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Reactive oxygen species (ROS) are produced in plants under various stress conditions and serve as important mediators in plant responses to stresses. Here, we show that the cytosolic glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenases (GAPCs) interact with the plasma membrane-associated phospholipase D (PLD $\delta$ ) to transduce the ROS hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) signal in *Arabidopsis thaliana*. Genetic ablation of *PLD $\delta$*  impeded stomatal response to abscisic acid (ABA) and H<sub>2</sub>O<sub>2</sub>, placing PLD $\delta$  downstream of H<sub>2</sub>O<sub>2</sub> in mediating ABA-induced stomatal closure. To determine the molecular link between H<sub>2</sub>O<sub>2</sub> and PLD $\delta$ , GAPC1 and GAPC2 were identified to bind to PLD $\delta$ , and the interaction was demonstrated by coprecipitation using proteins expressed in *Escherichia coli* and yeast, surface plasmon resonance, and bimolecular fluorescence complementation. H<sub>2</sub>O<sub>2</sub> promoted the GAPC–PLD $\delta$  interaction and PLD $\delta$  activity. Knockout of *GAPCs* decreased ABA- and H<sub>2</sub>O<sub>2</sub>-induced activation of PLD and stomatal sensitivity to ABA. The loss of *GAPCs* or *PLD $\delta$*  rendered plants less responsive to water deficits than the wild type. The results indicate that the H<sub>2</sub>O<sub>2</sub>-promoted interaction of GAPC and PLD $\delta$  may provide a direct connection between membrane lipid-based signaling, energy metabolism and growth control in the plant response to ROS and water stress.

## INTRODUCTION

Reactive oxygen species (ROS) are produced in plants in response to a wide variety of stresses, including drought, UV irradiation, high light, wounding, ozone, low and high temperatures, and pathogens (Desikan et al., 2001; Apel and Hirt, 2004; Suzuki et al., 2012). ROS were originally viewed as by-products of metabolic pathways, and a high concentration of ROS is toxic to the cells (Apel and Hirt, 2004; Quan et al., 2008; Finkel, 2011). It has now been well documented that ROS are generated as signals that alter various cellular and physiological processes in plant growth and development (Desikan et al., 2001; Apel and Hirt, 2004; Gechevet et al., 2006; Shao et al., 2008). Hydrogen

peroxide (H<sub>2</sub>O<sub>2</sub>) is the major and most stable species of ROS and plays a signaling role in plant response to stresses, such as mediating abscisic acid (ABA)-regulated stomatal closure (Pei et al., 2000; Zhang et al., 2001). H<sub>2</sub>O<sub>2</sub> is thought to affect target protein activities through modification of thiol groups of Cys residues (Hancock et al., 2005). However, it is unclear how such oxidative modification affects a signaling cascade that leads to alteration of cellular function and plant stress responses.

Recent studies indicate that phospholipase D (PLD) and its product phosphatidic acid (PA) play a role in ROS-mediated signaling (Sang et al., 2001; Yamaguchi et al., 2004; Zhang et al., 2009; Lanteri et al., 2011). The *Arabidopsis thaliana* genome contains 12 *PLDs*, *PLD $\alpha$ (3)*,  *$\beta$ (2)*,  *$\gamma$ (3)*,  *$\delta$* ,  *$\epsilon$* , and  *$\zeta$ (2)*, and these *PLDs* exhibit distinguishable biochemical properties and cellular functions. Knockout (KO) of *PLD $\alpha$ 1* decreases the production of ROS, and addition of PA induces recovery of ROS levels in the *PLD $\alpha$ 1* mutant (Sang et al., 2001). PA interacts with NADPH oxidase and increases its activity and ROS production (Zhang et al., 2009). PLD and PA are also implicated in promoting the elicitor-induced generation of ROS in suspension rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*) cells (Yamaguchi et al., 2004; Lanteri et al., 2011). On the other hand, H<sub>2</sub>O<sub>2</sub>-induced activation of PLD enhances elicitor-induced biosynthesis of phytoalexins in rice cells (Yamaguchi et al., 2004). Plasma membrane-associated PLD $\delta$  is activated by H<sub>2</sub>O<sub>2</sub>, and ablation of it renders *Arabidopsis* cells more sensitive to H<sub>2</sub>O<sub>2</sub>-promoted programmed cell death than the wild type (Wang and Wang, 2001; Zhang et al., 2003, 2005; Wang et al., 2006). These results

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
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
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suggest that whereas  $PLD\alpha 1$  promotes the ROS production,  $PLD\delta$  mediates plant responses to ROS. However, it is unknown how  $H_2O_2$  activates  $PLD\delta$  and whether  $PLD\delta$  is involved in mediating the  $H_2O_2$  effect in the ABA signaling.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in the glycolytic pathway, thus functioning to produce energy and supply intermediates for cellular metabolism (Plaxton, 1996). The *Arabidopsis* genome contains seven phosphorylating GAPDHs, five of which are located in plastids, whereas GAPC1 and GAPC2 are in the cytosol (Rius et al., 2008; Muñoz-Bertomeu et al., 2010). GAPDHs have been implicated in embryo development, pollen development, root growth, and ABA signal transduction (Rius et al., 2006, 2008; Muñoz-Bertomeu et al., 2009, 2010, 2011). The catalytic Cys residues of GAPDH can be oxidized by oxidants such as  $H_2O_2$ , leading to fully or partially reversible inactivation of GAPDH (Hancock et al., 2005; Hara et al., 2005; Holtgreffe et al., 2008). GAPC1 has been suggested to be a  $H_2O_2$  target potentially involved in mediating ROS response in *Arabidopsis* (Hancock et al., 2005; Holtgreffe et al., 2008). Here, we show that GAPC1 and GAPC2 bind to  $PLD\delta$ , that  $H_2O_2$  promotes the GAPC interaction with  $PLD\delta$ , and that the interaction mediates plant response to ABA and water deficits.

## RESULTS

### Ablation of $PLD\delta$ Compromises ABA- and $H_2O_2$ -Induced Stomatal Closure, but Not ABA-Promoted $H_2O_2$ Production

To determine if  $PLD\delta$  is activated by ABA, we isolated  $PLD\alpha 1$   $PLD\delta$  double KO  $pld\alpha 1 pld\delta$  (see Supplemental Figure 1 online) and assayed the PLD activity in response to ABA in wild-type,  $pld\alpha 1$ ,  $pld\delta$ , and  $pld\alpha 1 pld\delta$  using 1-palmitoyl-2-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl-*sn*-glycero-3-phosphocholine (NBD-PC)-labeled protoplasts (Figure 1A). The  $PLD\alpha 1$  KO mutant was used because  $PLD\alpha 1$  was reported to be responsible for a majority of PA produced in response to ABA (Zhang et al., 2009). PA production was increased twofold after wild-type protoplasts were incubated with ABA for 20 min (Figure 1A). The ABA-induced PA production in  $pld\alpha 1$  and  $pld\delta$  was ~62 and 28% lower, respectively, than in the wild type. No significant PA increase was observed in response to ABA in  $PLD\alpha 1$   $PLD\delta$  double KO cells (Figure 1A). The results indicate that in addition to  $PLD\alpha 1$ ,  $PLD\delta$  is also activated by ABA and that  $PLD\alpha 1$  and  $PLD\delta$  together account for virtually all ABA-induced PLD activity, with  $PLD\alpha 1$  providing twice as much PA as  $PLD\delta$  in response to ABA in *Arabidopsis*.

