Phospholipase D- and Phosphatidic Acid-Mediated Signaling in Plant Response to Abscisic Acid and Reactive Oxygen Species

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Phospholipase D- and Phosphatidic Acid-Mediated Signaling in Plant Response to Abscisic Acid and Reactive Oxygen Species

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Dissertation presented to the Graduate School of Arts and Sciences at the University of Missouri-St Louis in partial fulfillment of the requirements of Doctor of Philosophy

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ABSTRACT

Arabidopsis genome has 12 phospholipase D (PLD) genes that are classified into six types, PLDα, PLDβ, PLDγ, PLDδ, PLDe, and PLDζ, based on sequence similarities, domain structures, and biochemical properties. Phosphatidic acid (PA) produced by PLDs has been identified as important lipid signaling molecule in cell growth, development, and stress responses in both plants and animals. This study was undertaken to determine the role of PLD and PA in plant response to abscisic acid (ABA) and reactive oxygen species (ROS). The lipid mediator PA was found to interact with sphingosine kinases (SPHKs) in Arabidopsis. Two unique SPHK cDNAs were cloned and expressed. Both SPHKs are catalytically active, phosphorylating various long-chain sphingoid bases (LCBs). PA binds to and stimulates both SPHKs, and the interaction promotes lipid substrate binding to the catalytic site of the enzyme. SPHK-deficient and PLDα1-deficient mutants were employed to determine the cellular and physiological functions of the PA-SPHK interaction in plants. Compared to wild-type (WT) plants, SPHK and PLDα1 mutants all displayed decreased sensitivity to ABA-promoted stomatal closure. The data indicate that SPHK and PLDα1 act together in ABA response and that SPHK and phytosphingosine-1-phosphate (phyto-S1P) act upstream of PLDα1 and PA in mediating the ABA response. On the other hand, PA is involved in the activation of SPHK, forming a positive loop in signaling plant response to ABA. In addition, another PLD, PLDδ, was found to be involved in the ROS and ABA signaling pathways. PLDδ functions downstream of PLDα1 and H2O2 to mediate the ABA-induced stomatal closure. Furthermore, the study has identified that cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) acts as a molecular link between H2O2 and PLDδ activation. H2O2 inhibited GAPC activity but promoted GAPC-PLDδ interaction and the PLDδ activity. The loss of both GAPCs decreased plant insensitivity to ABA- and H2O2-induced stomatal closure, like PLDδ. In addition, GAPC-deficient plants produced less PA than wild-type in response to ABA and H2O2. These results indicate that GAPCs mediate H2O2-activation of PLDδ in Arabidopsis response to ABA. The interaction of a cytosolic metabolic enzyme GAPC and a membrane-associated PLDδ transduces ROS signals in plant response to ABA and oxidative stress. The physiological functions of
GAPC were characterized using the *GAPC* T-DNA insertion lines. There are two *GAPC* genes in *Arabidopsis*, which are involved in the glycolytic pathway, are potentially important to plant growth and development. *GAPC* deficiency did not cause growth inhibition or development problems for the plants. Instead, the GAPC mutants displayed larger size and accumulated more biomass when grown under normal condition. However, *GAPC*- and *PLDδ*-deficient mutants were less tolerant to salt and freezing stresses. In addition, *GAPC* double knockouts had a 4-5% decrease in seed oil content. These results indicate the importance of GAPC in plant stress tolerance and metabolism pathway.
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ABBREVIATIONS

ABA, Abscisic acid
ABI1, ABA INSENSITIVE 1
BiFC, Bimolecular fluorescence complementation
Co-IP, Co-immunoprecipitation
COM, Compelmetation
GAPC, Cytosolic glyceraldehydes-3-phosphate dehydrogenase
GAPDH, Glyceraldehyde-3-phosphate dehydrogenase
GFP, Green fluorescence protein
HIS, Histidine
KO, Knockout
LCB, Long-chain base
LCBP, Long-chain base phosphate
OE, Overexpressed
PA, Phosphatidic acid
PC, Phosphatidylcholine
PE, Phosphatidylethanolamine
PG, Phosphatidylglycerol
Phyto-S1P,
Phytosphingosine-1-phosphate
PI, Phosphatidylinositol
PLA, Phospholipase A
PLC, Phospholipase C
PLD, Phospholipase D
PS, Phosphatidylserine
ROS, Reactive oxygen species
SD, Standard deviation
SE, Standard error
SPHK, Sphingosine kinase
SPR, Surface plasmon resonance
T-DNA, Transferred DNA
UTR, Untranslated region
*, significant difference at P<0.05 based on Student t-test
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Chapter 1. Research Background, Goal and Specific Objectives

Research background
Phospholipids provide not only the major structural component of biological membranes, intermediates for oil biosynthesis, but also various mediators in cell signaling and other regulatory processes (Wang, 2004; Wang et al., 2006). Phospholipids consist of a glycerol backbone, a head group and two fatty acid acyl chains. A head group is attached to the glycerol backbone at sn-3 position and two fatty acid acyl chains are linked to the glycerol backbone at the sn-1 and sn-2 positions (Fig. 1) (Wang et al., 2006). Phospholipids can be hydrolyzed by phospholipases including phospholipase D (PLD), phospholipase C (PLC), and phospholipase A (PLA) (Wang et al., 2006). The first PLD cDNA was cloned from castor bean over 15 years ago (Wang et al., 1994). PLDs were cloned and studied in other species such as yeast and humans later based on the sequence information of castor bean PLD (Qin et al., 1997). PLD has been found in various species from viruses, bacteria, yeast, to plants and mammals (Qin et al., 1997). PLD has been implicated in various cellular processes, including signal transduction, cytoskeletal reorganization, vesicular trafficking, membrane remodeling, and lipid degradation (Wang et al., 2006; Wang, 2004; Wang, 2005; Bargmann and Munnik, 2006). In recent years, increasing evidence has shown that phospholipids function as signaling mediators in plant cells (Testerink and Munnik, 2005). The lipid mediators produced under certain conditions can bind to the target proteins to increase or inhibit the activities of target proteins, serve as substrate for the production of other lipid mediators, or act as membrane anchor to tether target proteins to cell membranes (Hong et al., 2010; Li et al., 2009). PA generated by PLD plays pivotal roles in different plant functions, ranging from responses to different stresses, nutrient deficiency, cell development, to seed quality (Hong et al., 2010; Li et al., 2009; Wang et al., 2002).

Different classes of lipids have been implicated as lipid messengers in plant growth, development, and stress responses, and recent results have begun to unveil complex interactions among different lipid signaling pathways (Peters et al., 2010). Under a given stress, more than one lipid mediators are often produced, and some are antagonistic
whereas others have similar functions. Both phospatidic acid (PA) and long-chain base-1-phosphate (LCBP) promote abscisic acid (ABA)-mediated stomatal closure and decrease reactive oxygen species (ROS)-induced cell death (Jacob et al., 1999; Zhang et al., 2003; Coursol et al., 2005; Shi et al., 2007). ABA and ROS are pivotal signals impacting various aspects of plant growth and stress responses. This raises intriguing questions of how these two lipid signaling processes interact to mediate plant stress responses.

**Different PLDs involved diverse stress responses**

PLD catalyzes the hydrolysis of phospholipids to produce PA and a free head group (Fig. 1), and this enzyme was first discovered in plants and has since been found to occur also in yeast, bacteria, fungi, and animals (Qin et al., 1997; Wang and Wang, 2001). The *Arabidopsis* genome has 12 genes encoding PLDs, which are grouped into six classes, PLDα(1-3), β(1, 2), γ(1-3), δ, ε, and ζ(1, 2) based on the protein sequences, protein domain structures, and enzyme biochemical properties (Wang et al., 2006). PLDα, β, γ, δ, and ε contain a Ca\(^{2+}\)/phospholipids-binding C2 domain whereas PLDζ1 and ζ2 contain the pleckstrin homology (PH) and phox homology (PX) domain (Wang et al., 2006) (Fig. 2). All the PLDs have two conserved HxKxxxD (HKD) motifs that are involved in catalytic activities (Wang et al., 2006; Li et al., 2009). Some of the C2-containing PLDs contain a polyphosphoinositide-binding region (PBR1) located between two HKD domains, which binds phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) (Zheng et al., 2002) (Fig. 2).

These sequence differences provide a structural basis for distinctively different biochemical properties for different PLDs. All the C2-containing PLDs require Ca\(^{2+}\) for activity, but PX and PH-containing PLDζs do not (Wang et al., 2006). In addition, the differences in the C2 sequences can explain in part of the different Ca\(^{2+}\) concentration requirements. PLDα1 is most active when assayed at millimolar Ca\(^{2+}\) whereas PLDβ1 and PLDγ1 require micromolar concentrations of Ca\(^{2+}\) for optimal activity and also require PIP\(_2\) as cofactor (Qin et al., 1997; Zheng et al., 2002; Pappan et al., 2004). PLDδ and PLDε both are active within a broad range of Ca\(^{2+}\) concentrations (µM-mM) (Hong et al.,
PLDδ requires oleate and PIP₂ for its activity, but PLDe is active under the reaction conditions of PLDα₁, β₁, γ₁, and δ (Wang and Wang, 2001; Qin et al., 2002; Hong et al., 2008). Arabidopsis PLDs also selectively hydrolyze common membrane phospholipids such as PC, PE and PG (Li et al., 2009). The varied co-factor requirements and substrate preferences for different PLDs indicate that specific PLDs are activated differently in the cell and may have unique cellular and physiological functions (Li et al., 2009).

Different PLDs are involved in various physiological processes, displaying unique and overlapping functions (Li et al., 2009). PLDα₁-deficient plants are altered in plant response to several stresses, including water loss (Sang et al., 2001b), ROS production (Sang et al., 2001a; Zhang et al., 2009), and salt tolerance (Bargmann et al., 2009; Yu et al., 2010). PLDδ is involved in freezing tolerance (Li et al., 2004), dehydration (Katagiri et al., 2001), salt tolerance (Bargmann et al., 2009), H₂O₂-induced programmed cell death (Zhang et al., 2003), microtubule organization and cytoskeletal rearrangement (Gardiner et al., 2001; Gardiner et al., 2003). PLDα3 is also involved in salt tolerance (Hong et al., 2008) whereas PLDe enhances Arabidopsis nitrogen signaling and growth (Hong et al., 2009). PLDζ₁ and ζ₂ are involved in lipid remodeling and root growth in plant responses to phosphate deprivation (Cruz-Ramirez et al., 2006; Li et al., 2006a, b). PLDζ₁ is implicated in root-hair patterning (Ohashi et al., 2003), and PLDζ₂ participates in vesicle trafficking to regulate auxin response (Li and Xue, 2007).

**PA as a pivotal class of lipid messengers**

One mechanism by which PLDs affects plant stress responses is to produce PA, which has been identified as a class of lipid messengers in plants and animals (Fig. 3). PA is minor phospholipid and constitutes less than 1% of total phospholipids in most plant tissues, but the cellular level of PA changes dynamically in plants under abiotic and biotic stresses (Wang et al., 2006). The amount of PA in Arabidopsis leaves increased more than 60% within 10 min of application of ABA (Zhang et al., 2004). Other stresses, including wounding, freezing, various osmotic stresses, oxidative stress, and drought, induce accumulation of PA (Li et al., 2009). Manipulations of various PLDs in
Arabidopsis have shed light on the regulatory functions of PA. Characterization of knockouts, knockdown, and overexpression lines of PLDs, has shown that PA produced from different PLDs has unique roles in plant response to different stresses, including water deficits, high salinity, freezing, phosphate deprivation, nitrogen availability, and plant-pathogen interactions (Sang et al., 2001b; Zhang et al., 2003; Hong et al., 2008, 2009; Bargmann et al., 2009; Peters et al., 2010).

One mode of PA actions is its direct interaction with target proteins (Fig. 3). In yeast and animal cells, PA binds to protein kinases, lipid kinases, protein phosphatases, transcriptional factors, and proteins involved in vesicular trafficking and cytoskeletal rearrangement (Gomez-Cambronero, 2010; Wang et al., 2006). In plants, PA has been found to interact with ABI1 PP2C phosphatase, PDK1 (phosphoinositide-dependent protein kinase1), PEPC (phosphoenolpyruvate carboxylase), protein phosphatase 2A subunit RCN1, CTR1 protein kinase, the actin capping protein AtCP, PA transport protein TGD2, NADPH oxidase, and MAPK6 (mitogen-activated protein kinase6) (Zhang et al., 2004; Anthony et al., 2004; Testerink et al., 2004; Huang et al., 2006; Testerink et al., 2007; Lu and Benning, 2009; Zhang et al., 2009; Yu et al., 2010) (Fig. 3). Several potential PA-interacting proteins were identified by PA-affinity chromatography followed by mass spectrometric analyses in plants, and additional experiments need to confirm the interaction and determine their roles in cell functions in plants (Testerink et al., 2004). PA-protein interaction may modulate the function of a protein in two ways, tethering it to the membrane to change their localization, and/or increasing or decreasing the enzyme catalytic activity. For example, PA specifically binds to PDK1 to activate AGC2-1 kinase to promote root hair growth (Anthony et al., 2004). PA is found to interact with Arabidopsis NADPH oxidase to promote NADPH oxidase activity (Zhang et al., 2009). PA-NADPH oxidase interaction plays positive roles in ABA-induced stomatal closure. PLDα1-deprived PA interacts with ABI1 and tethers ABI1 to the plasma membrane to inhibit ABI1 phosphatase activity, leading to stomatal closure in response to ABA (Zhang et al., 2004).

