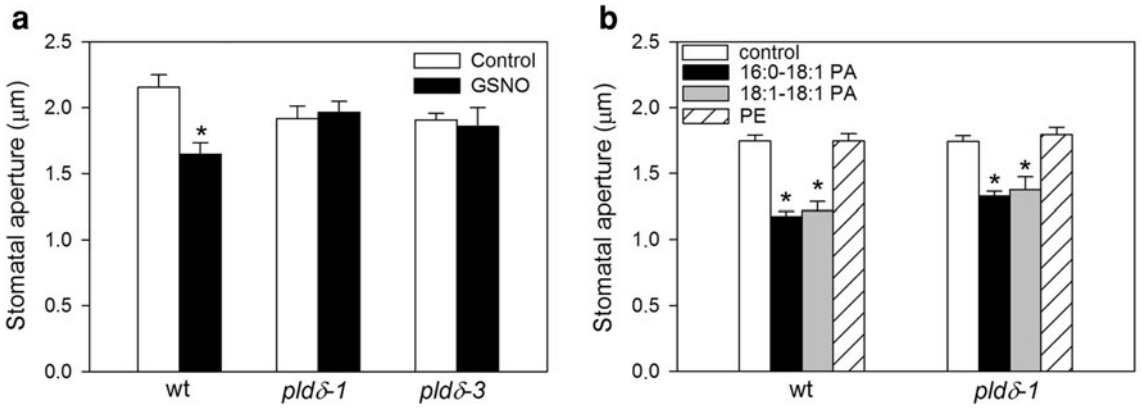


**Fig. 2** PLDδ mediates NO-induced stomatal closure. Wild-type (*wt*) and *pldδ* mutants (*pldδ-1 and pldδ-3*) epidermal peels were incubated in opening buffer under light for 3 h. **a** The peels were treated with buffer (*white bars*) or 100 μM of the NO donor GSNO (*black bars*), for 1 h. **b** The peels were treated with buffer (*control*), 50 μM PA species 16:0–18:1; 18:1–18:1; or 50 μM PE, for 1 h. Stomatal aperture values (see "Materials and methods") are expressed as mean ± SE. The results show the mean of 90–120 stomata measured from three independent experiments. *Asterisks* denote statistical difference respective control (Student's *t* test, $P < 0.05$)

epidermal peels and leaves. Figure 1c and d shows that *PLDδ* is expressed in leaves and guard cells and is up-regulated by ABA, which is consistent with a recently published report (Wang et al. 2011). Since PLDα1 is involved in ABA-induced stomatal closure, we also analyzed *PLDα1* expression. Figure 1c and d shows that *PLDα1* is expressed in leaves and guard cells, but is not up-regulated by ABA.

In order to evaluate the role of PLDδ in NO-induced stomatal closure, we used two *Arabidopsis* independent knock-out lines: *pldδ-1* previously characterized by Bargmann et al. (2009a, b) and *pldδ-3* (Fig. 1a, b). Disruption of the gene was assayed by RT-PCR from total RNA isolated from the leaves (Fig. 1c). *pldδ* plants developed normally, exhibiting no obvious phenotype when grown under standard greenhouse or growth chamber conditions (data not shown). Figure 2 shows that 100 μM of the NO donor GSNO promotes stomatal closure in wild-type plants. However, *pldδ* are impaired to close the stomata in response to GSNO (Fig. 2a). Previous reports showed that exogenously applied PA, the lipid product of PLD activity, induces stomatal closure (Jacob et al. 1999; Zhang et al. 2009). When PA was supplied to *pldδ-1* epidermal peels, the stomata were able to close (Fig. 2b), showing that PA can restore the *pldδ* mutants to wild-type phenotype (Fig. 2b). PE was used as a control of the lipid applied and showed no effect in wild-type or mutant guard cells (Fig. 2b). In addition, Fig. 2b shows that *pldδ* mutants have functional stomata.

In contrast to *pldδ*, *pldα1* plants close the stomata in response to NO treatment (Zhang et al. 2009). Thus, we used *pldα1* plants and the double-mutant *pldα1/pldδ-1* (Bargmann et al. 2009b) to evaluate NO response. We observed that 100 μM GSNO promotes stomatal closure in *pldα1* plants but *pldα1/pldδ-1* double-mutant plants are impaired to close the stomata in response to GSNO (Fig. 3). The response of the *pldα1/pldδ* double mutant is the same as the single *pldδ* mutant, suggesting that PLDδ is downstream of PLDα1 in ABA-signaling cascade. Thus, PLDδ is required for NO-induced stomatal closure.