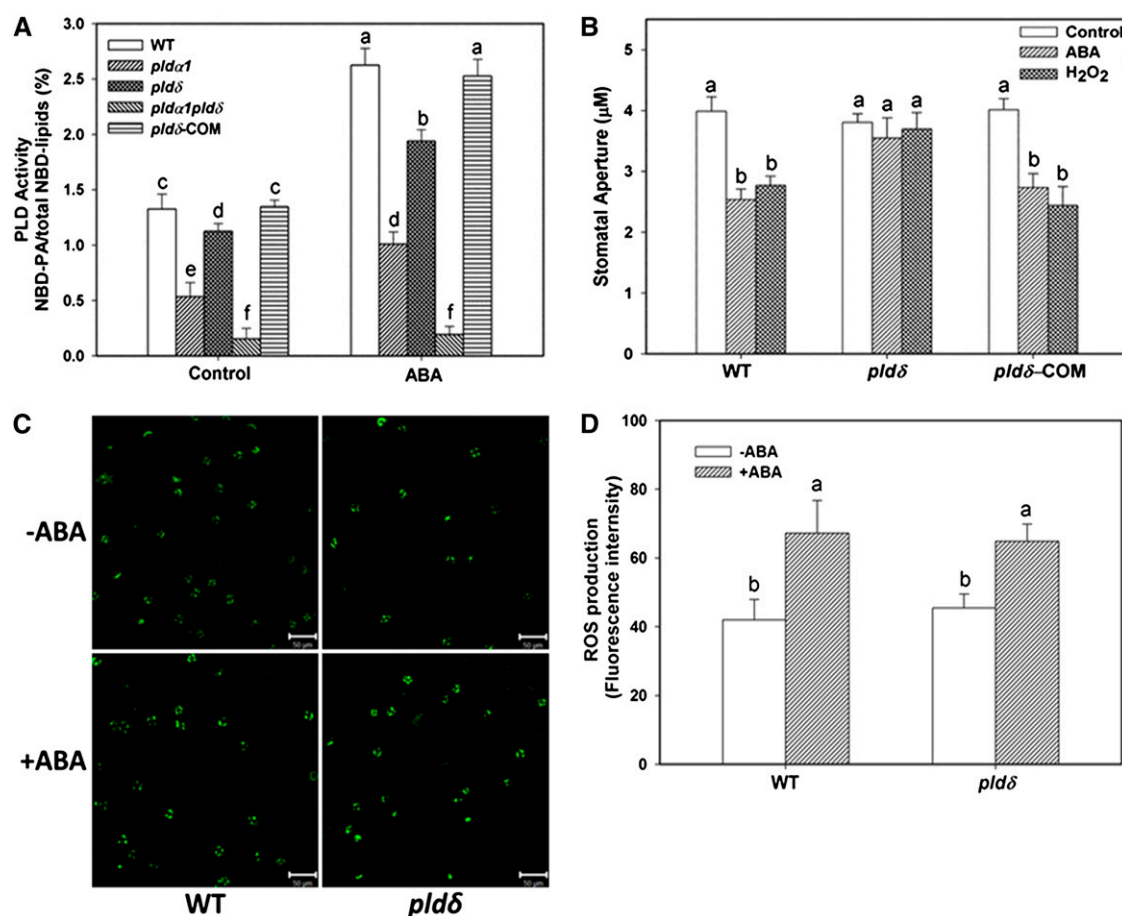
To determine the role of  $PLD\delta$  in ABA response, we investigated whether the loss of  $PLD\delta$  alters ABA-promoted stomatal closure and  $H_2O_2$  production in guard cells.  $pld\delta$  leaf peels exhibited decreased sensitivity to ABA-promoted stomatal closure (Figure 1B), a response similar to  $pld\alpha 1$  (Zhang et al., 2004; Zhang et al., 2009).  $H_2O_2$  has been shown to induce stomatal closure in  $pld\alpha 1$  (Zhang et al., 2009). However,  $H_2O_2$  failed to induce stomatal closure in  $pld\delta$  (Figure 1B). Introduction of  $PLD\delta$  driven by its own promoter into  $pld\delta$  restored the phenotype for

both ABA- and  $H_2O_2$ -induced stomatal closure, indicating that loss of  $PLD\delta$  is responsible for the ABA and  $H_2O_2$  response phenotype (Figure 1B). In addition, unlike  $pld\alpha 1$ , which decreased ABA-promoted  $H_2O_2$  production (Zhang et al., 2009), KO of  $PLD\delta$  did not affect the ABA-induced  $H_2O_2$  production. The basal level of ROS in  $pld\delta$  and wild-type cells were also similar, as revealed by the fluorescent dye 2',7'-dichlorofluorescein diacetate ( $H_2DCF$ -DA) intensity (Figures 1C and 1D). These results indicate that  $PLD\delta$  is not required for ABA-induced  $H_2O_2$  production but is involved in stomatal response to ABA and  $H_2O_2$ . The data suggest that  $PLD\delta$  acts downstream of  $H_2O_2$  in signaling ABA-induced stomatal closure.

### Direct Interaction between GAPC and $PLD\delta$

To determine how  $PLD\delta$  is involved in the  $H_2O_2$  response, we incubated purified  $PLD\delta$  with  $H_2O_2$  and the treatment had no impact on enzyme activity (Zhang et al., 2003). The transcript level of  $PLD\delta$  was not increased after ABA treatment for 40 min (see Supplemental Figure 2 online). These data indicate that the ABA-induced activation of  $PLD\delta$  in the early phase is not mediated by increased  $PLD\delta$  expression or the direct effect of  $H_2O_2$  on  $PLD\delta$ . To test whether a protein is involved in the  $H_2O_2$  activation of  $PLD\delta$ , we investigated the potential interaction of  $PLD\delta$  with GAPC, because GAPC was reported as a direct target of  $H_2O_2$  in *Arabidopsis* (Hancock et al., 2005; Holtgreffe et al., 2008). His-tagged GAPC1 was expressed in *Escherichia coli* and incubated with microsomal proteins from *Arabidopsis* leaves, and immunoblotting with  $PLD\delta$  antibodies detected  $PLD\delta$  in the GAPC1 coprecipitate (see Supplemental Figure 3 online). To verify the interaction, we purified His-tagged GAPC1 and GAPC2 proteins expressed in *E. coli* (see Supplemental Figure 4A online) and used them for reciprocal pulldown with glutathione S-transferase (GST)- $PLD\delta$ . GAPC2 pulled down  $PLD\delta$ .  $PLD\delta$  also pulled down GAPC1, as indicated by immunoblotting with anti-His or anti-GST antibodies (Figure 2A). In addition, the association of GAPCs and  $PLD\delta$  was increased in the presence of  $H_2O_2$  but decreased in the presence of the reducing reagent DTT (Figure 2A). To further validate the interaction, we coexpressed GAPC and  $PLD\delta$  in yeast (see Supplemental Figure 4B online) and grew the yeast cells with or without  $H_2O_2$ . GAPC1 and GAPC2 were detected in the complex with  $PLD\delta$  when  $PLD\delta$  was immunoprecipitated with FLAG antibody.  $PLD\delta$  also associated with GAPC1 or GAPC2 when GAPCs were immunoprecipitated with cMyc antibody. The presence of  $H_2O_2$  promoted the interaction between GAPC and  $PLD\delta$  (Figure 2B). These results indicate that the GAPC- $PLD\delta$  interaction is enhanced in an oxidative but weakened in a reducing environment.

To quantify the interaction between GAPC1 and  $PLD\delta$ , we used surface plasmon resonance (SPR) to determine the binding kinetics. Purified GAPC1 was immobilized on an nitrilotriacetic acid (NTA) chip followed by injection of purified GST or GST- $PLD\delta$ . The representative sensorgram showed an increase in response unit (RU) when GST- $PLD\delta$ , but not GST, was injected, indicating that  $PLD\delta$  interacts with GAPC1 (Figure 2C). When  $H_2O_2$ -treated GAPC1 was used, the GAPC1- $PLD\delta$  interaction was enhanced as RU was higher than when GAPC1 was not



**Figure 1.** Decreased Response of *pldδ* Plants to H<sub>2</sub>O<sub>2</sub> and ABA.

(A) ABA-induced PA production in leaf protoplasts of *pldα1*, *pldδ*, *pldα1 pldδ*, *PLDδ*-complementation (COM), and the wild type (WT). Values are means  $\pm$  SE ( $n = 3$ ).

(B) Stomatal closure induced by 25  $\mu$ M ABA or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. Values are means  $\pm$  SE ( $n = 50$ ).

(C) Representative image of ROS production in guard cells, visualized by fluorescent dye. +ABA, epidermal peels were loaded with H<sub>2</sub>DCF-DA for 10 min followed by addition of 25  $\mu$ M ABA for 5 min; -ABA, no ABA added. Bars = 50  $\mu$ m.

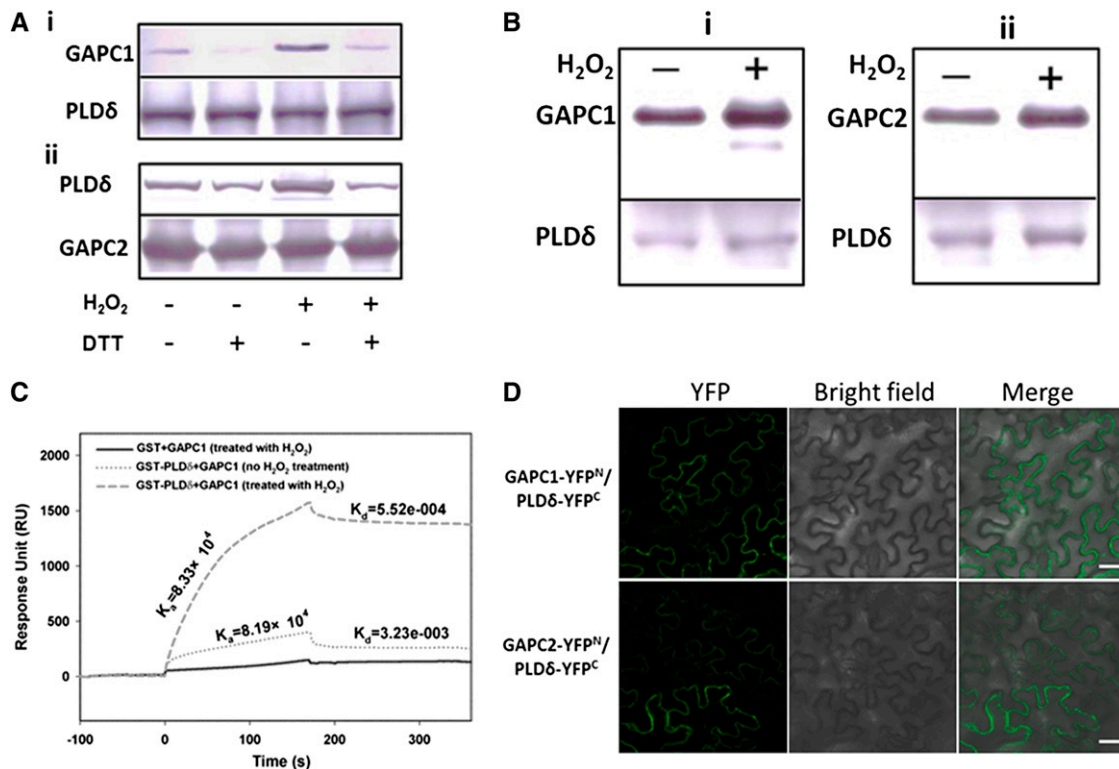
(D) Quantification of ROS production based on fluorescence intensity (mean pixel intensity). Values are means  $\pm$  SE ( $n = 50$ ). Columns with different letters are significantly different from each other (ANOVA,  $P < 0.05$ ).