PLD and PA in the production of and response to reactive oxygen species (ROS)
ROS are produced during cellular metabolism processes and biotic/abiotic stresses also induce ROS production in both animal and plant cells (Quan et al., 2008; Finel, 2011; Apel and Hirt, 2004). ROS are recognized as signaling molecules involved in various physiological processes including plant growth, development and stress responses in plants (Desikan et al., 2001; Gechev et al., 2006; Shao et al., 2008). PLD and PA have been reported to be involved in the regulation of ROS production and manipulations of PLD affected ROS production (Sang et al., 2001a; Zhang et al., 2009; Yamaguchi et al., 2004; Lanteri et al., 2011). *PLDa1*-deficient *Arabidopsis* had impaired ROS production and PA increased the levels of ROS in *Arabidopsis* leaves (Sang et al., 2001a). PLDα1 is activated in response to ABA to generate PA, which binds to and activates NADPH oxidase to produce ROS (Zhang et al., 2009). In suspension rice cells, PLD and PA have been shown to promote the production of ROS (Yamaguchi et al., 2004). In addition, PLD and PA play a role in ROS responses. In rice cells, H$_2$O$_2$-induced activation of PLD is involved in biosynthesis of phytoalexins (Yamaguchi et al., 2004). In *Arabidopsis*, PLDδ is activated by H$_2$O$_2$ and PLDδ-deprived PA is required to decrease the H$_2$O$_2$-promoted programmed cell death (Zhang et al., 2003). However, knockout of PLDδ does not alter H$_2$O$_2$ production under the stress conditions tested (Zhang et al., 2003). These results indicate that specific PLDs function in different steps in plant ROS signaling pathways.

**GAPDH as an oxidative stress sensor**

Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is a classic metabolic enzyme involved in glycolysis pathways (Plaxton, 1996). It catalyzes the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate to provide energy for the cell and intermediates for other metabolism pathways (Munoz-Bertomeu et al., 2009). Recently, numerous studies suggest that GAPDH is not only a metabolism enzyme but also participates in regulatory processes in animals and plants (Hara et al., 2009; Bae et al., 2006; Harada et al., 2007; Kim et al., 2003). GAPDH has cysteine residues which are critical to GAPDH enzyme activity. Meanwhile, these cysteine residues are sensitive to oxidative stress and oxidation of the cysteine residues inhibits the activity of GAPDH (Hara et al., 2009; Kim et al., 2003). Besides glycolysis function, GAPDH is implicated
to be a regulatory protein involved in many events including gene transcription, phosphotransferase, DNA replication, nuclear RNA export, endocytosis, microtubule bundling, and oncogenesis in animals (Hara et al., 2005; Sirover, 1997; Harada et al., 2007). Oxidation of catalytic cysteine residues inhibits GAPDH and causes GAPDH to relocate into the nucleus to regulate apoptotic process (Hara et al., 2005). Interestingly, GAPDH has been shown to interact with PLD2 and promote PLD2 activity under oxidative stress in PC12 cells (Kim et al., 2003), which raises the possibility that GAPDH function as an oxidative sensor and mediates the activation of PLD.

**SPHKs in plants**

Sphingosine kinase (SPHK) is a member of the DAG kinase family (Strub et al., 2010), and phosphorylates LCBs to LCBPs, such as sphingosine-1-phosphosphate (S1P) and phytos-1P (Fig. 4). SPHK activity and functions have been well characterized in animals and yeast (Worrall et al., 2003). In mammals, two SPHKs and their product S1P have important roles in regulations of many cellular processes including cell growth, suppression of apoptosis and pathophysiology of various diseases (Strub et al., 2010). Sphingosine is the predominant LCB in animal cells, but it is minor LCB in some plants and absent in *Arabidopsis* (Lynch et al., 2009; Michaelson et al., 2009). SPHK activity is mainly associated with the membrane fraction in *Arabidopsis* and could phosphorylate various LCBs to generate lipid mediators such as phyto-S1P (Coursol et al., 2005).

The *Arabidopsis* genome contains four genes with sequence similarities to mammalian SPHKs. At5g23450 encodes a long-chain base kinase AtLCBK1 (Nishira et al., 2000) whereas At5g51290 is regarded as a ceramide kinase (Liang et al., 2003). At2g46090 did not have sphingosine phosphorylating activity (Worrall et al., 2008). At4g21540 was annotated as one SPHK, and this sequence consists of two repeats that are most similar to mammalian SPHKs. A cDNA from the second repeat was reported to encode an active SPHK, designated SPHK1 (Worrall et al., 2008). SPHK1 utilizes sphingosine, phytosphingosine, and other LCBs as substrates and it is involved in ABA response in *Arabidopsis* (Worrall et al., 2008).
LCBs as lipid mediators

Like glycerophospholipids, sphingolipids serve not only as main component of cell membranes, but also important signaling molecules (Lynch et al., 2009; Pata et al., 2010). S1P is produced in animal cells by two sphingosine kinases and is degraded either by S1P lyase or S1P phosphatases (Fig. 4). S1P regulates a variety of developmental and disease processes in animals (Strub et al., 2010). Many lines of evidence indicate that S1P is an intracellular messenger acting directly on intracellular target proteins (Maceyka et al., 2011). In addition, S1P is exported out of cells to mediate signaling pathways through five specific G protein-coupled receptors (S1RP1-S1RP5) on the plasma membrane (Maceyka et al., 2011).

The function of sphingolipids in plants is not well defined, but accumulating evidence indicates that sphingolipid metabolites, including LCBs, LCBPs, and ceramides, are involved in signaling pathways in plants (Lynch et al., 2009; Pata et al., 2010). Characterization of Arabidopsis deficient in sphingolipid metabolism genes facilitates the understanding of signaling and physiological functions of sphingolipid in plants. For example, characterization of ceramide kinase mutant (acd5) shows that ceramide induces plant programmed cell death (PCD) whereas phosphorylated ceramide partially attenuates PCD (Liang et al., 2003). Mutation of a LCB1 subunit of serine palmitoyltransferase blocks accumulation of LCBs in Arabidopsis and indicates that LCBs are involved in initiating programmed cell death (PCD) through induction of reactive oxygen species (ROS) production in Arabidopsis (Shi et al., 2007). LCBPs decrease reactive oxygen species (ROS)-induced programmed cell death (PCD) whereas unphosphorylated LCBs promote ROS-mediated cell death (Shi et al., 2007).

SPHK/Phyto-S1P and PLD/PA in the ABA signaling pathway

One of the functions that have been studied for SPHK and phyto-S1P is their roles in mediating the ABA-promoted stomatal closure. ABA treatments increased SPHK activity in Arabidopsis and drought stress induced the production of a LCBP in Commelina communis (Coursol et al., 2003; Ng et al., 2001). Application of S1P induces stomatal closure and inhibits stomatal opening (Ng et al., 2001). Knockout of either SPHK1
decreased the sensitivity to ABA in *Arabidopsis*, whereas overexpression of *SPHK1* increased ABA sensitivity (Worrall et al., 2008). The involvement of LCBP in the ABA signaling in guard cells is further supported by analysis of the LCBP phosphatase mutant *spp1*. The *spp1* plants displayed increased sensitivity to ABA in stomatal closure due to the defect in LCBP degradation in the mutant (Nakagawa et al., 2011).

A number of studies have shown that PLD and PA play important roles in signaling ABA-mediated stomatal closure (Jacob et al., 1999; Zhang et al., 2004). PLD and PA promote open stomata to close and meanwhile prevent the closed stomata from opening (Jacob et al., 1999; Zhang et al., 2004). In *Arabidopsis*, *PLDα1*-deficient plants displayed insensitivity to ABA, whereas overexpression (OE) of *PLDα1* resulted in increased sensitivity to ABA (Sang et al., 2001b). *PLDα1* regulates ABA signaling pathways through different interactions. PA binds to ABI1 phosphatase 2C and this interaction inhibits the negative function of ABI1 in ABA response and mediates ABA-promoted stomatal closure (Zhang et al., 2004; Mishra et al., 2006). On the other hand, *PLDα1* interacts with Gα to mediate the ABA inhibition of stomatal opening (Zhao and Wang, 2004; Mishra et al., 2006). In addition, *PLDα1*-derived PA binds to and increases NADPH oxidase activity to promote the production of reactive oxygen species in ABA-mediated stomatal closure (Zhang et al., 2009).

**Goals and specific objectives**

A series of recent results have provided insights into how specific PLD and PA mediate the ABA promotion of stomatal closure in *Arabidopsis* (Li et al., 2009). In addition to PA, another lipid messenger, phytosphingosine 1-phosphate (phyto-S1P), has been found to promote the ABA effect on stomatal closure (Ng et al., 2001; Coursol et al., 2005; Worrall et al., 2008). In animal systems, S1P is an important lipid mediator and its production has been suggested to be regulated by PA (Olivera, 1996). Thus, sphingosine kinase (SPHK) could be one potential target for PLD and PA in ABA-mediated stomatal closures. Recent results indicate that activation of PLD plays a role in the production of
and response to ROS. In *Arabidopsis*, PLD and PA have been implicated in increasing NADPH oxidase activity and ROS production (Zhang et al., 2009). ABA is known to stimulate \( \text{H}_2\text{O}_2 \) production in guard cells. It has been shown that ablation of PLD\( \alpha_1 \) decreases \( \text{H}_2\text{O}_2 \) production in response to ABA, and that PLD\( \alpha_1 \)-derived PA directly interacts with NADPH oxidase. On the other hand, PLD\( \delta \) is activated by \( \text{H}_2\text{O}_2 \) in *Arabidopsis*, (Zhang et al., 2003). These results infer that PLD\( \alpha_1 \) promotes the ROS production and PLD\( \delta \) mediates plant responses to ROS.

The goal of this proposed research was to establish the interactions of PLDs and PA with other cell signaling compounds, to elucidate their mechanisms of action in the signaling pathways, and understand the physiological functions that impact plant growth and productivity. This research focused on two PLDs, PLD\( \alpha_1 \) and PLD\( \delta \), with the following supporting objectives:

1. Isolation of *SPHK* genes from *Arabidopsis* to express SPHK in *E.coli* and determination of their biochemical activity and interaction with PA. In *Arabidopsis*, annotated At4g21540 locus encodes two repeats of SPHK which have high similarity with mouse SPHK. To determine if At4g21540 locus encodes one or two SPHKs and the relationship of PLD\( \alpha_1 \)/PA and SPHK/phyto-S1P in ABA signaling pathway, two SPHK genes were amplified for following purposes: i) express SPHK proteins in *E.coli* to determine their SPHK activity and substrate usage; ii) determine SPHK is a molecular target of PA; iii) kinetics analyses of SPHK.

2. Isolation of *SPHK* T-DNA mutants and generation of *SPHK* OE lines to investigate their role in ABA-signaling pathway. Two homozygous T-DNA insertion mutants of *SPHK* were isolated and OE lines of both *SPHKs* were generated. ABA response assays including germination, root elongation and stomatal closure were done and it was found that manipulation of *SPHK* altered plant sensitivity to ABA.

3. Determination of the relationship of PLD\( \alpha_1 \)/PA and SPHK/phyto-S1P in the pathway of ABA-mediated stomatal closure *PLD\( \alpha_1 \)* and *SPHK* double knockout were
generated by crossing the single mutants and the PLD mutants were used to determine the relationship of the lipid messengers PLDα1/PA and SPHK/phyto-S1P in regulating ABA signaling. Physiological study, enzymatic analyses and lipidomics profiling were performed to determine that there is crosstalk between PLDα1 and SPHK in the ABA signaling pathway.

4. Determination of how PLDδ is involved in ROS response and identification of the mediator that leads to the activation of PLDδ by H2O2. PLDδ T-DNA mutant was used for detail analyses and PLDδ was found to mediate ROS signals to regulate stomatal aperture. In the search of PLDδ-interaction proteins, cytosolic glycerol-3-P-dehydrogenase (GAPC) was found to be co-pulled down with PLDδ by previous lab member, indicating that GAPC is a potential regulator of PLDδ. Additional interaction studies were carried out to confirm their interaction. Activity assays were done to determine that this interaction promoted PLDδ activity.

5. Isolation of GAPC mutants to elucidate the physiological functions of GAPC in Arabidopsis. The T-DNA seeds were obtained from ABRC (Ohio State University), and homozygous mutants were verified. Double knockouts of GAPC1/GAPC2 and triple knockout of GAPC1/GAPC2/PLDδ were generated. GAPC mutants were functionally characterized with PLDδ mutant to determine their roles in Arabidopsis. The mutants were tested for altered response to different stresses including ABA, drought, salinity, freezing and so on. The mutants displaying alteration compared to WT were further characterized under a specific condition. Furthermore, the GAPC mutants were analyzed for metabolism alterations to determine how GAPC affects the plant energy metabolism.
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**Table 1. Biochemical properties of *Arabidopsis* phospholipase Ds**

<table>
<thead>
<tr>
<th>PLD type</th>
<th>Activity requirements and substrate preference</th>
<th>Ca²⁺</th>
<th>PI(4,5)P₂</th>
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<th>Substrate preference</th>
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<tr>
<td>α1</td>
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<td>No</td>
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*PLDα₃ and PLDε are active under broad reaction conditions. Both are active under PLDα1 reaction condition which does not have PIP₂ and oleate (Hong et al., 2008; Hong et al., 2009). Other references are cited in the text. Table is modified from Li et al., 2009.*
Figure 1. Structure of phospholipid and the cleavage site of phospholipase D. Phospholipids consist of glycerol backbone, two fatty acyl chains and a head group. PLD hydrolyzes phospholipids at the phosphodiester bond to produce phosphatidic acid (PA) and a free head group. Figure is modified from Wang et al., 2006.
Figure 2. The domain structure of Arabidopsis PLDs. 12 PLDs in Arabidopsis which have been classified into 6 types: PLDα (3), PLDb (2), PLDγ (3), PLDδ, PLDe, and PLDζ (2). There are two groups of PLDs: C2-PLD and PX/PH-PLD. Animal PLDs are PX/PH PLD which has PX/PH domain but not C2 domain. PLDζ belongs to the PX/PH PLD. C2, Ca$^{2+}$ and phospholipid binding domain; PH, Pleckstrin homology domain; PX, phox homology domain; HKD, HxKxxxxD motif; DRY motif, involved in protein interaction. Figure is modified from Wang et al., 2006.
Figure 3. Generation of PA from phospholipids and PA target proteins functionally characterized in plants. PA is generated via two pathways during stress responses: PLD hydrolyzes phospholipid to generate PA, and PLC hydrolyzes phospholipid to generate DAG which can be phosphorylated by DAG kinase (DGK). PA has been found to interact with target proteins to regulate cellular functions. Examples of PA regulation of target proteins are discussed in the text and the references are cited in the text.* indicates that PA is generated from PLD while others are not determined. Figure is modified from Hong et al., 2010.
Figure 4. Phosphorylation of sphingosine and phytosphingosine by SPHK and the interaction SPHK and PA. SPHK catalyzes the formation of S1P or phyto-S1P from sphingosine or phytosphingosine. S1P or phyto-S1P can be degraded by S1P phosphatase (SPP) or S1P lyase (not shown). Figure is modified from Coursol et al., 2005.
Chapter 2. Phosphatidic Acid Binds and Stimulates Arabidopsis Sphingosine Kinases

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AUTHOR CONTRIBUTIONS
L.G. and X.W. designed the research; L.G. performed most experiments; K.T. cloned SPHK1 and performed the PA-SPHK1 interaction assays; G.M. confirmed the interaction between PA and SPHK1, did SPHK1 enzyme activity assay; L.G. and X.W analyzed the data and wrote the manuscript.