## *pldδ* mutants produce $H_2O_2$ and NO upon ABA treatments

ABA signaling in guard cells involves $H_2O_2$ and NO production, both required for ABA-induced stomatal closure (Bright et al. 2006). We assessed for the ability of *pldδ* plants to produce NO and $H_2O_2$ in response to ABA using

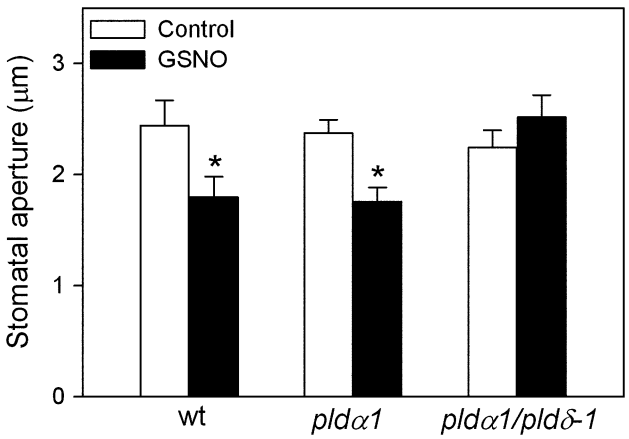


**Fig. 3** NO-induced stomatal closure on *PLD* mutants. Wild-type (*wt*), *pldα1 and pldα1/pldδ-1* epidermal peels were incubated in opening buffer under light for 3 h. The peels were treated with buffer (*white bars*) or 100 μM of the NO donor GSNO (*black bars*), for 1 h. Stomatal aperture values (see "Materials and methods") are expressed as mean ± SE. The results show the mean of 90–120 stomata measured from three independent experiments. *Asterisks* denote statistical difference respective control (Student's *t* test, $P < 0.05$)

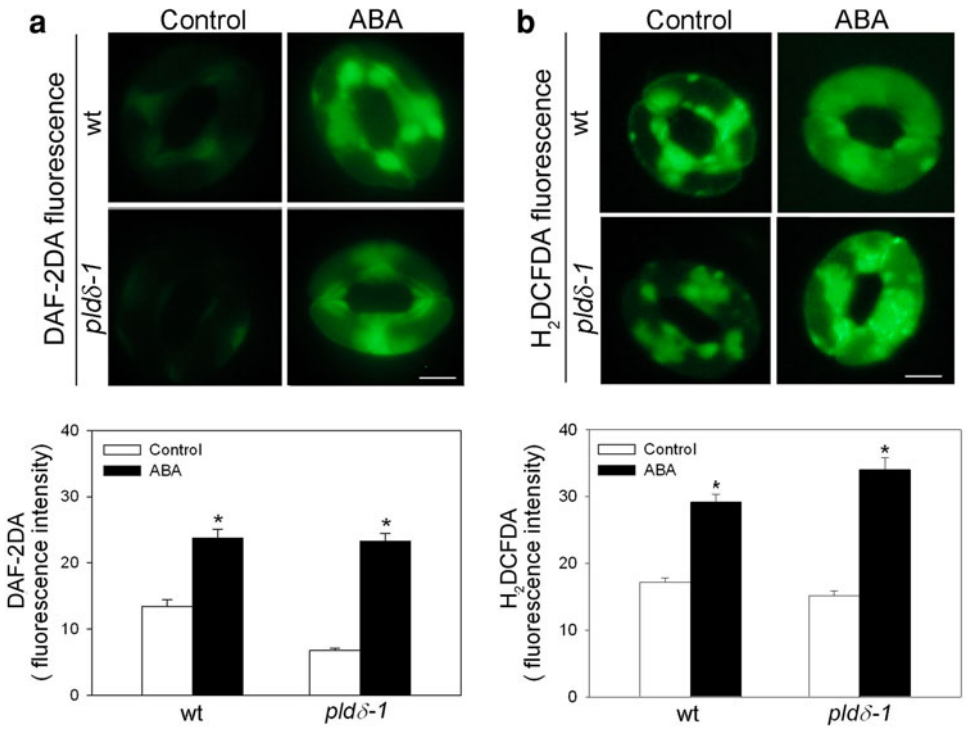


**Fig. 4** PLD$\delta$ is not required for ABA-dependent NO (**a**) and $H_2O_2$ (**b**) production in guard cells. Wild-type (*wt*) and *pld$\delta$-1* epidermal peels were incubated in opening buffer under light for 3 h, loaded with 10 μM fluorescent dye DAF-2DA (for NO detection) or 10 μM fluorescent dye $H_2$DCFDA (for $H_2O_2$ detection) for 10 min, washed for 20 min and subsequently treated with buffer (*control*) or 20 μM ABA for 10 min for NO detection and 5 min for $H_2O_2$ detection. *Upper panel* shows representative images of NO or $H_2O_2$ production in guard cells as indicated. *Size bar* 6 μm. *Lower panel* shows mean pixel intensities measured with image analysis software in areas without chloroplasts. Controls are showed in *white bars*. ABA treatment is represented in *black bars*. The results show the mean of 60–90 stomata measured from three independent experiments. *Error bars* indicate SE of the means. *Asterisks* denote statistical difference respective control (Student's *t* test, $P < 0.05$)