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incubated with H<sub>2</sub>O<sub>2</sub> (Figure 2C). H<sub>2</sub>O<sub>2</sub>-treated or untreated GAPC1 displayed comparable association rate constants ( $K_a = 8.19 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$  versus  $8.33 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ ). However, the dissociation rate constant was lower when GAPC1 was exposed to H<sub>2</sub>O<sub>2</sub> ( $K_d = 5.52 \times 10^{-4} \text{ s}^{-1}$  versus  $3.23 \times 10^{-3} \text{ s}^{-1}$ ). The maximum specific binding is 1564 RU for H<sub>2</sub>O<sub>2</sub>-treated GAPC1 and 286 RU for GAPC1 without H<sub>2</sub>O<sub>2</sub> treatment (Figure 2C). The equilibrium binding constant  $K_D$  is  $6.62 \times 10^{-9} \text{ M}$  for GAPC1-PLD $\delta$  interaction in the presence of H<sub>2</sub>O<sub>2</sub> and  $3.94 \times 10^{-8} \text{ M}$  for GAPC1-PLD $\delta$  interaction without H<sub>2</sub>O<sub>2</sub>. The results indicate that the GAPC1-PLD $\delta$  interaction is significantly enhanced by H<sub>2</sub>O<sub>2</sub> and that H<sub>2</sub>O<sub>2</sub> stabilizes the interaction by decreasing dissociation between GAPC1 and PLD $\delta$ .

To visualize the GAPC-PLD $\delta$  interaction in plant cells, we used bimolecular fluorescence complementation (BiFC) that brings

together two yellow fluorescent protein (YFP) fragments fused to two interacting proteins (Walter et al., 2004). GAPC1 or GAPC2 was fused to the N terminus of YFP (GAPC1-YFP<sup>N</sup> or GAPC2-YFP<sup>N</sup>), and PLD $\delta$  was fused to the C terminus of YFP (PLD $\delta$ -YFP<sup>C</sup>). These constructs were cointroduced into tobacco leaves. No fluorescence was observed when empty vectors YFP<sup>N</sup> and YFP<sup>C</sup> were cotransformed or when GAPC-YFP<sup>N</sup> and PLD $\delta$ -YFP<sup>C</sup> were transformed separately (see Supplemental Figure 5 online). In the positive controls, bZIP63-YFP<sup>N</sup> and bZIP63-YFP<sup>C</sup>, the transcription factor, formed dimers and brought YFP<sup>N</sup> and YFP<sup>C</sup> together to generate fluorescence in the nucleus (see Supplemental Figure 5 online). GAPC1-YFP<sup>N</sup> or GAPC2-YFP<sup>N</sup> coexpressed with PLD $\delta$ -YFP<sup>C</sup> produced fluorescence in the cell, indicating that both GAPCs interacted with PLD $\delta$  (Figure 2D).



**Figure 2.** Interaction of GAPC with PLD $\delta$ .

**(A)** Immunoblotting of proteins after coprecipitation using *E. coli*-expressed GST-PLD $\delta$  and His-GAPC1/2, as affected by H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) and DTT (100  $\mu$ M). i, Coprecipitation of His-GAPC1 with GST-PLD $\delta$ . GAPC1, immunoblotting of GAPC1 using anti-His antibody for the precipitates; PLD $\delta$ , the starting GST-PLD $\delta$  used for precipitation. ii, Coprecipitation of GST-PLD $\delta$  with His-GAPC2. PLD $\delta$ , immunoblotting of PLD $\delta$  using anti-GST antibody for the precipitates. GAPC2, the starting His-GAPC2 used for precipitation. DTT was added before the addition of H<sub>2</sub>O<sub>2</sub> when both were applied.

**(B)** Immunoblotting of coprecipitated GAPC and PLD $\delta$  that were coexpressed in yeast grown in the presence or absence of added H<sub>2</sub>O<sub>2</sub> (20  $\mu$ M). i and ii, Reciprocal pulldown of PLD $\delta$  and GAPC1 and GAPC2, respectively. PLD $\delta$  was fused with a FLAG tag and GAPC1 or GAPC2 with a cMyc tag. GAPC1 or GAPC2 band indicates immunoblotting with cMyc antibody against the sample precipitated with FLAG antibody-conjugated agarose beads. PLD $\delta$  band indicates immunoblotting with FLAG antibody against the sample precipitated with cMyc antibody for GAPC1 or GAPC2.

**(C)** Quantitative SPR analysis of PLD $\delta$  binding to GAPC1. GAPC1 (no H<sub>2</sub>O<sub>2</sub> treatment or pretreated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>) was first immobilized on the NTA chip followed by injection of GST or GST-PLD $\delta$ .

**(D)** Representative confocal images of BiFC. Green color represents YFP fluorescence, indicating interaction of GAPC with PLD $\delta$ . PLD $\delta$ -YFP<sup>C</sup> was cotransformed with GAPC1-YFP<sup>N</sup> or GAPC2-YFP<sup>N</sup> into tobacco leaves by infiltration. Bars = 50  $\mu$ m.

### GAPCs Promote the Activity of PLD $\delta$ under Oxidative Conditions

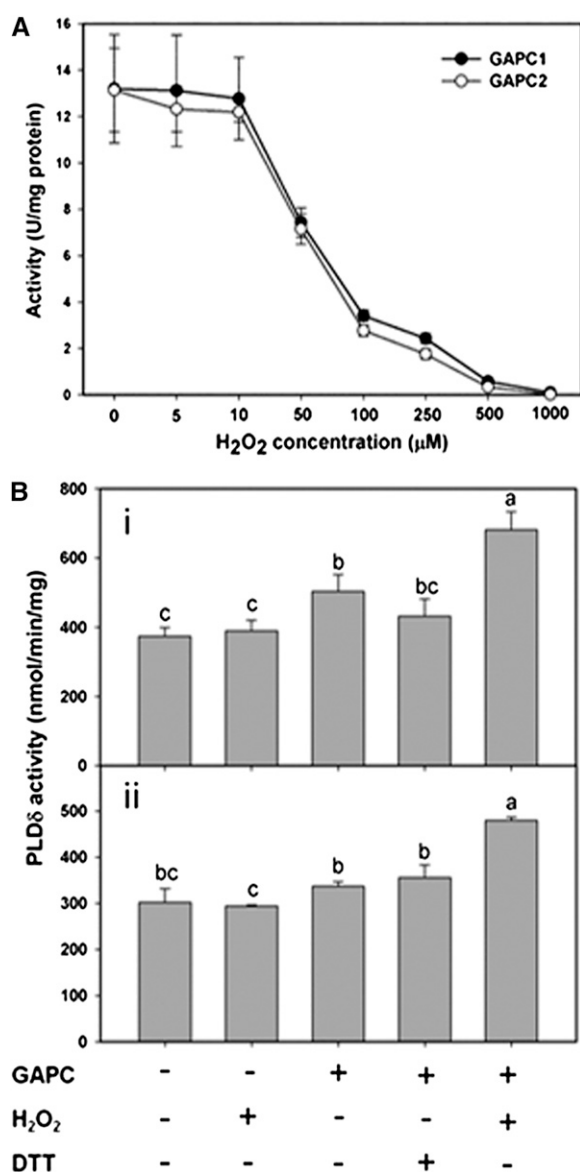
To determine the function of GAPC interaction with PLD $\delta$ , we first tested the sensitivity of GAPC1 and GAPC2 purified from *E. coli* to H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> inhibited GAPC activity in a dose-dependent manner, and virtually all GAPC1 or GAPC2 activity was inhibited at 500  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Figure 3A). When different concentrations of DTT were added to GAPCs first, followed by addition of 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>, the loss of GAPC activity was small, showing that H<sub>2</sub>O<sub>2</sub> oxidation of GAPCs can be protected by DTT reduction (see Supplemental Figure 6A online). After incubation with 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>, partial GAPC activity could be recovered by addition of DTT (see Supplemental Figure 6B online).

Purified PLD $\delta$  was then incubated GAPCs with or without H<sub>2</sub>O<sub>2</sub> to determine the effect of H<sub>2</sub>O<sub>2</sub> and GAPC on PLD $\delta$  activity. Without GAPC, addition of 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> did not affect PLD $\delta$

activity (Figure 3B), verifying that H<sub>2</sub>O<sub>2</sub> has no direct effect on PLD $\delta$  activity. Incubation of PLD $\delta$  with GAPC1 and GAPC2 increased PLD $\delta$  activity by 34 and 11%, respectively (Figure 3B). However, pretreatment of GAPC1 and GAPC2 with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> increased PLD $\delta$  activity by 82.1 and 58.9%, respectively (Figure 3B). The data indicate that H<sub>2</sub>O<sub>2</sub> inactivates GAPC but promotes the GAPC binding to PLD $\delta$ , and the binding increases PLD $\delta$  activity.