ABSTRACT
Phosphatidic acid (PA) and phytosphingosine-1-phosphate (phyto-S1P) have both been identified as lipid messengers mediating plant response to abscisic acid (ABA). To determine the relationship of these messengers, we investigated the direct interaction of PA with Arabidopsis sphingosine kinases (SPHKs) that phosphorylate phytosphingosine to generate phyto-S1P. Two unique SPHK cDNAs were cloned from the annotated At4g21540 locus of Arabidopsis and the two transcripts are differentially expressed in Arabidopsis tissues. Both SPHKs are catalytically active, phosphorylating various long-chain sphingoid bases (LCBs) and are associated with the tonoplast. They both interact with PA as demonstrated by lipid-filter binding, liposome binding, and surface plasmon resonance (SPR). SPHK1 and SPHK2 exhibited strong binding to 18:1/18:1, 16:0/18:1, and 16:0/18:2 PA, but poor binding to 16:0/16:0, 8:0/8:0, 18:0/18:0, and 18:2/18:2 PA. Surface dilution kinetics analyses indicates that PA stimulates SPHK activity by increasing the specificity constant through decreasing $K_m^B$. The results show that the annotated At4g21540 locus is actually comprised of two separate SPHK genes. PA binds to both SPHKs, and the interaction promotes lipid substrate binding to the catalytic site of the enzyme. The PA-SPHK interaction depends on the PA molecular species. The data suggests that these two Arabidopsis SPHKs are molecular targets of PA, and the PA stimulation of SPHK is part of the signaling networks in Arabidopsis.
INTRODUCTION

Phosphatidic acid (PA) has emerged as a class of pivotal lipid messengers in cell growth, development, and stress responses, and the regulatory functions of PA are being established in plants, animals, and fungi (1-3). PA is a minor membrane lipid, constituting less than 1% of total phospholipids in most plant tissues (4). However, the cellular level of PA in plants is dynamic, increasing rapidly under various conditions, including chilling, freezing, wounding, pathogen elicitation, dehydration, salt, nutrient starvation, nodule induction, and oxidative stress (1, 2, 5, 6). The functional significance of PA has been indicated by characterization of various phospholipase Ds (PLD) that produce regulatory PA and by measurements of PA changes under different stress conditions (1, 2). Characterization of genetic ablations, together with biochemical analyses, has shown that different PLDs have unique functions (1, 7). The differential activation, expression, and cellular locales, as well as substrate preferences of PLDs, indicate that the cellular location and timing of PA production are important determinants of PA’s function.

A series of recent results have provided mechanistic insights into how specific PLD and PA mediate the abscisic acid (ABA) promotion of stomatal closure in Arabidopsis (8, 9). Recently, PLDα1 and PA were found to regulate NADPH oxidase activity and the production of reactive oxygen species (ROS) in ABA-mediated stomatal closure (10). In addition to PA, another lipid messenger, long-chain base-1-phosphate (LCBP) including sphingosine-1-phosphate (S1P) and phyto-S1P has been found to promote the ABA effect on stomatal closure (11-13). Arabidopsis sphingosine kinase (SPHK) activity was mainly associated with the membrane fraction (13). Recent study suggests that sphingosine and S1P are not detectable in Arabidopsis leaves due to the lack of expression of sphingolipid Δ4-desaturase, indicating that sphingosine and S1P are unlikely to play a significant role in ABA-mediated stomatal closure (14). However, knockout of Arabidopsis SPHK1 rendered the stomatal closure less sensitive to ABA, whereas overexpression of SPHK1 increased stomatal closure and ABA sensitivity (15). These results suggest that other LCBPs are involved in ABA signaling in Arabidopsis (16). Phytosphingosine is one of such LCBs in Arabidopsis leaves and its phosphorylated form, phyto-S1P, is also
detectable in *Arabidopsis* leaves (17). Thus, ABA promotes the formation of PA and phyto-S1P, and both PLD/PA and SPHK/phyto-S1P positively regulate ABA-mediated stomatal closure (8, 13). However, the relationship between PA and phyto-S1P in plant signaling pathways is unknown.

One important mode of action by PA to regulate cell function is through its direct interaction with effector proteins (1). PA has been reported to bind to various proteins, including transcriptional factors, protein kinases, lipid kinases, protein phosphatases, and proteins involved in vesicular trafficking and cytoskeletal rearrangement (1). Several PA-interacting proteins have been identified in plants, including ABI1, PDK1, CTR1, TGD2, and NADPH oxidase (8, 10, 18, 19, 20). Additional PA-binding proteins were isolated by PA-affinity chromatography followed by mass spectrometric analyses (21). In animals, both SPHK and its product S1P are potentially important signaling molecules. Acidic phospholipids including PA have been suggested to stimulate SPHK activity (22). PLD activation up-regulated SPHK in mammalian cells (23). The PLD activator, PKC, was found to activate SPHK1 (24). PA has also been suggested to promote the intracellular translocation of cytosolic murine SPHK1 to membranes enriched in PA (25). These results suggest that SPHK is an effector protein of PA in animal cells.

To determine the relationship of the lipid messengers PA and phyto-S1P in regulating plant functions, we investigated the direct interaction of PA with *Arabidopsis* SPHK1 (At4g21540) that phosphorylates phytosphingosine to generate phyto-S1P. During the study, we found that the annotated At4g21540 locus of *Arabidopsis* actually encodes two SPHKs and both SPHKs are associated with the vacuolar membrane. PA binds to both *Arabidopsis* SPHKs and the interaction stimulates their activity by promoting the binding of lipid substrate to the catalytic site of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Cloning the SPHK1 and SHPK2 cDNAs**

The At4g21540 locus contains a tandem repeat, and the second repeat sequence was
previously cloned and named as \textit{SPHK1} (15). The coding region of \textit{SPHK1} was amplified from a stock DNA for \textit{At4g21540} obtained from ABRC (Stock# U16738) using specific primers \textit{AtSPHK1-F} 5'\textsc{-TAGGATCCATGGATCGTCAGCCGGAGAGGGA-3'} and \textit{AtSPHK1-R} 5'\textsc{-TACTCGAG TTATTCAGGAGAGAAGAGAGTGTCGCTC-3'} with engineered \textit{BamHI} and \textit{XhoI} (underlined) sites, respectively. The cDNA of the first repeat (\textit{SPHK2}) of \textit{At4g21540} was amplified from \textit{Arabidopsis} leaf cDNA using the primers: 5'\textsc{-ATGGAGAAATGATCAATTCTGTGTC-3'} (forward) and 5'\textsc{-AGCAAGATGGAGGAGACGAT-3'} (reverse). The cloned fragments were sequenced and a stop codon was found at the 3' end. Then the following primers were designed to clone the coding region of \textit{SPHK2}: \textit{AtSPHK2-F} 5'\textsc{-GCGGGAATCCATGGAGAAATGATCAATTCTGTGTC-3'} and \textit{AtSPHK2-R} 5'\textsc{-GCGCTCGAGTCAAATACGATGAGAGAAGAGTG-3'} with engineered \textit{BamHI} and \textit{XhoI} (underlined) sites, respectively. Phusion High-Fidelity DNA Polymerase (New England BioLabs) was used for PCR under the condition of 98°C 1min, 40 cycles of 98°C 10s, 60°C 20s, and 72°C 30s.

\textbf{Expression and Purification of SPHKs}

The cDNA of \textit{SPHK1} and \textit{SPHK2} were amplified using the primers described above and ligated to pET-28a-c(+) vector to produce SPHK1 and SPHK2 with 6 histidine residues at the N terminus. The recombinant plasmids were transformed into \textit{E. coli BL21(DE3)pLysS}. Expression of \textit{SPHKs} was induced by 0.4 mM isopropyl-1-thio-\textit{β}-D-galactopyranoside at room temperature for 8 h. Cells were harvested by centrifugation at 2,000g at 20°C for 10 min. SPHKs were purified using Ni-NTA agarose (Qiagen) according manufacturer’s instructions with modifications. The cells (harvested from 200 mL cell culture) were resupended in 10 mL lysis buffer and lysed by sonication in lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF). The lysate was centrifuged at 12,000g at 4°C for 20 min and the supernatant was incubated with Ni-NTA agarose for 2 h at 4°C with gentle rotation. The agarose beads were pelleted and washed 3 times with a wash buffer. Protein was eluted with an elution buffer and dialyzed with TBS buffer overnight. The dialyzed protein was 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 Blue staining. The prepared proteins for activity assay were kept in 50% glycerol at -80°C.

RNA Extraction and Real-time PCR

*Arabidopsis thaliana* (ecotype Col-0) plants were grown in a growth chamber with cool white light of 200 µmol m⁻²s⁻¹ under 12-h light/12-h dark and 23°C/19°C cycles. Total RNA was isolated from tissues of eight week-old *Arabidopsis* plants using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instruction. Total RNA was digested with RNase-free DNase I. The absence of genomic DNA contamination was confirmed by PCR using RNA as template without reverse transcription. The first-strand cDNA was synthesized from 1 µg of total RNA using an iScript cDNA synthesis kit in a total volume of 20 µL according to the manufacturer’s instructions (Bio-Rad). The efficiency of the cDNA synthesis was assessed by real-time PCR amplification of a control gene encoding UBQ10 (At4g05320). cDNAs were then diluted to yield similar threshold cycle (Ct) values (20) based on the Ct of the UBQ10. The level of individual gene expression was normalized to that of UBQ10 by subtracting the Ct value of UBQ10 from the tested genes. PCR was performed with a MyiQ system (Bio-Rad) using SYBR Green. Each reaction contained 7.5 µL 2×SYBR Green master mix reagent (Bio-Rad), 3.5 µL diluted cDNA, and 200 nM of each gene-specific primer in a final volume of 15 µL. The primers were as follows: UBQ10, `5'-CACACTCCACTTGGTCTTGCGT-3` (forward) and `5'-TGGTCTTTCCCGGTGAGAGTCTTCA-3` (reverse); SPHK1, AGACCTTGTTGAGAAGGAGGAG-3 (forward) and `5'-GGGTCTTTCCCGGTGAGAGTCTTCA-3` (reverse); SHK2, AGACCTTGTTGAGAAGGAGGAG-3 (forward) and `5'-GGGTCTTTCCCGGTGAGAGTCTTCA-3` (reverse). The real-time PCR condition was: 95°C for 3 min; and 50 cycles of 95°C for 30s, 57°C for 30 s, and 72°C for 30 s.

Subcellular Localization of SPHKs

*SPHK1* and *SPHK2* cDNA were cloned into p35S-FAST/eYFP, which was derived from p35S-FAST by introducing eYFP. Agro-infiltration for transient protein expression in
tobacco leaves was performed as described by Voinnet et al. (26). 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 MgCl₂, and 150 mg ml⁻¹ acetosyringone. 3-week-old Nicotiana benthamiana leaves were infiltrated with the bacteria solutions through abaxial air spaces. p35S-FAST/eYFP and p35S-FAST/PLDδ:eYFP were transformed as control. The eYFP fluorescence was examined in tobacco leaves using a Zeiss LSM 510 confocal/multi-photon microscope, with a 488 nm excitation mirror and a 505-530 nm and 530-560 nm emission filter to record images.

The above SPHK:eYFP constructs were transformed into Arabidopsis to obtain transgenic plants. To isolated subcellular fractions, total proteins from leaves of SPHK1 or 2 transgenic Arabidopsis plants were extracted with a chilled buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM PMSF, and 2 mM DTT. Total protein was centrifuged at 10,000g for 20 min at 4°C to remove tissue debris, and the supernatant was centrifuged at 100,000g for 45 min at 4°C. The resulting supernatant and pellet are referred to as the soluble and microsomal fractions. The plasma and intracellular membranes were prepared using an aqueous polymer two-phase system according to the method described by Fan et al. (27). To isolate tonoplasts, protoplasts were prepared from fully expanded leaves of 4- to 6-week-old Arabidopsis (28). Vacuoles were then purified from protoplasts following the protocol adapted from Jaquinod et al. (29). Marker enzymes for the plasma membrane, intracellular membrane and tonoplast are ATPase, cytochrome c reductase and α-mannosidase, respectively (27, 29). The concentration of proteins from different fractions was determined using the Bradford protein assay. Proteins from the different fractions were subjected to 10% SDS-PAGE followed by immunoblotting. SPHK1 was immunoblotted with anti-FLAG antibody and SPHK2 was detected with anti-GFP antibody.