the NO-specific fluorescence dye, DAF-2DA, and the $H_2O_2$ detection fluorescence dye, $H_2$DCFDA. As previously reported, ABA induces NO and ROS production in wild-type plants (Fig. 4a, b). Figure 4a shows that *pld$\delta$-1* guard cells produced similar NO levels as the wild type in response to ABA (2.2- and 2.6-fold increase for wild type and *pld$\delta$-1,* respectively). Figure 4b shows that ABA also induces $H_2O_2$ in wild-type and *pld$\delta$-1* guard cells (1.7- and 2.2-fold increase for wild type and *pld$\delta$-1,* respectively). In conclusion, *pld$\delta$* mutants are able to produce $H_2O_2$ and NO upon ABA treatments and further suggests that PLD$\delta$ acts downstream of NO in ABA-induced stomatal closure.

### PLD$\delta$ is required for ABA and $H_2O_2$-induced stomatal closure

We further studied the response of *pld$\delta$* stomata to ABA and $H_2O_2$ treatments. Wild-type, *pld$\delta$-1* and *pld$\delta$-3* epidermal peels were treated with 100 μM $H_2O_2$ or 20 μM ABA and then stomatal aperture was measured. As previously reported, wild-type plants close their stomata in response to $H_2O_2$ or ABA (Fig. 5). However, this effect was impaired in *pld$\delta$* plants, confirming that PLD$\delta$ acts downstream of $H_2O_2$ and ABA.

## Discussion

In this report, we show that PLD$\delta$ is involved in NO regulation of stomatal closure. Our previous results showed that PLD is activated upon NO treatments in *Vicia faba* guard cells (Distéfano et al. 2008). Seeking for the PLD isoenzyme responsible for NO-induced stomatal closure, we found that PLD$\delta$ is required for NO, $H_2O_2$, and ABA-induced stomatal closure but not for ABA-dependent NO and $H_2O_2$ production.

As it was previously reported (Wang et al. 2011), we showed that, in guard cells, *PLD$\delta$* expression is up-regulated by ABA. In contrast, the expression of the other PLD isoenzyme involved in stomatal regulation, PLD$\alpha$1 (Zhang et al. 2004), is not regulated by ABA. We showed that although NO treatment promotes stomatal closure in *pld$\alpha$1* plants, *pld$\delta$* and *pld$\alpha$1/pld$\delta$-1* plants were impaired to close the stomata. Thus, PLD$\alpha$1 and PLD$\delta$ are differently

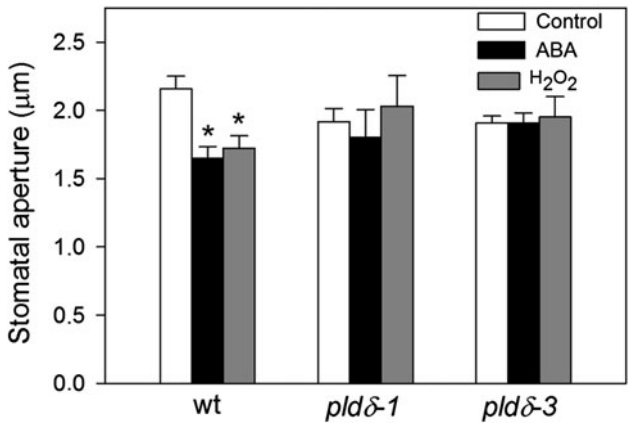


**Fig. 5** PLD$\delta$ mediates ABA and $H_2O_2$-induced stomatal closure. Wild-type (wt) and *pld$\delta$* (*pld$\delta$-1 and pld$\delta$-3*) epidermal peels were incubated in opening buffer under light for 3 h. Then the peels were treated with buffer (*white bars*), 20 μM ABA (*black bars*) or 100 μM $H_2O_2$ (*gray bars*) for 1 h. Stomatal aperture values (see "Materials and methods") are expressed as mean ± SE. The results show the mean of 90–120 stomata measured from three independent experiments. *Asterisks* denote statistical difference respective control (Student's *t* test, $P < 0.05$)