### GAPC Mediates the H<sub>2</sub>O<sub>2</sub> Activation of PLD $\delta$ in the Cell

To evaluate whether GAPC affects the activity of PLD $\delta$  in living cells, we compared PLD activity in GAPC-KO, PLD $\delta$ -KO, and wild-type protoplasts as affected by H<sub>2</sub>O<sub>2</sub>. Two homozygous T-DNA insertion KO lines of *Arabidopsis* were isolated for GAPC1 (*gapc1-1*, CS328689; *gapc1-2*, SALK\_129091) and for GAPC2 (*gapc2-1*, SALK\_016539; *gapc2-2*, SALK\_070902) (see Supplemental Figure 7 online). The GAPC1 transcript was lost in



**Figure 3.** Oxidized GAPC Promotes PLD $\delta$  Activity.

(A) H<sub>2</sub>O<sub>2</sub> inhibition of GAPC1 and GAPC2 activities.

(B) GAPC promotion of PLD $\delta$  activity under oxidative conditions. Equal molar ratios of PLD $\delta$  and GAPC proteins were used. PLD $\delta$  activity was assayed in the presence of GAPC1 (i) or GAPC2 (ii) under different conditions as indicated; 100  $\mu$ M DTT or 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> was used as indicated. Values are means  $\pm$  SE ( $n = 3$ ). Different letters indicate significant differences (ANOVA,  $P < 0.05$ ).

two GAPC1-KO lines, and GAPC2 transcript was also absent in two GAPC2-KO lines, suggesting that all four GAPC T-DNA lines are null mutants (Figure 4A). We then generated two double KO lines (*gapc1-1 gapc2-1* and *gapc1-1 gapc2-2*) by crossing the single mutants. Two lines of triple KO mutants (*gapc1-1 gapc2-1 pld $\delta$*  and *gapc1-1 gapc2-2 pld $\delta$* ) were also isolated by crossing the GAPC double KO with *pld $\delta$* . NAD-dependent GAPDH activity was determined in the single and double KO

lines of GAPC. The GAPDH activity in leaves was decreased by 21% (*gapc1-1*), 25% (*gapc1-2*), 23% (*gapc2-1*), and 21% (*gapc2-2*) for GAPC single mutants (Figure 4B). GAPC double KO plants *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2* had ~45% decrease in GAPDH activity (Figure 4B). The results indicate that GAPC1 and GAPC2 contribute almost equally to the activity, and together they account for nearly half of NAD-dependent GAPDH activity in *Arabidopsis* leaves.

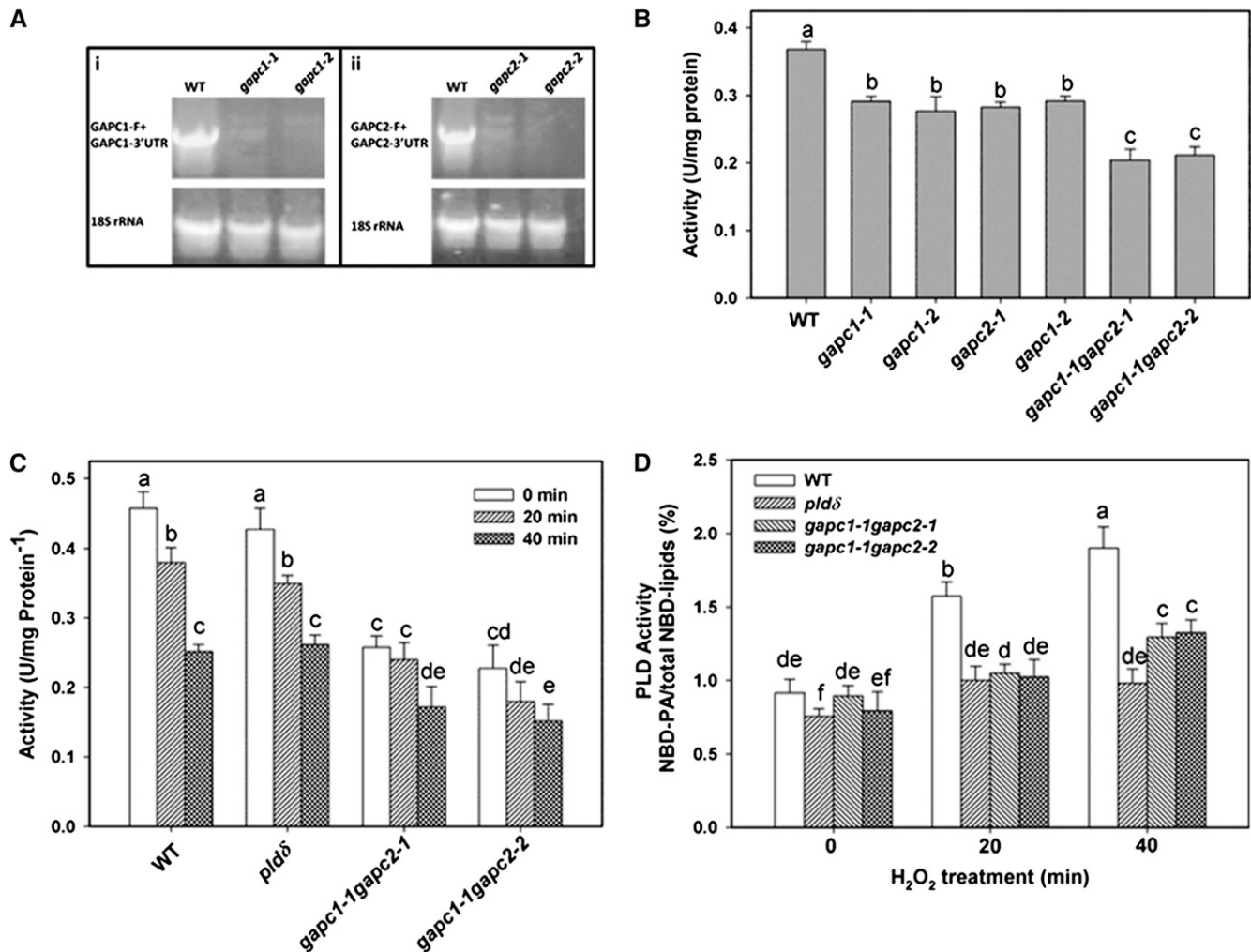
To determine if KO of both GAPCs affects PLD activation by H<sub>2</sub>O<sub>2</sub>, protoplasts of wild-type, *pld $\delta$* , and GAPC double mutants were labeled with NBD-PC and treated with H<sub>2</sub>O<sub>2</sub>. We first examined how GAPDH activity in protoplasts responded to H<sub>2</sub>O<sub>2</sub>. Protoplasts from GAPC double KOs had significantly lower GAPDH activity than the wild type or *pld $\delta$*  (Figure 4C). H<sub>2</sub>O<sub>2</sub> treatments for 20 min had no significant effect on GAPDH activity in the GAPC double KO but decreased GAPDH activity in the wild type and *pld $\delta$*  by 15%. Significant decreases in GAPDH activity occurred in all genotypes after 40 min of H<sub>2</sub>O<sub>2</sub> treatments (Figure 4C). The results indicate that H<sub>2</sub>O<sub>2</sub> inhibits GAPDH activity in the cell and also could mean that the loss of the GAPDH activity in the early phase (20 min) results primarily from H<sub>2</sub>O<sub>2</sub> inhibition of GAPCs.

Without addition of H<sub>2</sub>O<sub>2</sub>, the PLD activity, as measured by the formation of PA, in *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2* was comparable to that of the wild type (Figure 4D). The H<sub>2</sub>O<sub>2</sub> treatment increased PA production nearly twofold after 40 min in the wild type, whereas it increased PA production only 30% in *pld $\delta$* . The *gapc1 gapc2* double KOs and *gapc1 gapc2 pld $\delta$*  triple KOs exhibited similar attenuated PA increase as *pld $\delta$*  in response to H<sub>2</sub>O<sub>2</sub> (Figure 4D). The results indicate that PLD $\delta$  is the main PLD responsible for the H<sub>2</sub>O<sub>2</sub> activation of PLD and that GAPCs mediate the H<sub>2</sub>O<sub>2</sub>-induced increase of PLD $\delta$  activity.

### GAPCs Are Involved in ABA-Induced PA Production

To characterize the effect of GAPC and PLD $\delta$  on PA production in response to ABA, we measured the PA levels and composition in 4-week-old *Arabidopsis* leaves treated with ABA up to 20 min. PA level was induced by ABA in the wild type and reached a plateau at 10 min after ABA treatment. The total PA level was increased in *pld $\delta$* , *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* leaves after ABA treatment (Figure 5A). However, the amount of PA was significantly lower in *pld $\delta$* , *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* than in the wild type at 10 and 20 min after ABA treatment (Figure 5A).

The molecular species of PA in response to ABA at 10 min were analyzed for the wild type, *pld $\delta$* , *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2*. In wild-type *Arabidopsis* leaves, 34:2 (16:0/18:2), 34:3 (16:0/18:3), 36:4 (mainly 18:2/18:2), 36:5 (18:2/18:3), and 36:6 (18:3/18:3) are the most abundant PA species (Zhang et al., 2009). The levels of major PA species, including 34:1, 34:2, 34:3, 36:2, 36:4, and 36:5 PA, were significantly decreased in *pld $\delta$* , and the major overall decrease of total PA level was due to the decrease in 34:2, 34:3, 36:4, and 36:5 PA (Figure 5B). Similarly, the levels of PA species 34:2, 34:3, 36:2 and 36:4 PA were significantly reduced in *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2* compared with the wild type after 10 min of ABA treatment (Figure 5B). The PA acyl combinations affected by



**Figure 4.** H<sub>2</sub>O<sub>2</sub> Effects on GAPC and PLD $\delta$  Activities.

(A) RT-PCR detection of *GAPC1* and *GAPC2* expression in the leaves of wild-type (WT) and mutant plants. 18S rRNA was a control confirming the synthesis of cDNA.