Assaying Sphingosine Kinase Activity

Sphingosine, phytosphingosine, dihydrosphingosine (D-erythro-DHS), DL-threo-dihydrosphingosine (DL-threo-DHS), N,N-dimethylsphingosine (DMS) were purchased from Enzo Life Sciences. 4-Hydroxy-8-sphingenine (t18:1) and 4, 8-sphingadienine
(d18:2) were generous gifts from Dr Daniel Lynch (Williams College). SPHK activity was measured as previously described with some modifications (30). Briefly, purified SPHK was incubated at 37°C in 200 μL sphingosine kinase buffer in the presence of 50 μM sphingolipid added in micellar form with 0.25% (v/v) Triton X-100, and [γ-32P]ATP (10 μCi, 1 mM) in 10 mM MgCl2. Reactions were stopped by the addition of 800 μL chloroform:methanol:concentrated HCl (100:200:1; v/v/v). Chloroform (250 μL) and 2 M KCl (250 μL) were then added sequentially to generate a two-phase system. The labeled lipids in the organic phases were separated by TLC with chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1; v/v/v/v) and visualized with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). For quantification, LCBP was scraped and extracted from the TLC plate and quantified by scintillation counter. SPHK activity was expressed as nanomoles of LCBP formed per min and per milligram of protein. Michaelis-Menten plots and enzyme kinetic parameters were analyzed using SigmaPlot Enzyme Kinetics Module.

**Lipid-SPHK Binding by Blotting**

The filter binding was performed as described with some modifications (31). Lipids (10 μg) were spotted on a nitrocellulose filter, followed by incubation with purified His-tagged SPHK in TBST (0.1% Tween 20) overnight at 4°C. The filter was washed 3 times with TBST (0.1 % Tween 20). The filter was then incubated with anti-His antibody, followed by incubation with a second antibody conjugated with alkaline phosphatase. SPHK protein that bound to lipids on filters was visualized by staining alkaline phosphatase activity.

**Liposome Binding Assay**

Liposome binding assay was performed as described (10). PC and PA were mixed in the molar ratio of 2:1 in chloroform with the final concentration of lipids per sample being at 640 nmole. The lipids were dried under nitrogen and rehydrated for 1 hr using extrusion buffer containing 250 mM raffinose, 25mM Tris pH 7.5, and 1 mM DTT. Liposomes were produced using lipid extruder (0.2 μm filters, Avanti Polar Lipids) following the manufacturer’s protocol. Liposomes were diluted in three volumes of a binding buffer
containing 125 mM KCl, 25 mM Tris pH 7.5, 1 mM DTT and 0.5 mM EDTA and centrifuged at 50,000g for 15 min. The liposome pellet was resuspended in 1 ml of binding buffer, and 1.2 µg purified SPHKs was added and incubated for 1h at room temperature. The His-tagged SPHK protein used in the assay was preclarified by centrifugation at 16,000g for 30 min to remove any insoluble protein. Liposomes were harvested by centrifuging at 16,000g for 30 min and washed three times in the binding buffer. Liposomes were resuspended in SDS-PAGE sample buffer and were loaded on a SDS-PAGE gel. The proteins were subjected to SDS-PAGE and then transferred on a PVDF membrane, followed by immunoblotting using anti-His antibodies.

Surface Plasmon Resonance Analyses

SPR binding assays were performed using a Biacore 2000 system according to the manufacturer’s instructions with some modifications. Liposomes were prepared by mixing PC and PA at a 2:1 molar ratio as described above. Liposomes were resuspended in a running buffer (0.01 M HEPES, 0.15 M NaCl, 50 µM EDTA, pH 7.4). The purified His-tagged SPHK1 was dialyzed in the running buffer overnight at 4°C, and then the protein was centrifuged at 13,000g to remove insoluble protein. The protein concentration was measured using the Bradford assay. Biacore Sensor Chip NTA designed to bind His-tagged proteins for interaction analyses was used to immobilize protein. For each experiment, the running buffer containing 500 µM NiCl₂ was injected to saturate the NTA chip with nickel. His-tagged SPHK1 protein (2 µM) was immobilized on the sensor chip via Ni²⁺/NTA chelation. Lipid-SPHK interaction was monitored as di16:0 PA/di18:1 PC or di18:1 PA/di18:1 PC liposomes (100 µM) were injected in sequence over the surface of the sensor chip. The liposome made with di18:1 PC only was used as control. Sensor chip was regenerated by stripping nickel from the surface with a regeneration buffer (0.01 M HEPES, 0.15 M NaCl, 0.35 M EDTA, pH 8.3). During the evaluation, the sensorgrams from the beginning of association to the end of dissociation for each protein-liposome interaction were analyzed and plotted by SigmaPlot 10.0. Kinetic constants including Bₘₐₓ, association (kₚₚ), and dissociation rate (kₚₜ) were analyzed using the BIAevaluation Software.

Preparation of Triton X-100/Phytosphingosine Mixed Micelles
Triton X-100/lipid micelles were prepared according to the method described by Qin et al. (32). Phytosphingosine dissolved in ethanol was dried under a stream of nitrogen, and de-ionized water was added to give a final concentration of 1 mM. The phytosphingosine suspension was sonicated on ice until clear. To obtain specific substrate concentrations at the desired mole fraction (MF), the phytosphingosine solution was diluted with Triton X-100 stock solution (40 mM) using the following formula: mole fraction$_{\text{phytosphingosine}}$ = [phytosphingosine]/([phytosphingosine]+[Triton X-100 (free)]); [Triton X-100 (free)] = [Triton X-100 (total)]-critical micelle concentration of Triton X-100 (0.24 mM). When the effect of PA mole concentration was to be tested, PA was added at this point, using the following formula: mole fraction$_{\text{PA}}$ = [PA]/([phytosphingosine] + [PA] + [Triton X-100 (free)]). The Triton X-100/phytosphingosine mixture was vortexed briefly and let stand at room temperature for half an h.

RESULTS

**At4g21540 locus encodes two SPHK genes**

Four genes showing homology to human and mouse SPHK genes have been annotated in the *Arabidopsis* genome. At5g23450 encodes a long-chain base kinase, designated as AtLCBK1 (33) whereas At5g51290 was reported to be a ceramide kinase (34). At2g46090 did not have sphingosine phosphorylating activity (15). At4g21540 potentially encodes two SPHKs in tandem but was annotated as one SPHK in database (Fig. 1A). A cDNA from the second repeat was previously reported to encode an active SPHK while the cloning of the first repeat remained unsuccessful (15).

Utilizing primers corresponding to the first and second repeats, corresponding cDNAs were cloned and further verified by DNA sequencing (Fig. 1B). Sequencing of the first repeat revealed a stop codon at the 3’ end that is 788 bp upstream of the start codon of the second repeat SPHK1. Thus, the annotated At4g21540 is actually comprised of two separate SPHK genes (Fig. 1A). Since the second repeat was already named SPHK1(15), we thus designated the first repeat SPHK2. Both genes have 10 exons and 9 introns, and the size of exons from 2 to 9 is the same for two genes. *Arabidopsis*
SHPK1 and SHPK2 share 72.6% identity of amino acid sequences. Like mouse SHPK1, both SHPK1 and SHPK2 have 5 conserved C domains in the deduced amino acid sequence (Fig. 1C).

**SHPK1 and SHPK2 display a distinguishable pattern of expression and are associated with the tonoplast**

The expression of **SHPK1** and **SHPK2** in different *Arabidopsis* tissues was examined by real-time PCR (Fig. 2A). Both **SHPK1** and **SHPK2** were detectable in all tissues examined and they had similar levels of expression in flowers, siliques, young leaves and roots. However, **SHPK1** had a much higher expression level in inflorescence, older leaves, and stems than **SHPK2** (Fig. 2A). These distinguishable patterns of expression further support the finding that **SHPK1** and **SHPK2** are encoded by two separate genes.

To determine the intracellular location of these enzymes, SHPK1 and SHPK2 were fused with yellow fluorescence protein (eYFP) at the C-terminus and transiently expressed in tobacco leaves while eYFP and PLDδ:eYFP were used as control. eYFP alone was detected in the nucleus and cytoplasm as expected, as the eYFP fluorescence surrounded the chloroplast in the cytoplasm (Fig. 2B, panel a). PLDδ was previously documented to be associated with the plasma membrane (35), and the distribution of PLDδ:eYFP associated with the plasma membrane was consistent with the previous results (Fig. 2B, panel b). The subcellular distribution of SHPK1:eYFP and SHPK2:eYFP both were different from that of eYFP or the plasma membrane-associated PLDδ:eYFP. Using chloroplast (red color) as a reference, SHPK1:eYFP and SHPK2:eYFP fluorescence was separated from the plasma membrane by chloroplasts (Fig. 2B, panel c and d), indicating that they are not associated with the plasma membrane. *Arabidopsis* SHPK1 was previously reported to be localized on tonoplast (36). SHPK1:eYFP and SHPK2:eYFP exhibited the same pattern of localization, suggesting that both are localized on the tonoplast.

To further verify the subcellular association of SHPK1 and SHPK2, we generated transgenic *Arabidopsis* expressing SHPK1:eYFP and SHPK2:eYFP and isolated subcellular fractionations from leaves of the transformed plants. Isolation of the
membranes was confirmed by assaying the marker enzymes. Vanadate-sensitive ATPase showed the highest activity in the plasma membrane fraction but little activity for other fractions. NADH-Cyt c reductase had highest activity in the intracellular membranes and α-mannosidase activity was mainly associated with tonoplast-enriched fraction, indicating successfully isolation of different membrane fractions with low contamination (Table 1). Proteins from different fractions were separated by SDS-PAGE followed by immunoblotting. SPHK1 and SPHK2 were present primarily in the microsomal fraction and only trace amounts of SPHKs were detected in the cytosolic fraction (Fig. 2C). When the microsomal fraction was separated into the plasma and intracellular membranes, SPHKs were associated with the intracellular membranes and not with the plasma membrane (Fig. 2C). In addition, we isolated vacuoles from leaf protoplasts. Both SPHKs were present in the tonoplast fraction (Fig. 2C). These results consistently indicate that both SPHKs were associated with the tonoplast (Fig. 2C).

**SPHK1 and SPHK2 are both catalytically active**

We expressed both SPHK1 and SPHK2 protein in *E. coli* to determine whether they were active enzymes. Proteins at about 53 kD were produced from the cDNA of *SPHK1* and *SPHK2* and the size was as predicted based on the cDNA-coding regions (Fig. 3A, B). Both SPHK1 and SPHK2 phosphorylated phytosphingosine to produce phyto-S1P. The increase in phyto-S1P production was proportional to the reaction time within 15 min (Fig. 3C). It was reported previously that SPHK1 expressed in human embryonic kidney 293 (HEK 293) cells used various LCBs as substrates (15). In our study, both purified SPHK1 and SPHK2 were able to utilize various LCBs including sphingosine, phytosphingosine, t18:1, d18:2 and D-erythro-DHS as substrates (Fig. 3D). However, SPHK1 and SPHK2 displayed different activities towards these substrates. SPHK1 had higher activity on sphingosine, phytosphingosine and t18:1 while SPHK2 was more active on d18:2 (Fig. 3D). In addition, SPHK2 exhibited much less activity towards DL-threo-DHS than did SPHK1 (Fig. 3D). DMS, a potent inhibitor for mammalian SPHKs, was not phosphorylated by either of SPHKs under our experiment condition (Fig. 3D).

**PA binds to SPHK1 and SPHK2**
To determine the potential interaction of PA with SPHK1 and SPHK2, we performed a filter-binding assay utilizing nitrocellulose filter spotted with different lipids. Both SPHK1 and SPHK2 exhibited binding to egg yolk PA but not other phospholipids, including PC, PE, PG, PI, PS, LPC and LPE (Fig. 4A). Different PA molecular species showed different binding patterns as 8:0/8:0, 18:0/18:0 or 18:2/18:2 PA did not bind to either SPHK, whereas 16:0/16:0, 18:1/18:1, 16:0/18:1 and 16:0/18:2 PA exhibited binding (Fig. 4A). SPHK1 was further used to examine the PA-SPHK interaction by a liposome binding assay. The liposomes were made with 18:1/18:1 PC only as control or with a mixture of 18:1/18:1 PC and different PA species in a molar ratio of 2:1. No SPHK1 was pelleted with PC-only liposomes suggesting the binding to be specific to PA containing liposomes. Only trace quantities of SPHK1 were pelleted with liposomes containing 18:0/18:0 or 18:2/18:2 PA, whereas substantially more SPHK1 was associated with liposomes containing 16:0/18:1, 16:0/18:2 16:0/16:0, or 18:1/18:1 PA (Fig. 4B). The result of liposome binding was consistent with that of lipid-filter binding assay.

PA-SPHK interaction was further validated with SPR which is a highly sensitive method for quantitative detection of molecular interaction. Purified SPHK1 was first immobilized on a NTA chip followed by injection of liposomes made of PC only or PA plus PC. In the representative sensorgram, response unit (RU) increased when the liposome was composed of PA (16:0/16:0 or 18:1/18:1) plus PC. By comparison, there was almost no increase of RU when PC only liposome was injected, indicating that PA interacts with SPHK1 specifically (Fig. 4C). Compared to 16:0/16:0 PA binding to SPHK1, 18:1/18:1 PA displayed a higher association rate constant ($K_a=590.50 \text{ M}^{-1}\text{s}^{-1}$ vs. $16.93\text{ M}^{-1}\text{s}^{-1}$) and a lower dissociation rate constant ($K_d=2.88\times10^{-4} \text{ s}^{-1}$ vs. $3.20\times10^{-3} \text{ s}^{-1}$). The maximum specific binding is estimated to be 8904 RU for 16:0/16:0 PA and 4426 RU for 18:1/18:1 PA. The equilibrium binding constant $K_D$ is calculated to be $1.89\times10^{-4}$ M for 16:0/16:0 PA-SPHK1 interaction and $4.88\times10^{-7}$ M for 18:1/18:1 PA-SPHK1 interaction, indicating a low affinity between 16:0/16:0 PA and SPHK1 but a high affinity between 18:1/18:1 PA and SPHK1.