regulated during stomatal closure. In addition to regulation of gene expression, these two PLDs have distinctive substrate selectivity, subcellular locations, $Ca^{2+}$ sensitivity and temporal activation (Wang et al. 2006). PLDα1 and PLD$\delta$ are activated in response to ABA, however, PLDα1 activation is earlier and produces twice as much PA as does PLD$\delta$ in ABA-treated leaf protoplast (Zhang et al. 2009; Guo et al. 2012). Moreover, PLD$\delta$ is the main PLD responsible for $H_2O_2$-stimulated PA production (Guo et al. 2012). These results suggest that both PLDs are involved in PA production during ABA signaling, however, the timing, strength and location of PA would generate different downstream responses (Wang et al. 2006). PA derived from PLDα1 could activate NADPH oxidase, which in turn, and via NO production could activate PLD$\delta$, triggering PA production in a different location.

According to the data obtained by Zhang et al. (2009), *pldα1* close the stomata in response to $H_2O_2$ or NO, but failed to produce $H_2O_2$ and NO, in response to ABA. Our results showed that *pldα1* does close the stomata in response to NO treatments, as reported earlier (Zhang et al. 2009). However, *pld$\delta$* or *pldα1/pld$\delta$* mutants do not close the stomata upon NO treatments. Thus, while PLD$\delta$ is downstream of NO-induced stomatal closure, PLDα1 acts downstream of ABA but upstream of $H_2O_2$ and NO (Zhang et al. 2009). These results suggest that PLD$\delta$ might have a role on regulation of stomatal closure downstream of ABA-induced ROS and NO production via PLDα1 activation; however, direct evidences will be needed to confirm this hypothesis. Our results provide experimental evidences supporting an earlier suggestion by Zhang et al. (2005) who proposed that PLDα1 and PLD$\delta$ are in the same signaling pathway activated by ABA. At the same time of this report, Guo et al. (2012) established that PLD$\delta$ is downstream of $H_2O_2$ in mediating ABA-induced stomatal closure, further supporting our results and the above-proposed hypothesis. However, Uraji et al. (2012) suggested that PLDα1 and PLD$\delta$ have a cooperative function in ABA-induced stomatal closure, since simple mutants close the stomata upon ABA treatment, but the double mutant does not. Methodological reasons may account this situation, however, further studies are needed in order to explain these differences.

It has been reported that PA inhibits the function of the protein phosphatase ABI1 which is a negative regulator of ABA responses involved in the ABA receptor complex (Ma et al. 2009; Park et al. 2009). PA binds ABI1 decreasing phosphatase activity and tethering ABI1 to plasma membrane to prevent ABI1 translocation from cytosol to the nucleus (Zhang et al. 2004). ABI1 have been placed downstream of NO in ABA signal transduction pathway (Desikan et al. 2002). Disruption of PA–ABI1 binding did not affect $H_2O_2$ and NO production in response to ABA but did impaired $H_2O_2$ and NO-induced stomatal closure (Zhang et al. 2009); it can also be speculated that PLD$\delta$-derived PA inactivates ABI1. In addition, mitogen-activated protein kinases (MAPKs), which are involved in ABA-promoted stomatal closure, have been implicated as targets of PA (Testerink and Munnik 2011) and stimulated by NO (Neill et al. 2008). Thus, MAPKs could be targets regulated by NO-induced PLD$\delta$-mediated stomatal closure. Another target of PLD$\delta$-derived PA involved in stomatal closure could be the $K^+_{in}$ channels, since they are regulated by PA and NO (Jacob et al. 1999; Garcia-Mata et al. 2003).

Besides PLDα1 and PLD$\delta$, at least two other isoenzymes, PLDε or PLDα3, could be involved in drought stress (Sang et al. 2001; Katagiri et al. 2001; Hong et al. 2008, 2009). However, their role in regulation of stomatal movements remains to be studied. Interestingly, *pldα1*, *pld$\delta$*, *pldε*, and *pldα3* mutants have a wild-type phenotype under normal growth conditions. However, during different kind of stresses, the mutants have particular phenotypes. The lack of redundancy during stress condition indicates that individual PLD(s) are tightly controlled and can be differentially activated. As NO and nitrosative species concentrates in membranes, nitrosylation or nitration of phospholipases could be a mechanism of regulation. So far, there are no reports of in vivo or in vitro nitrosylation or nitration of these enzymes in neither animals nor plants. This is subject for a future research.

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