(B) GAPDH activity in the total protein extracted from the leaves of wild-type and mutant plants.

(C) GAPDH activity using protein extracted from protoplasts after 1 mM H<sub>2</sub>O<sub>2</sub> treatment.

(D) H<sub>2</sub>O<sub>2</sub>-promoted PA production in protoplasts. Values are means  $\pm$  SE ( $n = 3$ ). Different letters mark significant differences from each other (ANOVA,  $P < 0.05$ ).

PLD $\delta$  and GAPC expression are the molecular species typically derived from hydrolysis of extraplastidic phospholipids (Welti et al., 2002), consistent with the extraplastidic location of these enzymes. The results show that the ablation of either PLD $\delta$  or GAPCs decreases the ABA-induced PA production. The attenuation of ABA-induced activation of PLD $\delta$  in GAPC double KOs is consistent with the results that GAPCs are required for the activation of PLD $\delta$  activity (Figure 4D).

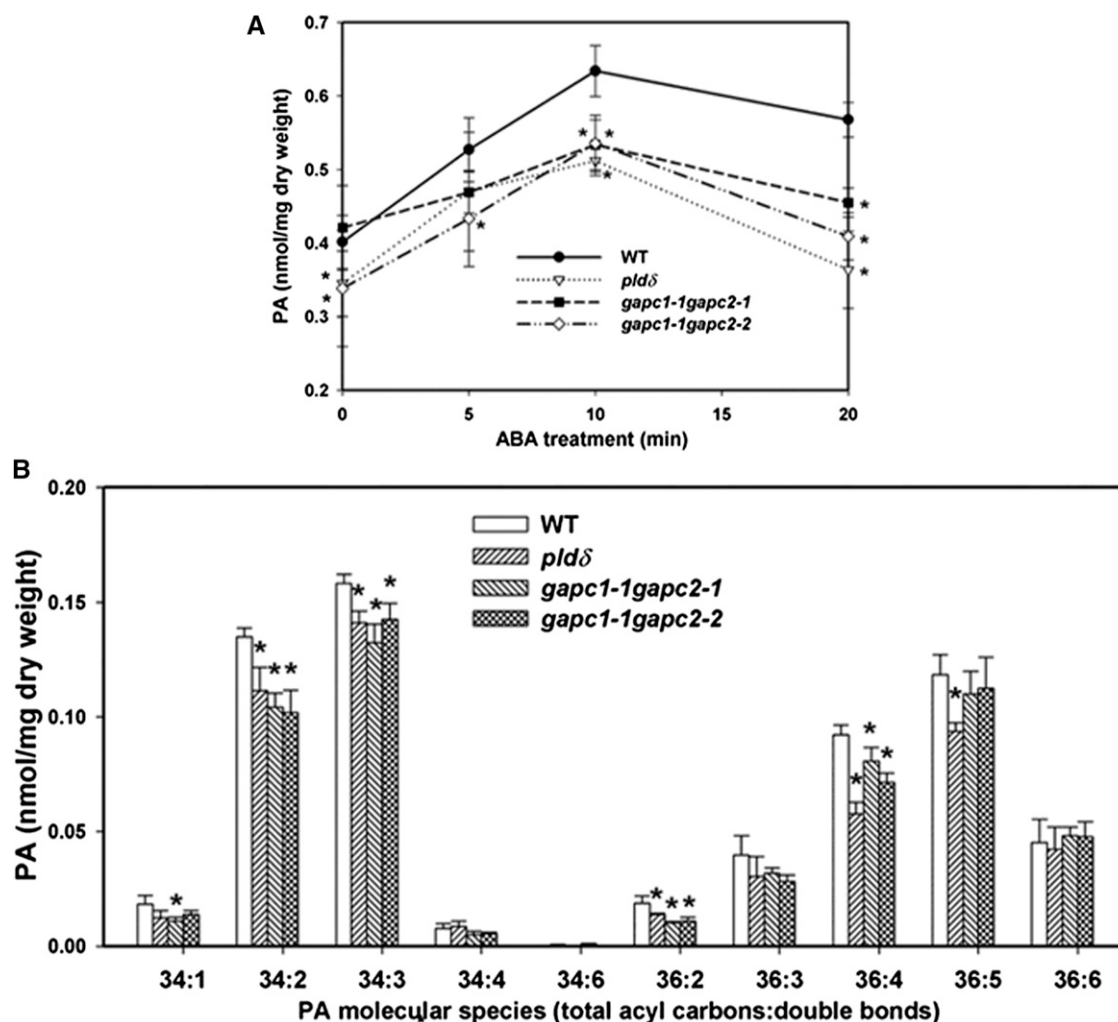
#### Loss of GAPCs or PLD $\delta$ Renders Plants Less Responsive to Water Deficits

To determine if GAPC–PLD $\delta$  interaction is involved in the process of mediating plant response to ROS, we measured

stomatal closure in response to ABA and H<sub>2</sub>O<sub>2</sub> in leaves deficient in both GAPCs or GAPC and PLD $\delta$ . Stomata of *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2* were less sensitive to ABA or H<sub>2</sub>O<sub>2</sub>, as indicated by greater stomatal aperture in these mutants than that of the wild type after the treatment of ABA or H<sub>2</sub>O<sub>2</sub> (Figure 6A). Two triple mutants (*gapc1-1 gapc2-1 pldδ* and *gapc1-1 gapc2-2 pldδ*) were also less sensitive to ABA- and H<sub>2</sub>O<sub>2</sub>-promoted stomatal closure (Figure 6A).

To determine how the effect of GAPCs and PLD $\delta$  on ABA and H<sub>2</sub>O<sub>2</sub> signaling impacts plant response to water deficits, we evaluated the effect of GAPCs and PLD $\delta$  KOs on *Arabidopsis* plants grown under three field water capacity (FC) conditions: 100% FC for well-watered control, and 60 and 30% FC for mild and acute drought stress, respectively (see Supplemental Figure





**Figure 5.** PA Content of *GAPC* and *PLDδ* Mutant Leaves in Response to ABA.

(A) Total PA content of leaves harvested at different times after spraying with ABA (100  $\mu$ M). WT, the wild type.

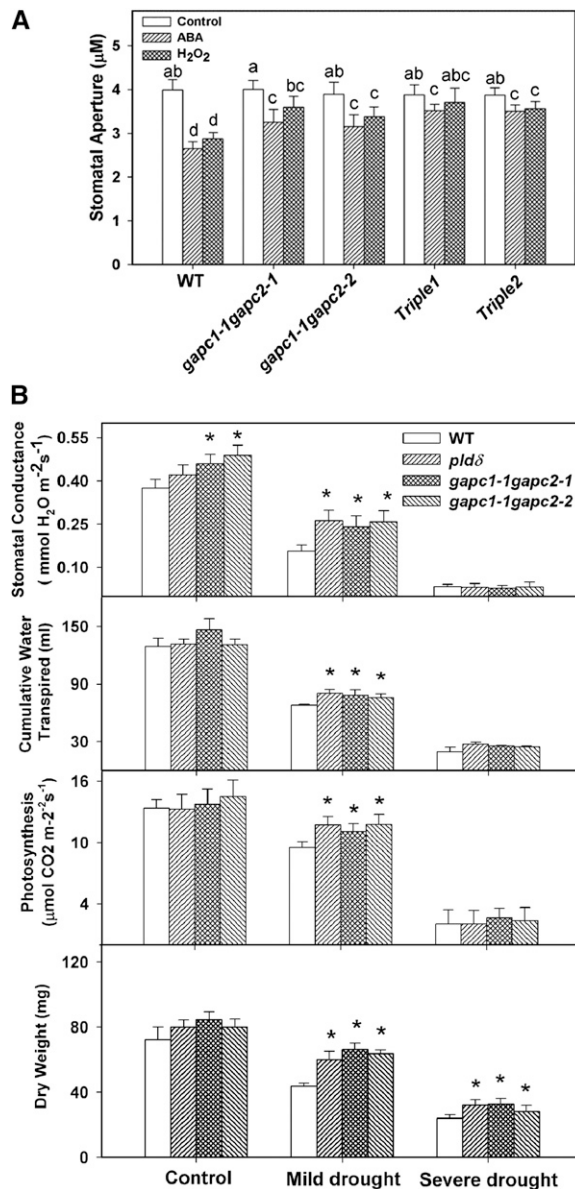
(B) PA molecular species in leaves of the wild type and mutants treated with ABA for 10 min. Values are means  $\pm$  SE ( $n = 5$ ). Asterisks indicate significant difference from the wild type at the same time point of ABA treatment ( $P < 0.05$ ,  $t$  test).