PA Stimulates SPHK Activity

To determine the effect of PA binding on SPHK, we tested the activity of SPHK1 under a
range of PA concentrations. Including 10 nM PA in the assay augmented SPHK1 activity by 1.5 fold (Fig. 5A). The stimulation of enzyme activity continued in a dose-dependent manner, but reached a plateau up at 50 µM PA at which a 2.5-fold increase in kinase activity was observed (Fig. 5A). When different PA species were tested for their effect on SPHK1 activity at 50 µM, egg yolk PA, 18:1/18:1 PA, 16:0/18:1 PA, and 16:0/18:2 PA significantly increased SPHK1 activity more than 2 fold whereas 16:0/16:0 PA, 18:0/18:0 PA, 18:2/18:2 PA, or egg yolk PC had no significant effect on SPHK1 activity (Fig. 5B). The pattern of stimulation of SPHK1 activity by different PA species was in agreement with that of PA binding, suggesting that PA-SPHK interaction stimulates SPHK activity.

**PA Stimulates SPHK Activity by Promoting Substrate Binding**

In order to determine the kinetic behavior of SPHK and the mechanism of stimulation of SPHK activity by PA, we used a surface dilution kinetic system because SPHK catalyzes the reaction at a water-lipid interface. The surface dilution model takes into account both two-dimensional surface interaction and three-dimensional bulk interaction between an enzyme and lipid substrate (37). The principle of surface dilution kinetics is presented in Equation 1 and the rate expression for surface dilution kinetic model is given in Equation 2 (Fig. 6A). Triton X-100 is one of the most commonly used detergents for surface dilution kinetics as it forms uniformly mixed micelles with different lipids including sphingolipids (38). SPHK1 activity was measured using increasing Triton X-100 concentrations in the mixed micelles along with purified SPHK1. The result showed that Triton X-100 served as a typical neutral dilutor at a concentration range of 0.8 mM to 10 mM for SPHK1 (Fig. 6B).

When phytosphingosine concentration was kept at 50 µM, the maximum activity was achieved at 0.8 mM Triton X-100 (Fig. 6B). To determine the surface dilution kinetic parameters, SPHK1 activity was determined as a function of the sum of the molar concentration of Triton X-100 and phytosphingosine at a series of set molar fractions (MF) (Fig. 7A). As the surface concentration of phytosphingosine decreased, the apparent $V_{\text{max}}$ decreased (Fig. 7A). Double-reciprocal plot of the results in Fig. 7A indicated that SPHK1 exhibited saturation kinetics when the bulk concentration of Triton X-100 and phytosphingosine was varied at each fixed MF of phytosphingosine (Fig. 7B). According
to Equation 2, the intercept of the 1/V intercept axis is equal to 1/V\textsubscript{max} and the intercept of the 1/B axis is equal to -1/K\textsubscript{m}\textsuperscript{B}. 1/V intercepts obtained in Fig. 7B versus the reciprocal of the MF of phytosphingosine was replotted to determine the V\textsubscript{max} and K\textsubscript{m}\textsuperscript{B} of SPHK1 (Fig. 7C). The V\textsubscript{max} and K\textsubscript{m}\textsuperscript{B} were determined to be 12.94 nmol/min/mg and 5.49×10\textsuperscript{-3} MF, respectively. The slope versus 1/B from Fig. 7B was replotted to determine the dissociation constant K\textsubscript{s}\textsuperscript{A}. K\textsubscript{s}\textsuperscript{A} was calculated to be 18.68 nM by using the slope of the line in Fig. 7D and the V\textsubscript{max} and K\textsubscript{m}\textsuperscript{B} determined in Fig. 7C.

To understand the mechanism by which PA stimulates SPHK activity, we compared the two constants, K\textsubscript{s}\textsuperscript{A} and K\textsubscript{m}\textsuperscript{B}, in the absence or presence of PA. The effect of PA on K\textsubscript{s}\textsuperscript{A} of SPHK1 activity was measured as a function of the sum of the molar concentration of Triton X-100 and phytosphingosine at three set MF of PA (0, 0.002 and 0.02) with the phytosphingosine MF fixed at 0.01 (Fig. 8A). Increasing mole fractions of PA increased the apparent V\textsubscript{max} but the apparent K\textsubscript{s}\textsuperscript{A} was not significantly changed (Fig. 8A). The result indicates that PA does not promote the bulk binding of SPHK1 to the mixed micelles. The effect of PA on K\textsubscript{m}\textsuperscript{B} for SPHK1 activity was measured as a function of the MF of phytosphingosine at the three set PA mole fractions (Fig. 8B). The apparent V\textsubscript{max} increased with the increase of PA mole fractions and the apparent K\textsubscript{m}\textsuperscript{B} decreased by more than 50% in the presence of PA (Fig. 8B). The specificity constant (apparent V\textsubscript{max}/ K\textsubscript{m}\textsuperscript{B}) was increased by 2.44 fold in the presence of 0.005 mole fraction of PA (Fig. 8B).

Overall, the surface-dilution kinetics analyses indicates that PA stimulates SPHK1 activity by promoting the binding of substrate to the catalytic site of the enzyme, but PA does not affect the binding of SPHK1 to the mixed micelle surface.

**DISCUSSION**

Results of this study indicate that the annotated At4g21540 locus is actually comprised of two separate SPHK genes which are both transcribed in *Arabidopsis thaliana*. The conclusion is supported by molecular cloning, sequence analyses, and the distinguishable patterns of expression of SPHK1 and SPHK2 in *Arabidopsis* tissues. The stop codon of SPHK2 is 788 bp upstream of the start codon of SPHK1, and the 788 bp region may
serve as the promoter of the second SPHK1. Subcellular localization indicates that both SPHK1 and SPHK2 were localized on tonoplasts, which is consistent with the finding that SPHK activity is mainly associated with membranes (15). *Arabidopsis SPHK1* and *SPHK2* were expressed in *E. coli* and both purified SPHK1 and SPHK2 were active in producing phyto-S1P. The substrate specificity of SPHK1 from the E. coli-expressed enzyme is the same as the SPHK1 expressed in human embryonic kidney 293 cells, phosphorylating sphingosine, phytosphingosine, and other plant LCBs (15). The catalytic activity of SPHK1 is slightly higher than that of SPHK2 towards several LCBs. In addition, the level of expression of SPHK1 is higher than that of SPHK2 in most tissues examined except in the silique. These results indicate that SPHK1 is more prevalent than SPHK2 in producing LCBP in vegetative tissues.

In plants, PA and phyto-S1P play important roles in transducing the ABA effect in stomatal closure. PA acts as an important regulator of various proteins by interacting with effector proteins. However, the mechanism by which PA regulates target protein function is not well understood. The present study shows that PA binds to SPHK1 and SPHK2. The binding has been demonstrated by different approaches, including lipid filter assay, liposomal binding, and SPR. PA-protein interaction can affect the protein function by changing the protein membrane association and/or directly modulating the activity of its effector enzymes. This modulation can be either activation or inhibition, depending upon the target proteins (1). The localization of signaling kinases is regarded as key to their signaling functions (39). Mouse SPHK activity was found to be simulated by acidic phospholipids including PA (22), and PA stimulated mouse SPHK1 activity by promoting the association of mouse SPHK1 to membranes which were rich in PA (25). However, unlike mammalian SPHKs, these two *Arabidopsis* SPHKs are associated with the tonoplast. In addition, the basal level of PA in *Arabidopsis* cells is estimated to be 50 to 100 µM, which is considerably above PA's critical micelle concentration (CMC), which is in the sub-nanomolar range (1). Above the CMC, the concentration of PA monomer is constant, independent of the total concentration of the lipid. Thus, the accumulation of PA above the critical level during cell activation affects the concentration of membrane-associated PA, but not monomeric PA. This suggests that PA binding to target proteins occurs at the membrane, but not in solution. Our kinetic analyses data indicates that PA
increases *Arabidopsis* SPHK1 activity by promoting the binding of lipid substrates to the catalytic site of the enzyme without altering the bulk binding of the enzyme to the micelle surface. The result is consistent with the observation that SPHKs are already associated with the tonoplast which is rich in phospholipids and sphingolipids (40). PA stimulates SPHK activity at a 10 nM to 200 µM range, a level of PA achievable in plant cells.

In addition, the present results show that the PA binding and stimulation of SPHK depends on the PA molecular species. PA is composed of different molecular species due to variation in two fatty acyl chains. A recent study indicated that 18:1/18:1, 18:2/18:2, 16:0/18:1, 16:0/18:2 and 18:0/18:2 PAs bound to the NADPH oxidase RbohD, but 16:0/16:0 and 18:0/18:0 PAs did not (10). In the PA-SPHK interaction, the present results showed that 18:2/18:2 did not bound to SPHK, whereas 16:0/16:0 PA displayed binding although the binding is much weaker than that of 18:1/18:1 PA to SPHK. The SPHK binding to different PA species was also qualitatively different from ABI1 that displayed much stronger binding to 18:1/18:1 PA than 16:0/16:0, 18:0/18:0, or 18:2/18:2 PAs tested (8). SPHKs, ABI1, and RbohD all are involved in mediating ABA response and stomatal movement. The differential interaction with different PA species could mean that the PAs that interact with the different target proteins may result from different sources. The molecular interaction with different PAs may underlie a mechanism for the diverse function of PAs in mediating cellular response. Although more than 20 proteins have been found to interact with PA, the protein structure required for the PA-protein interaction is unknown. It has been proposed that lysine and arginine residues increase the charge of PA and induce an electrostatic/hydrogen bond switch to stabilize the protein-lipid interaction (41, 42). The requirements of different PA acyl species by different proteins suggest that not only the head group but also the acyl groups are involved in PA-protein interaction. To fully understand the function of PA in cell regulation, it is necessary to elucidate the structural requirements for such PA-protein interaction and how that interaction modulates the function in the ensuing lipid-protein complex.
REFERENCES

FIGURE LEGENDS

FIGURE 1. Cloning of two sphingosine kinase genes from *Arabidopsis*

A, diagram showing genomic structure of At4g21540 locus containing SPHK1 and SPHK2. Black region is SPHK2 and red region is SPHK1.

B, gene structure of SPHK1 and SPHK2, gray boxes are exons and white boxes indicate introns. The nucleotide length is shown within the box.

C, comparison of amino acid sequences of *Arabidopsis* SPHK1 and SPHK2 with mouse SPHK1. Shaded dark represents identical residues, and light blue represents conserved residues. The conserved domains (C1-C5) are underlined.

FIGURE 2. Gene expression and subcellular localization of SPHKs

A, expression of SPHK1 and SPHK2 in *Arabidopsis* tissues as determined by real-time PCR normalized to *UBQ10*. RNA was extracted from different tissues of eight week-old plants. Values are means ± SE (n=3).

B, subcellular localization of SPHK1 and SPHK2. Using eYFP and PLDδ:eYFP as control. Green color represents eYFP fluorescence and red color marks chloroplasts as a reference. The constructs were transiently transformed into tobacco leaves by infiltration.

C, immunoblotting of SPHK1 and SPHK2 in subcellular fractions of *Arabidopsis* leaves. 25 µg of protein per lane was loaded for total and soluble proteins, and 8 µg for membrane fractions. WT, wild-type total protein from leave; Total, total protein from transgenic *Arabidopsis* leaves; S, soluble fraction; M, microsomal fraction; PM, plasma membrane; IM, intracellular membrane; T, tonoplast. SPHK1 was immunoblotted with anti-FLAG antibody and SPHK2 was immunoblotted with anti-GFP antibody.

FIGURE 3. Expression and activity assays of SPHKs

A, immunoblotting of SPHK1 and SPHK2 expressed in *E. coli*. Total protein (10 µg) was loaded on a SDS-PAGE gel. SPHK1 and SPHK2 were immunoblotted with anti-
polyHistidine antibody conjugated with alkaline phosphatase.

**B**, Coomassie blue staining of purified SPHK1 and SPHK2 from *E. coli* separated on a 10% SDS-PAGE gel.

**C**, SPHK1 and SPHK2 activity as a function of reaction time. Purified SPHK1 or SPHK2 (3.2 µg) was incubated with 50 µM phytosphingosine for the indicated time. Values are means ± SE (n=3).

**D**, phosphorylation of different LCBs (50 µM) by purified SPHK1 and SPHK2. 0.25 µM enzyme was incubated with substrate for 15 min. Values are means ± SE (n=3).

**FIGURE 4. PA binding to SPHKs**

**A**, lipid binding specificity of SPHK1 and SPHK2 on filters. Different lipids and PA species (10 µg) were spotted onto a nitrocellulose membrane and incubated with equal amounts of purified SPHK1, SPHK2, or total protein from *E. coli* transformed with empty vector. EY, egg yolk PA.

**B**, SPHK1 binding to liposomes containing PC only or PC plus different PA species. Purified SPHK1 (20 µg) was incubated with different liposomes for 1 h at room temperature. The vesicles were pelleted by centrifugation. The protein was visualized by immunoblotting with anti-His antibody.

**C**, SPR quantitative analyses of PA binding to SPHK1. Liposomes containing PC only or PC plus 16:0/16:0 PA or 18:1/18:1 PA were used. SPHK1 was first immobilized on the NTA chip followed by injection of liposomes.

**FIGURE 5. PA stimulates SPHK activity**

**A**, the effect of varied PA (18:1/18:1) concentrations on SPHK1 activity. Different concentrations of PA from 10 nM to 500 µM were tested for the effect on SPHK1 activity with 0.25% Triton X-100.

**B**, the effect of different PA species on SPHK1 activity was tested with 0.25% Triton X-100. EY PC that did not bind to SPHK1 was used as a control.
FIGURE 6. Surface dilution kinetic model and effect of Triton X-100 on SPHK1 activity
A, Equation 1 depicts the surface dilution model, and Equation 2 is the rate expression for surface dilution kinetic model.

B, SPHK1 activity measured with increasing molar concentrations of Triton X-100 in the mixed micelles. The molar concentration of phytosphingosine was held at 50 µM. Values are means ± SE (n=3).