8 online). Under well-watered conditions, *pldδ*, *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* did not show significant difference from the wild type in cumulative water transpired and photosynthetic rate, but *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2* had higher stomatal conductance than the wild type (Figure 6B). At 60% FC, *pldδ*, *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* displayed higher stomatal conductance, higher cumulative water transpiration, and higher photosynthetic rate than wild-type plants (Figure 6B). At the severe water deficit (30% FC), stomatal conductance was very low in all genotypes, but *pldδ*, *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* mutant lines still exhibited the tendency to have more cumulative water transpiration than the wild type (Figure 6B).

As the FCs decreased, wild-type, *pldδ*, *gapc1-1 gapc2-1*, and *gapc1-1 gapc2-2* mutants accumulated less biomass, as plant growth was inhibited in response to water deficits. *pldδ*, *gapc1-1*

*gapc2-1*, and *gapc1-1 gapc2-2* accumulated more biomass than the wild type under both mild and acute drought conditions. At 60% FC, the three mutants accumulated ~30% more dry matter than the wild type. The greater biomass in the mutants than the wild type was consistent with higher stomatal conductance and photosynthetic rate. The decreased drought inhibition of plant growth in the mutants suggests that the loss of *PLDδ* or *GAPCs* renders plants less responsive to adjusting growth under water deficits. However, the mutants lost much more water and had lower instant water use efficiency (WUE) than the wild type (Figure 7A). When they were grown in separate pots without maintaining FC or watering, the *PLDδ* and *GAPC* mutants wilted faster than the wild type (Figure 7B), consistent with the measurements that *PLDδ*- and *GAPC*-deficient plants lost more water.





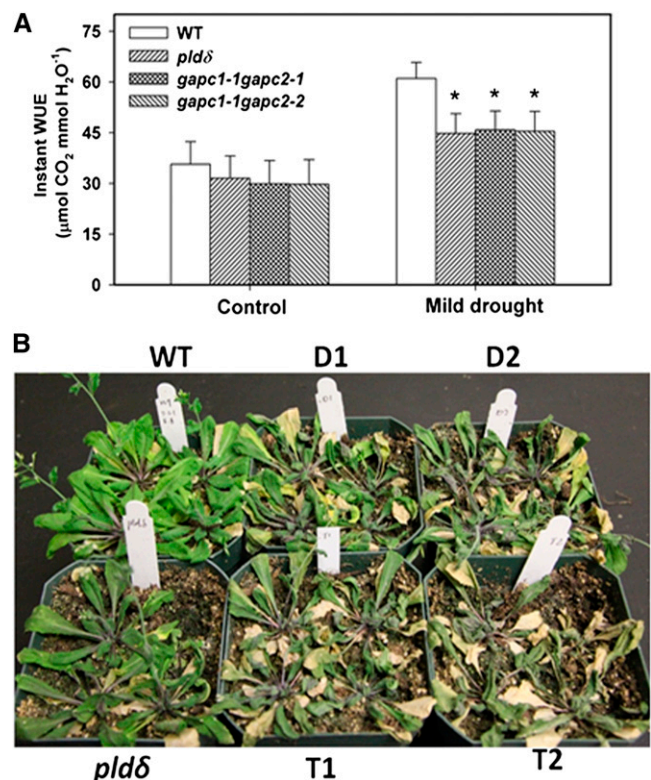
**Figure 6.** Response of GAPC and PLD $\delta$  Mutants to ABA and Water Deficits.

**(A)** Changes in stomatal aperture after ABA (25  $\mu$ M) or H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) treatment. Values are means  $\pm$  SE ( $n$  = 50). Different letters mark significant differences from each other (ANOVA,  $P$  < 0.05). WT, the wild type. **(B)** Stomatal conductance, cumulative water transpiration, photosynthesis, and dry weight. Asterisks mark significant difference from the wild type under the same growth condition. Values are means  $\pm$  SE ( $n$  = 16).

## DISCUSSION

This study demonstrates that PLD $\delta$  plays a role in mediating ABA-induced stomatal closure, but it acts in a distinctively different step from PLD $\alpha$ 1 in the ABA signaling pathway (Figure 8). PLD $\alpha$ 1 promotes NADPH oxidase activity and H<sub>2</sub>O<sub>2</sub> production (Zhang et al., 2009), whereas PLD $\delta$  mediates H<sub>2</sub>O<sub>2</sub> response but

not H<sub>2</sub>O<sub>2</sub> production. Both PLD $\alpha$ 1 and PLD $\delta$  are activated in response to ABA to generate PA. This raises the question of whether PA generated by PLD $\alpha$ 1 and PLD $\delta$  targets the same or different proteins. Our analyses of PLD $\delta$ - and PLD $\alpha$ 1-deficient mutants show that PLD $\alpha$ 1 produces twice as much PA as does PLD $\delta$  in response to ABA and that PLD $\delta$  is the main PLD responsible for H<sub>2</sub>O<sub>2</sub>-stimulated PA production. Also, temporal comparisons of PA formation in these mutants indicate that PLD $\alpha$ 1 is activated earlier than PLD $\delta$ . In addition, PLD $\alpha$ 1 and PLD $\delta$  have different subcellular locations and different substrate selectivities with PLD $\alpha$ 1 and PLD $\delta$  preferring PC and phosphatidylethanolamine, respectively (Figure 8; Wang et al., 2006). It is conceivable that the different magnitude, timing, and location of PA production as affected by PLD $\alpha$ 1 and PLD $\delta$  will

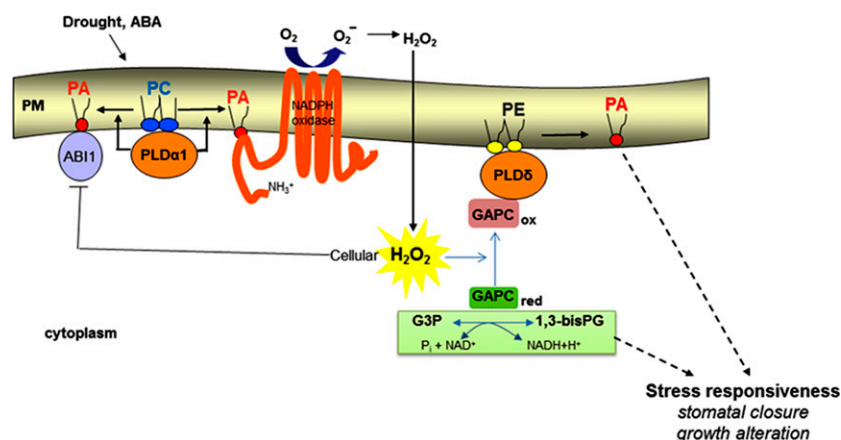


**Figure 7.** Increased Water Loss in GAPC-KO and PLD $\delta$ -KO *Arabidopsis* Plants.

**(A)** Instant WUE of wild-type (WT) and mutant plants under 100 and 60% FC. *Arabidopsis* seedlings were transplanted to pots and maintained at 100% FC and 60% FC. Instant WUE was calculated as the ratio of the photosynthetic rate to stomatal conductance; measurements were taken after the first 4 d after the onset of required stress. Asterisks indicate significant difference from the wild type. Values are means  $\pm$  SE ( $n$  = 16; \* $P$  < 0.05,  $t$  test).

**(B)** Increased dehydration of GAPC-KO and PLD $\delta$ -KO plants when FC was not maintained. Plants (25 d old) were fully watered and then left unwatered for 16 d when the photograph was taken. D1 and D2 are GAPC1 and 2 double KO *gapc1-1 gapc2-1* and *gapc1-1 gapc2-2*, respectively. T1 and T2 are GAPC1, GAPC2, and PLD $\delta$  triple KO *gapc1-1 gapc2-1 pldδ* and *gapc1-1 gapc2-2 pldδ*, respectively.

[See online article for color version of this figure.]



**Figure 8.** A Proposed Model for the Role of PLD/PA in Regulating ROS Production and Response under Water Deficits.

This model depicts only the known targets of PLD/PA in ABA-mediated stomatal closure; other ABA regulators are not included in this model. GAPCox refers to oxidized, catalytically inactive GAPC that interacts with PLD $\delta$  and promotes PLD $\delta$  activity. GAPCred refers to reduced, active GAPC that converts glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3-bisPG) with NADH production. PLD $\alpha$ 1 uses preferably phosphatidylcholine (PC), whereas PLD $\delta$  prefers phosphatidylethanolamine (PE) as substrate. Solid arrows indicate established links, and dashed arrows denote putative links. PM, plasma membrane.

impact their product PA interaction with target proteins. PA has been shown to bind to ABA INSENSITIVE1, NADPH oxidase, and sphingosine kinase. These proteins are involved in the ABA-mediated stomatal closure and targets of PLD $\alpha$ 1 (Figure 8) (Zhang et al., 2004; Zhang et al., 2009; Guo et al., 2011). In addition, mitogen-activated protein kinases (MAPKs), which are involved in various cellular processes, such as H<sub>2</sub>O<sub>2</sub>-induced cell death and ABA-promoted stomatal closure (Zhang et al., 2003; Zhang et al., 2006; Jammes et al., 2009; Yu et al., 2010), have been implicated as targets of PA. PLD $\delta$ -KO cells had a decreased MAPK activity in response to H<sub>2</sub>O<sub>2</sub> (Zhang et al., 2003); thus, MAPKs could be targets regulated by PA involved in PLD $\delta$ -mediated stomatal closure.