FIGURE 7. Activity of SPHK1 toward phytosphingosine in mixed micelles with Triton X-100
A, SPHK1 activity measured as a function of the sum of the molar concentrations of Triton X-100 (TX) plus phytosphingosine at a series of set mole fractions of phytosphingosine. Data represents the average of three replicates.

B, reciprocal plot of the data in A.

C, replot of 1/V intercepts obtained in B versus the reciprocal of the mole fraction of phytosphingosine.

D, replot of slopes obtained in B versus the reciprocal of the mole fraction of phytosphingosine.

FIGURE 8. Effect of PA on the kinetic behavior of SPHK1
A, SPHK1 activity measured as a function of phytosphingosine molar concentrations at set mole fractions of PA. The molar fraction of phytosphingosine was 0.01. Data represents the average of three replicates.

B, SPHK1 activity measured as a function of phytosphingosine mole fractions at set mole fractions of PA. The molar concentration of phytosphingosine was 50 µM. Data represents the average of three replicates.
TABLE 1 Marker enzyme activity in membrane fractions of *Arabidopsis* leaves

<table>
<thead>
<tr>
<th>SPHK1 transgenic Arabidopsis</th>
<th>SPHK2 transgenic Arabidopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker Enzyme</td>
<td>M</td>
</tr>
<tr>
<td>ATPase¹</td>
<td>13.84</td>
</tr>
<tr>
<td>Cyt c reductase²</td>
<td>95.24</td>
</tr>
<tr>
<td>α-mannosidase³</td>
<td>23.21</td>
</tr>
</tbody>
</table>

¹nmol phosphate min⁻¹ mg protein⁻¹.
²μmol Cyt c min⁻¹ mg protein⁻¹.
³nmol p-nitrophenol min⁻¹ mg protein⁻¹.

Data represents the mean of three measurements.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
A

Bulk step

\[ E + A \xleftrightarrow[k_{-1}]{k_1} EA \]

Surface step

\[ EA + B \xleftrightarrow[k_{-2}]{k_2} EAB \xleftrightarrow[k_{-3}]{k_3} EA + Q \]  (Eq. 1)

\[ V = \frac{V_{max}[A][B]}{K_s A K_m B + K_m B[A] + [A][B]} \]  (Eq. 2)

B

Figure 6
Figure 7
Figure 8
Chapter 3. Inter-relationship between Sphingosine Kinase and Phospholipase D in Signaling Arabidopsis Response to Abscisic Acid

This chapter is accepted by Journal of Biological Chemistry

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AUTHOR CONTRIBUTIONS
L.G. and X.W. designed the research; L.G. performed most experiments; G.M. cloned \( SPHK1 \), isolated homozygous Salk_000250 T-DNA line and did stomatal assay; J.M. analyzed the LCBP content; M.L., A.T and R.W. analyzed the PA content. L.G. and X.W analyzed the data and wrote the manuscript.

ABSTRACT
Phosphatidic acid (PA) and phytosphingosine-1-phosphate (phyto-S1P) both are lipid messengers involved in plant response to abscisic acid (ABA). Our previous data indicate that PA binds to sphingosine kinase (SPHK) and increases its phyto-S1P-producing activity. To understand the cellular and physiological functions of the PA-SPHK interaction, we isolated \( Arabidopsis \) \( thaliana \) SPHK mutants \( sphk1-1 \) and \( sphk2-1 \) and characterized them, together with \( phospholipase \) \( D \alpha 1 \) knockout, \( plda1 \), in plant response to ABA. Compared to wild-type (WT) plants, the \( SPHK \) mutants and \( plda1 \) all displayed decreased sensitivity to ABA-promoted stomatal closure. Phyto-S1P promoted stomatal closure in \( sphk1-1 \) and \( sphk2-1 \), but not in \( plda1 \), whereas PA promoted stomatal closure in \( sphk1-1 \), \( sphk2-1 \), and \( plda1 \). The ABA activation of \( PLD\alpha 1 \) in leaves and protoplasts was attenuated in the \( SPHK \) mutants, and the ABA activation of SPHK was reduced in \( plda1 \). In response to ABA, the accumulation of long-chain base phosphate (LCBP) was decreased in \( plda1 \) whereas PA production was decreased in \( SPHK \) mutants, compared WT. Collectively, these results indicate that SPHK and \( PLD\alpha 1 \) act together in ABA response and that SPHK and phyto-S1P act upstream of \( PLD\alpha 1 \) and PA in mediating the ABA response. PA is involved in the activation of SPHK, and activation of \( PLD\alpha 1 \) requires SPHK activity. The data suggest that SPHK/phyto-S1P and \( PLD\alpha 1/PA \) are co-dependent in amplification of response to ABA, mediating stomatal closure in Arabidopsis.

INTRODUCTION
Phosphatidic acids (PA) produced by phospholipase Ds (PLDs) have been identified as important lipid signaling molecules in cell growth, development, and stress responses in both plants and animals (1, 2). In *Arabidopsis*, the level of PAs increases rapidly under various conditions, including chilling, freezing, wounding, pathogen elicitation, dehydration, salt, nutrient starvation, nodule induction, and oxidative stress (1, 2, 3, 4). PLD and PAs are involved in the response of guard cells to abscisic acid (ABA) (5, 6, 7, 8). ABA failed to induce stomatal closure in *PLDα1*-deficient plants, whereas overexpression of *PLDα1* resulted in increased sensitivity to ABA (8). PLDα1 mediates ABA signaling via PA interacting with ABI1 phosphatase 2C (7). This interaction impedes the negative function of ABI1 in ABA response and mediates ABA-promoted stomatal closure (7, 9). On the other hand, PLDα1 interacts with the GDP-bound Ga to regulate stomatal opening (9). PLDα1 has also been implicated in ROS production in *Arabidopsis* through the regulation of NADPH oxidase activity to promote stomatal closure (8). These studies indicate that PA is an important second messenger in the regulation of multiple mediators that determine stomatal aperture in response to ABA.

ABA is an important endogenous phytohormone regulating developmental processes and stress responses in plants (10, 11). In response to drought stress, ABA level increases rapidly and initiates a network of signaling pathways in guard cell leading to stomatal closure (11). A number of intermediate components of ABA signaling pathway have been identified by forward and reverse genetic approaches (10-14). Recently, proteins, known as pyrabactin resistance 1 (PYR1), pyr1-like proteins (PYLs), or Regulatory Components of ABA Receptor (RCARs) have been identified as ABA receptors (15, 16, 17, 18). ABA binds to the receptor PYR/PYL/RCARs, resulting in inhibition of the negative regulator ABI1, allowing SNF1-related kinase 2 (SnRK2) activation, mediating downstream signaling (11). PYR/PYLs are soluble proteins present in the cytosol and nucleus (17). Other proteins that interact with ABA were reported to be localized in the plastids or on the plasma membrane (19, 20, 21). The role of cell membrane in ABA perception and signaling is not fully understood (22).

Sphingolipids are essential components of eukaryotic membranes and their metabolites also function as important regulators of many cellular processes (23, 24).
Phosphorylated sphingolipids, such as sphingosine-1-phosphate (S1P), are potent messengers in the regulation of a variety of processes in animals, including cell proliferation and survival (25). A number of genes involved in sphingolipid biosynthesis have been identified and characterized in *Arabidopsis* (26, 27). These studies indicate important roles for sphingolipids in plant growth, development, and response to stresses. Phosphorylated long-chain bases (LCBP), such as S1P and phytosphingosine-1-phosphate (phyto-S1P), have been implicated in the regulation of ABA-mediated stomatal behavior through G proteins in plants (28, 29, 30, 31). A recent study suggests that sphingosine and S1P are absent in *Arabidopsis* leaves due to the lack of expression of sphingolipid ∆4-desaturase (32). However, plants have other LCBPs, including phyto-S1P, a LCBP produced by sphingosine kinase (SPHK) (30). Phyto-S1P is implicated as a signaling molecule regulating ABA-dependent stomatal movement (30).

SPHK activity was recently established in *Arabidopsis*, and two genes *SPHK1* (At4g21540) and *SPHK2* (At4g21534) have been cloned and characterized (30, 31, 33). Both SPHKs were active and able to use various long-chain bases (LCBs) as substrates (31, 33). SPHK activity was shown to be rapidly induced by ABA and the production of phyto-S1P was involved in promotion of stomatal closure in response to ABA (29, 30). Overexpression of *SPHK1* increased ABA sensitivity during stomatal closure and germination (31). However, the physiological function of SPHK2 is unknown, and the mode of regulation of SPHK activation remains elusive. We recently showed that PA interacted with SPHK1 and SPHK2 and promoted their activity *in vitro* (33). This study was undertaken to determine the cellular and physiological functions of the PA-SPHK interaction. The results show that PA interacts directly with SPHK in *Arabidopsis* and that PLDα1 and PA act downstream of SPHK. Together, PLDα1/PA and SPHK/phyto-S1P function in a positive feedback loop to amplify the ABA signal for stomatal closure in *Arabidopsis*.

**EXPERIMENTAL PROCEDURES**

**Knockout Mutant Isolation and Complementation**

*Arabidopsis thaliana* (Col-0) wild type (WT) and two T-DNA mutant (Salk_000250
and Salk_042034) lines were obtained from ABRC at Ohio State University. A PCR-based approach was used to verify the insertion of T-DNA and the homozygous T-DNA lines. T-DNA left border primer (LBa1) is 5’-TGGTTCACGTAGTGCCCATCG-3’. Gene specific primers for Salk_000250 are 5’-CAGATTCCCTCCTGCCTCTTTC-3’ (RP2) and 5’-GGGAGCTAGGATTTGAAGG-3’ (LP2). Gene specific primers for Salk_042034 are 5’-ATTCCCTTGGTTGTGTGTGTG-3’ (RP1) and 5’-AACGGATTCAACACACACAGC-3’ (LP1). *pld*α1 (Salk_053785) was isolated and confirmed previously (7). *PLDα1* and *SPHK* double mutants were generated by crossing *pld*α1 with Salk_000250 and Salk_042034. To rescue the *SPHK* mutants, genomic sequence including both *SPHK1* and *SPHK2* was cloned using two primers (5’-AGCCTTTTGGGTGGTGCACG-3’ and 5’-AGCTAAACAAAATACTCTG-3’) and inserted into binary vector PEC291 for transformations of the *SPHK* mutants.

**Plant Growth Conditions and Treatments**

Plants were grown in soil in a growth chamber with cool white light of 160 μmol m⁻² s⁻¹ under 12 h light/12 h dark and 23°C/19°C cycles. The seed germination assay and root elongation assay were performed on agar plates containing ½ Murashige and Skoog (MS) medium supplemented with 1% sucrose. Desiccated seeds were sterilized in 70% ethanol followed by 20% bleach, rinsed three times with sterilized water, and placed on plates with or without ABA. The plates were kept at 4°C for 2 days before moving to the growth chamber under the same conditions described previously. For root elongation measurements, 4 day-old seedlings were transferred to ½ MS medium with 0 to 10 μM ABA; root lengths were recorded daily.

**RNA Extraction and Real-time PCR**

Real-time PCR was performed as described previously (34). Briefly, total RNA was digested with RNase-free DNase I and 1 μg RNA was used for synthesis of the first-strand cDNA using an iScript cDNA synthesis kit in a total reaction volume of 20 μL according to the manufacturer’s instructions (Bio-Rad). The primer sequences were described previously (33). The efficiency of the cDNA synthesis was assessed by real-time PCR amplification of a control gene encoding UBQ10 (At4g05320). cDNAs were then diluted to yield similar threshold cycle (Ct) values based on the Ct of the *UBQ10*. The level of individual gene expression was normalized to that of *UBQ10* by
subtracting the Ct value of UBQ10 from the tested genes. PCR was performed with a MyiQ system (Bio-Rad) using SYBR Green. Each reaction contained 7.5 µL 2×SYBR Green master mix reagent (Bio-Rad), 3.5 µL diluted cDNA, and 200 nM of each gene-specific primer in a final volume of 15 µL. The following standard thermal profile was used for all PCRs: 95°C for 3 min; and 50 cycles of 95°C for 30s, 55°C for 30 s, and 72°C for 30 s.

**Stomatal Aperture Measurements**

Stomatal aperture was measured according to procedure described by Zhang et al. (7). In brief, epidermal peels were floated in incubation buffer (10 mM KCl, 0.2 mM CaCl₂, 0.1 mM EGTA, 10 mM Mes-KOH, pH 6.15) for 2.5 h under cool white light at 23°C to induce stomatal opening. 25 µM ABA, 10 µM phyto-S1P, 10 µM phyto-S1P with 0.1% 1-butanol and 50 µM PA were applied separately. Epidermal peels 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).

**Purification of SPHK from Protoplasts and Immunoprecipitation**

Mesophyll protoplasts were isolated from 4 week-old *Arabidopsis* leaves overexpressing *SPHK2* according to a procedure previously described (35). Protoplast labeling and protein extraction was performed as described previously (7). Protoplasts were labeled with 0.5 mg/mL 1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine (NBD-PC, Avanti) for 80 min and washed two times with the protoplast W5 buffer (35) to remove unlabeled NBD-PC. NBD-PC-labeled protoplasts were treated with 50 µM ABA for 0-30 min, followed by lysis in protoplast lysis buffer (20 mM Tris-HCl pH 7.5, 20 mM KCl, 1 mM EDTA, 10 mM DTT, 0.5% Triton X-100, 50% glycerol, 10 g/mL antipain, 10 g/mL leupeptin, 10 g/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride) on ice for 5 min. Spermidine (5 mM) was added to the lysate followed by centrifugation at 10,000 g for 10 min. The cellular extract was incubated with ANTI-FLAG beads (Sigma) at 4°C for 3 h. The beads were pelleted by centrifugation and washed three times. Washed beads were extracted with chloroform: methanol (2:1). The extracts were dried under a stream of N₂, dissolved in chloroform, and separated by TLC (silica gel 60 F254; Merck, Darmstadt, Germany). NBD-PA, scraped from TLC plates, was
quantified using a fluorescence spectrophotometer, by comparing fluorescence intensities to those on a standard curve constructed with known amounts of NBD-PA.

**Fluorescence-based In Vivo Assay of Sphingosine Kinase Activity**

Protoplasts were prepared from fully expanded leaves of 4 week-old *Arabidopsis*. Protoplasts were incubated in 0.1 mg/mL NBD-sphingosine for 80 min on ice and washed briefly. Washed protoplasts were kept at room temperature for 30 min. To determine *in vivo* sphingosine kinase activity based on the production of NBD-sphingosine-1-phosphate (NBD-S1P), 100 µM ABA was added to NBD-sphingosine-labeled protoplasts (3×10⁵ for each assay) and incubated in a glass tube at room temperature for the indicated time (0-20 min). 800 µL chloroform:methanol:concentrated HCl (100:200:1; v/v/v) was added to extract the lipids. 250 µL chloroform and 250 µL 2 M KCl were added sequentially. The sample was vortexed and centrifuged to generate a two-phase system. The lower chloroform phase was collected into a clean glass tube. Samples were dried under nitrogen and then resuspended in 50 µL chloroform. Lipid samples were spotted onto TLC plates and separated with chloroform:acetone:methanol:acetic acid:water (10:4:3:2:1; v/v/v/v). Lipids were visualized under UV illumination. The regions corresponding to NBD-S1P and NBD-sphingosine were marked, scraped from the plates, placed in 600 µL chloroform:methanol:water (5:5:1), vortexed, and centrifuged for 5 min at 15,000 g. The fluorescence (excitation 460 nm, emission 534 nm) of the eluted lipids was measured in a fluorescence spectrophotometer.

To assay the activity of the purified SPHK1 and SPHK2 using NBD-sphingosine as substrate, 1-10 µg NBD-sphingosine was incubated in sphingosine kinase buffer (20 mM Tris PH 7.4, 20% glycerol, 1 mM mercaptoethanol, 1 mM EDTA and 0.25% (v/v) Triton X-100, 1 mM ATP and 10 mM MgCl₂) with 10 µg SPHK1 or SPHK2 purified from *E. coli* for 10 min at 37°C. Lipid extraction and separation by TLC was described above.

**Fluorescence-based In Vivo Assay of Phospholipase D Activity**

A PLD activity assay was performed according to a procedure described previously (7). Protoplasts prepared from leaves of 4 week-old plants were incubated in 0.5 mg/mL NBD-PC for 80 min on ice. To determine PLD activity, as affected by ABA
treatment at different time points *in vivo*, 100 µM ABA was added to the NBD-PC-labeled protoplasts, and 100 µL aliquots (~1.5×10⁵ for each assay) were transferred to a new tube at the end of each treatment. 0.4 mL hot isopropanol (75°C) was added, and the mixture incubated for 10 min at 75°C to inactivate PLD. Lipids were extracted with 0.5 mL chloroform:methanol:water (5:5:1). The phases were separated and 100 µL chloroform were added to the aqueous phase, vortexed, centrifuged at 15,000 g for 2 min, and the lower chloroform phases were pooled. Each sample was dried under a nitrogen and 20 µL chloroform:methanol (95:5) were added. NBD-PC and NBD-PA were separated by TLC developed in chloroform:methanol: NH₄OH (65:35:5) and visualized under UV illumination. The regions corresponding to NBD-PC and NBD-PA were marked and scraped from the plates. The scraped silica gel was placed in 600 µL chloroform:methanol (2:1), vortexed, and centrifuged for 5 min at 15,000 g. The eluted lipids were quantified by fluorescence spectrophotometry (excitation 460 nm, emission 534 nm).

**ESI-MS/MS Analyses of Lipid Molecular Species**

Lipids were extracted and PA analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) as described by Xiao *et al.* (36). Expanded leaves of 4 to 5 week-old plants were sprayed with 100 µM 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°C) immediately after sampling. The experiment was repeated 3 times with 5 replicates of each treatment each time.

**HPLC/ESI-MS/MS Analyses of LCBPs**

Sample preparation and analyses of LCB(P)s was carried out according to the method described by Markham *et al.* with some modifications (37). Briefly, 4 to 5 week-old plants were sprayed with 100 µM ABA with 0.01% Triton X-100. The excised leaves were extracted 5 times with solvent H (lower phase of isopropanol/hexane/water, 55:20:25 (v/v/v)) with agitation in 60°C water bath for 15 min. The extract was transferred to a new glass tube and the combined extract was dried under a stream of nitrogen. Further steps of sample preparation and mass spectrometry analyses were carried out as described previously (37).
RESULTS

Manipulations of SPHKs and their Expression in Response to ABA

To determine the function of SPHK1 and SPHK2 in Arabidopsis, we isolated two T-DNA insertion mutant lines for SPHK1 and SPHK2. Sphk1-1 (Salk_042034) and sphk2-1 (Salk_000250) each has a T-DNA insertion before the (SPHK1 or SPHK2) start codon (Figure 1A). Both lines were homozygous confirmed by PCR (Figure 1B). Plants of sphk1-1 and sphk2-1 grow and developed normally as WT under normal condition in soil (Supplemental Figure 1). The mutant sphk2-1 displayed almost no detectable SPHK2 transcript, whereas its SPHK1 expression level was comparable to WT, as quantified by real-time PCR. In sphk1-1, the SPHK1 transcript was decreased by 81% compared to WT whereas the transcript of SPHK2 was also comparable to WT (Figure 1C). The expression of SPHK1 and SPHK2 was restored to WT level in both sphk1-1 and sphk2-1 that were genetically rescued by the genomic sequence including both SPHK1 and SPHK2 (Figure 1C). SPHK2-OE lines driven by 35S promoter were generated in our previous study, and the production of the introduced SPK2 was detected by immunoblotting (33). Real-time PCR revealed that the expression level of SPHK2 was increased by 7 and 11 fold in SPHK2-OE2 and SPHK2-OE5 (Figure 1C).

SPHK activity was shown to be quickly induced by ABA in a previous study (29). To determine whether the transcript levels of SPHK1 or SPHK2 are increased in response to ABA, we sprayed WT Arabidopsis leaves with ABA and checked the expression levels of SPHK1 and SPHK2 by real-time PCR. The transcript level of ABI1 began to increase 5 min after ABA treatment, but the transcript level of SPHK1 and SPHK2 did not change significantly (Figure 1D). The results suggest that SPHK1 and SPHK2 are not induced at the transcriptional level by ABA (Figure 1D).

PA Interacts with SPHK and Promotes the Activity of SPHK in Arabidopsis

Our previous study using E. coli-expressed proteins showed that PA bound to SPHK1 and SPHK2, and the interaction promoted the SPHK activity in vitro (33). To demonstrate their interaction and function in plants, we isolated protoplasts from the SPHK2-OE line which expressed FLAG-tagged SPHK2. NBD-PC-labeled protoplasts were washed and treated with 50 μM ABA followed by lysis and
immunoprecipitation with ANTI-FLAG beads. The lipid was extracted from the immunoprecipitated fraction and separated by TLC. NBD-PA was co-precipitated with SPHK2 (Figure 2A, inset). ABA treatment for 30 min increased the amount of NBD-PA pulled down with SPHK2 approximately 6 fold, suggesting that ABA activated PLDα1 and increased the amount of PA interacting with SPHK2 in Arabidopsis cells (Figure 2A).

To determine whether PA promotes SPHK activity in the cell, we developed an assay, using NBD-sphingosine-labeled protoplasts, for production of NBD-S1P in vivo. First, we used SPHK purified from E. coli to confirm that Arabidopsis SPHK could phosphorylate NBD-sphingosine. Both SPHK1 and SPHK2 phosphorylated NBD-sphingosine to NBD-S1P (Figure 2B). We then labeled protoplasts with NBD-sphingosine followed by treatment with ABA or PA. Lipid extracts were separated by TLC and photographed under UV light (Supplemental Figure 2). ABA treatment increased SPHK activity; the highest level of NBD-S1P was produced after 2.5 min of ABA treatment (Figure 2C). The level of NBD-S1P in SPHK2-OE protoplasts was 36% higher, whereas the level in sphk1-1 and sphk2-1 protoplasts was, respectively, 19% and 40% lower than WT at 2.5 min of ABA treatment (Figure 2C).

The ABA-induced activity of SPHK was also impaired in pldα1; the level of NBD-S1P produced in pldα1 was approximately 33% lower than that in WT. The results indicate that PLDα1 is involved in activating SPHK in response to ABA (Figure 2C). To determine if the PLD product PA could stimulate SPHK in the cell, we added PA (18:1/18:1) to the protoplasts. Addition of PA increased NBD-S1P production by more than 60% in protoplasts of WT and pldα1 at 5 min after treatment (Figure 2D). Similar to the ABA treatment, the increased SPHK activity in the PA treatment was the highest in SPHK2-OE and lower in sphkl-1 and sphk2-1 protoplasts. However, unlike the ABA treatment, PA-treated WT and pldα1 protoplasts exhibited the same magnitude and pattern of NBD-S1P increase (Figure 2D). These data support the conclusion that SPHK is a target of PA and PLD-produced PA is involved in the SPHK activation in response to ABA.

**SHPK Acts Upstream of PLDα1 in the Signaling Pathway of the ABA-mediated Stomatal Closure**
To determine the relationship of SPHK/phyto-S1P and PLDα1/PA in the ABA signaling pathway, we measured stomatal aperture in response to phyto-S1P in SPHK and PLDα1 mutants. Phyto-S1P produced by SPHK was shown previously to induce stomatal closure (30). We used phyto-S1P to treat epidermal peels and found that phyto-S1P caused stomatal closure in WT, sphk1-1 and sphk2-1 but not in pldα1 or the double knockout mutants of plda1sphk1-1 or plda1sphk2-1 (Figure 3A). The result suggests that SPHK and phyto-S1P act upstream of PLDα1 and PA.

We then treated the epidermal peels with PA to determine the effect of PA on stomatal closure in these mutant lines. PA (18:1/18:1) was able to cause stomatal closure in WT, plda1, sphk1-1 and sphk2-1 (Figure 3B). This result is consistent with the finding (Figure 3A) that PLDα1 and PA act downstream of SPHKs to promote stomatal closure. To augment the finding, we added 1-butanol, which decreases PA production by PLD, to the Arabidopsis epidermal peels treated with phyto-S1P. 1-Butanol partially blocked the phyto-S1P-promoted stomatal closure in WT, sphk1-1, and sphk2-1, but had no effect on plda1 (Figure 3A). The results support the notion that PLD/PA is involved in mediating phyto-S1P signal in stomatal closure.

**ABA-promoted PLDα1 Activation Is Attenuated in SPHK Mutants**

The above results indicate that both SPHK and PLDα1 are involved in the same signaling pathway in ABA-promoted stomatal closure, with SPHK and phyto-S1P acting upstream of PLDα1. To define the effect of SPHK on PLD activity and PA production in response to ABA, we measured PA production in vivo using NBD-PC-labeled leaf protoplasts exposed to ABA or phyto-S1P. The production of PA increased almost two fold in WT in 40 min after the start of ABA treatment (Figure 4A, 4B). However, the increase in PA in both sphk1-1 and sphk2-1 was significantly smaller than that in WT. Compared to WT, after 40 min of ABA treatment, PA production in sphk1-1 and sphk2-1 was 17% and 30% lower, respectively (Figure 4B). In plda1, the PA level was lower than WT and SPHK mutants, and there was no significant increase in PA (Figure 4B), supporting the previous conclusion that PLDα1 is the major PLD responsible for ABA-induced PA production (7).

We reasoned that if PLDα1 acts downstream of SPHK, phyto-S1P should be able to activate PLDα1. To test this hypothesis, we first tested whether phyto-S1P could
stimulate PLDα1 directly in vitro. Additions of different concentrations of phyto-S1P failed to increase PLDα1 directly, indicating other cellular effectors are involved in the PLD activation by phyto-S1P (Supplemental Figure 3). We then treated the protoplasts with phyto-S1P and measured PA production in protoplasts (Figure 4C). The production of PA was increased by approximately two fold by phyto-S1P in WT and both SPHK mutants. PA reached the highest level after 10 min of incubation. Knockout of PLDα1 abolished the ABA or phyto-S1P-induced increase in PA (Figure 4B, 4C). The response of PLD activity to phyto-S1P indicates that SPHK and phyto-S1P are involved in activation of PLDα1 to produce PA in response to ABA.

**ABA Induces Different PA Changes in WT, sphk1-1, sphk2-1 and SPHK2-OE Lines**

To characterize the effect of SPHKs on PA production in response to ABA, we quantitatively profiled the changes in PA species in Arabidopsis leaves sprayed with ABA using ESI-MS/MS. Knockout of PLDα1 reduced greatly the PA production in response to ABA (8). The total amount of PA in sphk1-1 and sphk2-1 was not significantly different than that of WT without ABA treatment (Figure 5A). In WT, PA reached the highest level at 10 min after ABA treatment and then went down to the pretreatment level after 40 min (Figure 5A). The total PA level was also increased in sphk1-1, sphk2-1, and SPHK2-OE leaves after ABA treatment (Figure 5A). The PA level was higher than WT after ABA treatment in SPHK2-OE. However, the amount of PA was significantly lower in sphk1-1 and sphk2-1 treated by ABA for 5 and 10 min than in WT (Figure 5A). The results indicate that decreased SPHK expression attenuates ABA-induced activation of PLDα1, in agreement with the results for the in vivo PLD activity assay (Figure 4B).