The analyses of GAPC and PLD $\delta$  interaction further augment the role of PLD $\delta$  and PA in mediating ROS response. This study documented the direct interaction between PLD $\delta$  and GAPCs qualitatively and quantitatively using different approaches. H<sub>2</sub>O<sub>2</sub> inhibits GAPC activity by oxidizing the catalytic Cys residues in the enzyme (Hancock et al., 2005). Our results indicate that H<sub>2</sub>O<sub>2</sub> promotes the GAPC interaction with PLD $\delta$  by decreasing the dissociation of the GAPC-PLD $\delta$  binding. KOs of GAPCs attenuated the ABA- or H<sub>2</sub>O<sub>2</sub>-promoted production of PA in the cell, providing in vivo support for the role of GAPCs in the H<sub>2</sub>O<sub>2</sub> activation of PLD $\delta$ . It may be noted that the level of H<sub>2</sub>O<sub>2</sub> used in this study is within physiological range reported for *Arabidopsis* leaves, in which H<sub>2</sub>O<sub>2</sub> levels varied from 60  $\mu$ M to more than 5 mM under different stress conditions or different assays (Karpinski et al., 1999; Veljovic-Jovanovic et al., 2001; Queval et al., 2008). In our study, GAPC activity in vitro was significantly inhibited at 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> and almost completely lost at 500  $\mu$ M H<sub>2</sub>O<sub>2</sub>. When H<sub>2</sub>O<sub>2</sub> was applied to protoplasts, we used 1 mM H<sub>2</sub>O<sub>2</sub> to ensure the oxidation of GAPCs because plant cells have a high capacity to degrade H<sub>2</sub>O<sub>2</sub> by several scavenging enzymes.

Plants deficient in GAPCs or PLD $\delta$  were less sensitive to ABA-promoted stomatal closure and had higher transpirational water

loss than the wild type under drought stress. Without either GAPC or PLD $\delta$ , plants are less responsive to drought-induced growth inhibition. These results indicate that GAPC-PLD $\delta$  interaction mediates ROS signaling and increases plant responsiveness to water deficits. Under the controlled water deficits with specific FCs maintained, the GAPC- or PLD $\delta$ -deficient plants actually accumulated more biomass than the wild type. The data are consistent with the observation that GAPC- or PLD $\delta$ -deficient plants have higher stomatal conductance and a higher rate of photosynthesis than the wild type, probably due to more opened stomata to allow more CO<sub>2</sub> uptake and increased nutrient transport than the wild type. However, the increase in biomass accumulation was at the expense of increased water use. Indeed, without maintaining a specific soil water level, the GAPC- or PLD $\delta$ -deficient plants wilted faster than the wild type when plants were withheld water. Earlier studies showed that KO of PLD $\delta$  decreased plant tolerance to severe stresses, such as freezing, UV irradiation, and salt tolerance in *Arabidopsis* (Katagiri et al., 2001; Zhang et al., 2003; Li et al., 2004; Bargmann et al., 2009). Decreasing growth under water deficits is one of the key strategies for plants to cope with stress and survival. The results indicate that the loss of GAPC or PLD $\delta$  compromises plant ability to sense the water stress and to adjust cellular and physiological response accordingly.

The glycolytic enzyme GAPDH occurs in both the cytosol and plastids, and the specific contributions of the two glycolytic pathways to plant metabolism and growth are not well defined (Plaxton, 1996; Muñoz-Bertomeu et al., 2009). Recent studies show that KO of both plastid-localized GAPCps causes severe development and growth defects, including arrested root development, dwarfism, and male sterility in *Arabidopsis* (Muñoz-Bertomeu et al., 2009, 2010). Genetic ablation of another glycolytic enzyme, phosphoglycerate mutase, also indicates a critical role of glycolysis in stomatal movement, vegetative growth, and pollen production in *Arabidopsis* (Zhao and Assmann, 2011). By

comparison, our study reveals that the KO of both cytosolic GAPCs results in no overt growth defects under normal condition in *Arabidopsis*. Instead, the GAPC-deficient plants exhibited less growth inhibition than the wild type under drought under the controlled drought conditions. These results suggest that GAPCs are required for plant growth responsiveness to drought, and we propose that the H<sub>2</sub>O<sub>2</sub>-promoted interaction of GAPCs with PLD $\delta$  is involved in the stress signaling leading to growth inhibition (Figure 8). An alternative hypothesis could be that the decrease in GAPC would alter the flux through carbon metabolism and affect the growth phenotype without GAPC binding to PLD $\delta$ . If so, one would expect that under drought stress, the increased H<sub>2</sub>O<sub>2</sub> in plants would inhibit GAPCs, leading to growth inhibition. But this is not the case because GAPC-KO plants display less growth inhibition than the wild type. In addition, GAPC-KO mutants share a similar phenotype as PLD $\delta$ -KO; ablation of either GAPCs or PLD $\delta$  renders plants less responsive to water deficits, and the drought-induced growth inhibition requires the presence of both PLD $\delta$  and GAPCs. Thus, our results are consistent with the proposition that the interaction between GAPCs and PLD $\delta$  is involved in mediating H<sub>2</sub>O<sub>2</sub> signals in plant response to water deficits. However, further studies are needed to understand the requirement for and mechanism of the GAPC–PLD $\delta$  interaction in modulating plant growth under stress and the metabolic role of GAPCs in plant growth and stress responses.

The identification of GAPC interaction with PLD $\delta$  unveils a regulatory function of the carbon metabolic enzymes GAPCs in plants and potentially a molecular node linking stress signaling and metabolic alterations. Some classical metabolic enzymes can have crucial regulatory roles in the cell. For example, hexokinase has been found in the nucleus, where it forms a protein complex mediating glucose signaling in yeast and plant (Ahuatzi et al., 2004; Cho et al., 2006). In animal cells, GAPDH is involved in nonmetabolic processes, including gene transcription, DNA replication, nuclear tRNA export, and DNA repair, and these studies indicate that GAPDH has direct relationship to the pathology of various diseases (Sirover, 1997; Hara et al., 2005; Bae et al., 2006; Harada et al., 2007). Oxidized GAPDH is thought to be translocated to the nucleus to regulate gene expression to initiate apoptotic cell death (Hara et al., 2005; Bae et al., 2006). This study shows that the cytosolic GAPCs interact with the plasma membrane-bound PLD $\delta$  and the interaction is promoted by ROS. We propose that the GAPC–PLD $\delta$  interaction in response to ROS provides a molecular link between stress signaling and the alteration of cellular metabolism and growth (Figure 8). Further investigations on the specificity, mechanism, and downstream targets of these interactions will provide mechanistic insights to how plants adjust metabolism and growth in response to different stresses.

## METHODS

### Isolation of KOs and *pld $\delta$* Complementation

*Arabidopsis thaliana* (Columbia-0) wild-type and T-DNA insertion lines were obtained from the ABRC at Ohio State University. *pld $\alpha$ 1* (SALK\_053785) was isolated and confirmed previously (Zhang et al., 2004). The homozygous line of *pld $\delta$*  (SALK\_023247) was confirmed by PCR. The primers for PCR screening are listed in Supplemental Table 1 online. Four T-DNA lines

(*gapc1*, CS328689, SALK\_129091; *gapc2*, SALK\_016539 and SALK\_070902) were screened, and the homozygous lines were verified by PCR (see Supplemental Figure 6 online). The open reading frame of *GAPC1* and *GAPC2* shares 89.7% identity, while the 3' untranslated regions of both genes are not conserved. Thus, primers in the 3' untranslated region of *GAPC1* and *GAPC2* were used to distinguish the *GAPC1* and *GAPC2* transcripts. To complement *pld $\delta$* , a genomic sequence including the promoter of PLD $\delta$  was cloned (primers listed in see Supplemental Table 1 online) and inserted into binary vector PEC291 for transformations of *pld $\delta$* .

### Plant Growth Conditions and Physiological Analysis

Plants were grown in soil in a growth chamber with cool white light of 160  $\mu\text{mol m}^{-2} \text{s}^{-1}$  under 12-h-light/12-h-dark and 23°C/19°C cycles. Drought stress was created by a gravimetric approach (Sheshshayee et al., 2005; Peters et al., 2010). Ten-day-old *Arabidopsis* seedlings were transplanted to pots containing soil saturated to maximum field capacity (100% FC). Soil saturation was achieved by adding a known amount of water based on weight of soil and water holding capacity. The pots were covered with thick polyethylene sheets to prevent evaporation. One set of plants was maintained at 100% FC (control), and the other two sets were maintained at 60% (mild stress) and 30% (acute stress). The pots were weighed every day, and the difference in weight in subsequent days was corrected by adding water to maintain specific FCs. The amount of water added over the experimental period was summed up to give the cumulative water transpired. Stomatal conductance and photosynthetic rate were recorded on fully expanded leaf using a portable gas exchange system (LICOR6400-XT; LICOR Biosciences). Instant WUE was calculated as ratio of photosynthetic rate to stomatal conductance. Measurements were taken on the first 4 d after the onset of drought stress. At the end of the stress, the shoots were harvested, dried, and weighed.

### Stomatal Aperture Measurements

Stomatal aperture was measured using epidermal peels according to a described procedure (Zhang et al., 2004). Briefly, epidermal peels were floated in incubation buffer (10 mM KCl, 0.2 mM CaCl<sub>2</sub>, 0.1 mM EGTA, and 10 mM MES-KOH, pH 6.15) for 2.5 h under cool white light at 23°C to induce stomatal opening. ABA or H<sub>2</sub>O<sub>2</sub> was applied separately to epidermal peels, which were incubated for 2.5 h under cool white light at 23°C to induce stomatal closure. Stomata were imaged under a microscope with a digital camera and analyzed with ImageJ software (NIH).

### ROS Detection

The endogenous ROS levels in guard cells were detected using epidermal peels treated with the dye H<sub>2</sub>DCF-DA (Sigma-Aldrich) (Zhang et al., 2009). Epidermal peels were floated in incubation buffer for 2 h and then loaded with 50  $\mu\text{M}$  H<sub>2</sub>DCF-DA (50 mM stock in DMSO) for 10 min, followed by 20 min washing in incubation buffer. The 25  $\mu\text{M}$  ABA was added for desired time of treatment. Epidermal peels were observed with a confocal microscope (Zeiss LSM 510) (green fluorescence: excitation of 488 nm and emission of 525 nm).

### GAPC Cloning, Protein Purification, and Activity Assay

The cDNAs of *GAPC1* and *GAPC2* were amplified and ligated to pET-28a-c(+) vector to produce GAPC1 and GAPC2 with six His residues at the N terminus. The recombinant plasmids were transformed into *Escherichia coli* BL21(DE3)pLysS. Induction and purification of protein was as described (Guo et al., 2011). Purified proteins were dialyzed in tris-buffered saline buffer with DTT overnight. Dialyzed proteins were centrifuged at 12,000g for 20 min, and protein concentration was determined using the Bradford protein assay. Purified proteins were analyzed by 10% SDS-

PAGE, followed by Coomassie Brilliant Blue staining. The prepared proteins were used for activity assay or kept in 50% glycerol at  $-80^{\circ}\text{C}$ . NAD-dependent GAPDH activity assay was done using purified bacterially expressed GAPC (2 to 5  $\mu\text{g}$ ) or total protein (25 to 50  $\mu\text{g}$ ) extracted from *Arabidopsis* leaves with modification according to the method described previously (Rius et al., 2008).

### Protein Coprecipitation and Coimmunoprecipitation Assays

GST-PLD $\delta$  construct and expression of PLD $\delta$  were described previously (Qin et al., 2002). To pull down GAPC, GST-PLD $\delta$ -bound beads ( $\sim 15\ \mu\text{g}$  purified proteins) were incubated with total protein extracted from *E. coli* expressing GAPC1 or GAPC2 at  $4^{\circ}\text{C}$  for 3 h with gentle rotation (Zhao and Wang, 2004). To pull down PLD $\delta$ , GAPC-bound agarose beads ( $\sim 10\ \mu\text{g}$  purified proteins) were incubated with total protein extracted from *E. coli* expressing GST-PLD $\delta$  at  $4^{\circ}\text{C}$  for 3 h with gentle rotation. The beads were collected and washed three times and subjected to 10% SDS-PAGE followed by immunoblotting. To coexpress PLD $\delta$  and GAPC in yeast for coimmunoprecipitation, PLD $\delta$  and GAPC1 or GAPC2 were cloned into pESC-HIS vector and transformed into YPH yeast strain (Stratagene). PLD $\delta$  and GAPC1 or GAPC2 were coexpressed in yeast after induction by addition of galactose, and the yeasts were grown overnight at  $30^{\circ}\text{C}$ . Then,  $20\ \mu\text{M}$   $\text{H}_2\text{O}_2$  was added when oxidative condition was required. Primers used for cloning are listed in Supplemental Table 1 online. Total protein was extracted from harvested yeast and used for coimmunoprecipitation analysis.

### SPR Analysis

SPR binding assays were performed as described with some modifications (Guo et al., 2011). The purified proteins were dialyzed in the running buffer (0.01 M HEPES, 0.15 M NaCl, and  $50\ \mu\text{M}$  EDTA, pH 7.4) overnight at  $4^{\circ}\text{C}$  and then the proteins were centrifuged at  $13,000g$  to remove insoluble protein before use. For each experiment, the running buffer with  $500\ \mu\text{M}$   $\text{NiCl}_2$  was injected to saturate the NTA chip with nickel. His-tagged GAPC1 protein (200 nM) was immobilized on a Biacore Sensor Chip NTA via  $\text{Ni}^{2+}$ /NTA chelation. PLD $\delta$ -GAPC1 interaction was monitored as GST-PLD $\delta$  (200 nM) was injected in sequence over the surface of the sensor chip. The purified GST protein was used as control. During the evaluation, the sensorgrams from the beginning of association to the end of dissociation for each interaction were analyzed and plotted by SigmaPlot 10.0 (Systat Software, Inc.). Kinetic constants including  $B_{\text{max}}$ , association ( $k_{\text{on}}$ ), and dissociation rate ( $k_{\text{off}}$ ) were analyzed using BIAevaluation software (GE Healthcare).

### BiFC

The BiFC vectors were constructed, described, and provided by Walter et al. (2004). GAPC1 or GAPC2 cDNA was cloned into pSPYNE vector (GAPC-YFP<sup>N</sup>), and PLD $\delta$  cDNA was cloned into pSPYCE vector (PLD $\delta$ -YFP<sup>C</sup>). The constructs were transformed into C58C1 *Agrobacterium tumefaciens* strain and grown to stationary phase. Bacterial cells were collected and resuspended in solution containing 10 mM MES, pH 5.7, 10 mM  $\text{MgCl}_2$ , and  $150\ \text{mg mL}^{-1}$  acetosyringone. Three-week-old *Nicotiana benthamiana* leaves were infiltrated with the mixed bacteria (GAPC-YFP<sup>N</sup> and PLD $\delta$ -YFP<sup>C</sup>) solutions (Voinnet et al., 2003). YFP fluorescence was examined in tobacco leaves using a Zeiss LSM 510 confocal microscope, with a 488-nm excitation mirror and a 505- to 530-nm filter to record images.

### Assaying PLD Activity

For in vivo PLD activity assay, protoplasts prepared from leaves of 4-week-old plants were incubated in 0.5 mg/mL NBD-PC for 80 min on ice (Zhang et al., 2004). To determine PLD activity using fluorescent lipids, as affected

by ABA treatment at different time points in vivo,  $100\ \mu\text{M}$  ABA was added to the NBD-PC-labeled protoplasts, and  $100\ \mu\text{L}$  aliquots ( $1.5 \times 10^5$  for each assay) were transferred to a new tube at the end of each treatment. Then, 0.4 mL hot isopropanol ( $75^{\circ}\text{C}$ ) was added, and the mixture was incubated for 10 min at  $75^{\circ}\text{C}$  to inactivate PLD. Lipids extraction, separation, and quantification were done according to the procedure as described (Zhang et al., 2004). To test the effect of GAPC on PLD $\delta$ , PLD $\delta$  activity was assayed using dipalmitoylglycerol-3-phospho-[methyl- $^3\text{H}$ ]choline as substrate according to the procedure described previously (Qin et al., 2002).

### Electrospray Ionization–Tandem Mass Spectrometry Analysis of Lipid Molecular Species

Lipids were extracted and PA analyzed by electrospray ionization–tandem mass spectrometry (Xiao et al., 2010). Expanded leaves of 4- to 5-week-old plants were sprayed with  $100\ \mu\text{M}$  ABA with 0.01% Triton X-100. The leaves were excised and immersed in 3 mL of isopropanol with 0.01% butylated hydroxytoluene (preheated to  $75^{\circ}\text{C}$ ) immediately after sampling. The experiment was repeated three times with five replicates of each treatment each time.

### Statistical Analysis

Experimental values represent mean values and standard errors.  $n$  represents the number of independent samples. P values were calculated with Student's  $t$  test (two-tailed) using Microsoft Excel or analysis of variance (ANOVA).

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PLD $\alpha 1$ , At3g15730; PLD $\delta$ , At4g35790; GAPC1, At3g04120; GAPC2, At1g13440; and UBQ10, At4g05320.

### Supplemental Data

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

**Supplemental Figure 1.** Confirmation of Homozygous T-DNA Insertion PLD Mutants by PCR.

**Supplemental Figure 2.** Expression Level of PLD $\delta$  in Response to ABA.

**Supplemental Figure 3.** PLD $\delta$ -GAPC Association as Identified by GAPC1 Coprecipitation of PLD $\delta$  from Microsomal Proteins of *Arabidopsis* Leaves.

**Supplemental Figure 4.** Purification and Immunoblotting of PLD $\delta$  and GAPCs Produced in *E. coli* and Yeast.

**Supplemental Figure 5.** Negative and Positive Control for BiFC.

**Supplemental Figure 6.** DTT Protection of GAPC Activity.

**Supplemental Figure 7.** Isolation of GAPC T-DNA Homozygous Lines.

**Supplemental Figure 8.** Growth Phenotype of the Wild Type and GAPC and PLD $\delta$  Mutants under Control and Drought Conditions.

**Supplemental Table 1.** Primers Used in This Study.

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## AUTHOR CONTRIBUTIONS

L.G. and X.W. designed the research. L.G. performed most experiments. Y.Z. and W.Z. identified *pldδ* stomatal phenotype. X.P. and S.P.D. performed the interaction and GAPDH activity assays. R.N. performed the physiological study in Figure 6B. L.G. and X.W. analyzed the data and wrote the article.

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**Cytosolic Glyceraldehyde-3-Phosphate Dehydrogenases Interact with Phospholipase D $\delta$  to Transduce Hydrogen Peroxide Signals in the *Arabidopsis* Response to Stress**

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