The change of PA species in response to ABA at 10 min was analyzed for WT, plda1, sphk1-1, sphk2-1 and SPHK2-OE. The major PAs in WT Arabidopsis leaves are 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) (8, 38). The levels of all PA species were decreased in plda1 and the major overall decreases were due to decreases in 34:2 PA and 34:3 PA, two very abundant PAs in Arabidopsis leaves (Figure 5B). In comparison, the levels of most PA species (except 36:6 and 36:5 PA) were higher in WT than in sphk1-1 and sphk2-1 after 10 min of ABA treatment (Figure 5B). Overexpression of SPHK2 mainly
resulted in higher levels of 34:2 PA and 34:3 PA compared to WT and other PA species did not change significantly (Figure 5B). The results show that the activation of \textit{SPHK1} and \textit{SPHK2} affects levels of 34-carbon PAs more than other PAs.

**LCBP Profiling Reveals Regulation of SPHK by PA**

To determine the effect of PLD\(\alpha_1\)/PA on the level of different LCBPs in \textit{Arabidopsis}, LCBP species were profiled to measure LCBP changes in response to ABA. We first analyzed the LCBPs in \textit{Arabidopsis} leaves from WT and mutant lines. The total content of four major LCBP species (d18:0-P, d18:1-P, t18:0-P and t18:1-P) was comparable in WT, \textit{pld} \(\alpha_1\), and \textit{sphk1-1} (Figure 6A). The LCBP level in \textit{sphk2-1} was about 57\% lower than that in WT, indicating that ablation of \textit{SPHK2} dramatically decreased LCBP production in \textit{Arabidopsis} leaves (Figure 6A). Total LCBP level was increased by 40\% when \textit{SPHK2} was overexpressed in \textit{Arabidopsis} (Figure 6A). The lower level of total LCBP in \textit{sphk2-1} was mainly due to the decrease of t18:0-P and t18:1-P (Figure 6B). ABA treatment increased the LCBP content by 58\% in WT leaves at 2 min after ABA treatment, but no such ABA-induced increase occurred in \textit{sphk1-1}, \textit{sphk2-1}, or \textit{pld} \(\alpha_1\) (Figure 6C).

LCBP species displayed different patterns of changes in response to ABA treatment (Figure 6D). The increase in t18:0-P is transitory and occurred early, peaking at 2 min after treatment. The increase in d18:1-P peaked at 5 min whereas d18:0-P increased steadily over the 15 min tested. The mutant \textit{sphk1-1} displayed transitory changes similar to WT, except that the magnitude of increase was smaller. However, \textit{sphk2-1} did not exhibit the same level of transitory change in t18:0-P as WT, but d18:0-P and d18:1-P changed, peaking 5 min after treatment (Figure 6D). LCBP production in \textit{pld} \(\alpha_1\) was not induced by ABA as much as in WT, indicating knockout of \textit{PLDa1} reduced SPHK activation by ABA (Figure 6D). This indicates that PA is involved in SPHK activation in response to ABA (Figure 8).

**SPHK2-KO and OE Alter \textit{Arabidopsis} Sensitivity to ABA**

To determine the effect of \textit{SPHK1} and \textit{SPHK2} mutations on \textit{Arabidopsis} response to ABA, we assayed ABA responses of \textit{sphk1-1} and \textit{sphk2-1} together with \textit{SPHK2-OE} lines. Stomatal aperture was decreased by ABA in WT. However, \textit{sphk1-1} and \textit{sphk2-1} were less sensitive to ABA-promoted stomatal closure (Figure 7A). Double mutants
plda1sphk1-1 and plda1sphk2-1 were insensitive to ABA-caused stomatal closure like pldal (Figure 7A). Introducing a genomic sequence containing both SPHK1 and SPHK2 under their native promoters into sphk1-1 and sphk2-1 restored the stomatal response to ABA for both mutants, indicating that loss of SPHK1 and SPHK2 is responsible for the ABA response phenotype (Figure 7A).

Knockdown of SPHK1 or SPHK2 decreased while overexpression of SPHK2 increased ABA sensitivity during ABA-inhibited root elongation (Figure 7B). The root length of the two SPHK mutants was longer than that of WT under 5 µM or 10 µM ABA. Overexpression of SPHK2 increased ABA sensitivity during ABA-inhibited root elongation as the root lengths in the OE lines were shorter than that of WT (Figure 7B). Manipulation of SPHK1 and SPHK2 also altered ABA sensitivity during seed germination and post-germination growth. Sphk1-1 and sphk2-1 germinated earlier than WT on ½ MS plates with different concentrations of ABA whereas the germination of SPHK2-OE seeds was delayed and its postgermination growth was inhibited (Figure 7C, 7D). The data suggest that SPHK2 is involved in the control of three ABA responses in Arabidopsis.

DISCUSSION

SPHK1 and SPHK2 are two genes closely linked on chromosome 4 in Arabidopsis based on molecular cloning, sequence analyses and distinguishable expression patterns (33). We isolated two T-DNA mutants, sphkl-1 and sphk2-1, for SPHK1 and SPHK2 separately. Real-time PCR indicated that SPHK1 expression level was dramatically reduced in sphkl-1 whereas the transcript of SPHK1 was slightly induced in sphk2-1. In addition, the expression level of SPHK2 in sphkl-1 is not significantly different from that of WT. These data provide further evidence that SPHK1 and SPHK2 are two separate genes. SPHK1 was reported to have a role in two ABA signaling pathways in regulation of stomatal aperture and seed germination (31). The present study shows that both SPHK mutants display decreased sensitivity to ABA-promoted stomatal closure, ABA-inhibited root elongation and ABA-inhibited seed germination. In addition, SPHK2-OE lines were more sensitive to ABA in three ABA-mediated responses, indicating that SPHK2 is involved in ABA-mediated
signaling pathways (Figure 8).

Quantitative analyses of LCBP showed that the total LCBP level remained the same as WT in \textit{sphk1-1} but decreased about 57\% in \textit{sphk2-1}. The decreased LCBP content mainly came from t18:0-P and t18:1-P. There was still 43\% of LCBP in \textit{sphk2-1} compared to WT, which is presumably a result of \textit{SPHK1} and other kinases including AtLCBK1 and AtCERK (39, 40). These data indicate that whereas SPHK2 contributes more than SPHK1 to LCBP production in leaves, SPHK1 and SPHK2 have unique and overlapping functions in LCBP synthesis in \textit{Arabidopsis} leaves. Availability of \textit{SPHK1xSPHK2} double knockout mutants will be helpful to further determine the functions of both SPHKs. But isolating such mutants by crossing \textit{sphk1-1} and \textit{sphk2-1} has been unsuccessful because \textit{SPHK1} and \textit{SPHK2} are closely linked (33).

Our previous \textit{in vitro} study showed that PA binds to SPHKs and stimulates their activity, suggesting that \textit{Arabidopsis} SPHKs are molecular targets of PA (33). The present study using protoplasts provides \textit{in vivo} evidence that PA binds to and stimulates SPHK. More evidence was garnered from the SPHK activity assay and quantitative profiling of LCBPs from leaves. Addition of PA promoted the production of NBD-S1P in WT protoplasts and SPHK activity was attenuated in \textit{pld\alpha_1} when protoplasts were treated with ABA. LCBP analyses indicated that LCBP content increased by 58\% in WT \textit{Arabidopsis} leaves after a 2 min ABA treatment. Knockout of PLD\alpha_1 resulted in less than 10\% increase of LCBP in response to ABA treatment, indicating PLD\alpha_1 and PA were involved in promotion of SPHK activity in response to ABA (Figure 8).

Phyto-S1P (t18:0-P) was capable of promoting stomatal closure (30). Phyto-S1P is one of the major LCBPs found in \textit{Arabidopsis} leaves; it can serve as a signaling molecule to mediate ABA response. Our data show that ABA induced the increased production of all 4 LCBPs in \textit{Arabidopsis} leaves. Whether the other three LCBPs are involved in ABA-mediated signaling pathway needs to be determined. LCBPs have broad cellular functions in animals, and more functions of LCBPs in plants also should be explored.

The phenotypic analyses of stomata in this study also indicates that PLD/PA and
SPHK/phyto-S1P are involved in the same pathway in regulation of stomatal closure (Figure 8). pldα1 was insensitive to phyto-S1P-promoted stomatal closure. PLD enzyme activity assay showed that phyto-S1P activated PLDα1 in Arabidopsis cells, placing PLDα1 downstream of SPHK in ABA signaling pathway. Lipid profiling also revealed that all the PA species were increased in response to ABA in WT leaves. Our previous study indicated that not all PA species interacted with SPHK and promoted its activity. Among the PA species tested, 16:0/16:0 PA, 18:1/18:1 PA, 16:0/18:1 PA and 16:0/18:2 PA were able to bind to both SPHK1 and SPHK2 (33). 18:1/18:1 (36:2), 16:0/18:1(34:1) and 16:0/18:2 (34:2) PA naturally exist in Arabidopsis leaves and their levels are induced by ABA treatment. PA can be produced by multiple enzymes in response to different stimuli (1). PA regulates multiple proteins mediating ABA signaling, including ABI1, NADPH oxidases, and SPHKs (7, 8, 33). Many other PA-interacting proteins such as PDK1, CTR1 and TGD2 have also been identified in plants (41, 42, 43). Available data suggest that regulation of different proteins by PA depends on PA species and sources, timing, and localization of PA production.

In summary, the present physiological, genetic, and enzymatic analyses combined with lipid profiling clearly indicate a co-dependency between the two lipid signaling reactions, SPHK/phyto-S1P and PLD/PA (Figure 8). PA produced by PLDα1 interacts with SPHK and is required for SPHK activation in response to ABA. Increased phyto-S1P activates PLDα1, leading to an increase in PA level. PA functions as a signaling molecule to regulate downstream proteins including ABI1 and NADPH oxidase in ABA-mediated stomatal closure. The ABA signal is transduced to downstream pathways and regulates ion channels, leading to stomatal closure (Figure 8). It will be of interest in future studies to determine whether the interplay between PLDα1/PA and SPHK/phyto-S1P is involved in other signaling and regulatory pathways in plant growth, development, and response to stresses.
REFERENCES

FIGURE LEGENDS

FIGURE 1. Isolation of T-DNA insertion lines and expression of two SPHKs in Arabidopsis leaves

A, diagram showing the T-DNA insertion sites in Salk_042034 (sphk1-1) and Salk_000250 (sphk2-1). T-DNA is located in front of start codon of SPHK1 and SPHK2 separately. Thin lines represent non-coding regions and boxes represent exons.

B, PCR genotyping of two T-DNA insertion lines. The presence of T-DNA band and lack of SPHK1 or SPHK2 band indicate that each is a homozygous T-DNA insertion mutant. PCR was conducted using genomic DNA with a pair of gene specific primers (LP1+RP1 for SPHK1 and LP2+RP2 for SPHK2) or a combination of T-DNA left border primer (LBa1) and gene specific primers (RP1 for SPHK1 and RP2 for SPHK2).

C, expression level of SPHK1 and SPHK2 in WT, SPHK mutants, complementation, and overexpression lines determined by real-time PCR normalized to UBQ10. RNA was extracted from young leaves of 4 week-old Arabidopsis. The experiment was repeated three times. Values are means ± SE (n = 3) for one representative experiment.

D, effect of ABA on SPHK1 and SPHK2 expression measured by real-time PCR normalized to UBQ10. The ABA response gene ABI1 was used as a positive control. RNA was extracted from leaves sprayed with 100 µM ABA with 0.01% Triton X-100. The experiment was repeated three times. Values are means ± SE (n = 3) for one representative experiment.

FIGURE 2. PA interacts with SPHK and is involved in activation of SPHK in response to ABA

A, quantification of NBD-PA bound to SPHK2 pulled down by Anti-FLAG resin beads. Inset represents image of NBD-PA immunoprecipitated with SPHK2 on TLC plate. Control indicates protoplasts (incubated with ABA for 30 min) isolated from WT Arabidopsis.
**B.** SPHK activity assay using NBD-sphingosine as substrate. NBD-S1P was produced by both SPHKs as indicated on TLC plate. “-” indicates negative control without addition of protein.

**C.** quantification of NBD-S1P production in protoplasts treated with 50 µM ABA. Protoplasts were isolated from WT, plda1, sphk1-1, sphk2-1 and SPHK2-OE lines.

**D.** quantification of NBD-S1P production in protoplasts treated with 50 µM PA. The level of NBD-S1P was calculated as the percentage of NBD-S1P over the total NBD-labeled lipids. Values in C and D are means ± SE (n = 3).

**FIGURE 3. PLDα1 and PA mediate the phyto-S1P effect on the signaling pathway in ABA-mediated stomatal closure**

**A.** effect of phyto-S1P on stomatal closure in WT and mutants. The epidermal peels were incubated in stomatal incubation buffer containing 10 µM phyto-S1P or 10 µM phyto-S1P plus 0.1% 1-butanol. Asterisks indicate that the mean value is significantly different from that of the samples treated with phyto-S1P at P<0.05 based on Student’s t test.

**B.** PA (18:1/18:1) induces stomatal closure in WT and mutants. Epidermal peels were treated with 50 µM PA. All values are means ± SE (n = 50) in the stomatal assays.

**FIGURE 4. Activation of PLDα1 by ABA requires SPHK**

**A.** representative image of fluorescent-based assay of PLD activity using NBD-PC-labeled protoplasts treated with 50 µM ABA.

**B.** quantification of ABA-induced PA production in protoplasts isolated from WT, plda1, sphk1-1, and sphk2-1. Protoplasts were labeled with NBD-PC followed by treatment with ABA. WT control was treated with 0.1% ethanol.

**C.** quantification of phyto-S1P-promoted PA production in protoplasts isolated from WT, plda1, sphk1-1, and sphk2-1. The level of PA was calculated as the percentage of NBD-
PA over the total NBD-labeled lipids. Data in B and C are means ± SE (n = 3) for one representative experiment.

**FIGURE 5. ABA-induced PA changes in Arabidopsis leaves**

A, change in total PA content in leaves harvested at different times after spraying with ABA (100 µM).

B, comparison of PA molecular species in leaves of WT, mutants, and SPHK2-OE lines treated with ABA for 10 min. The experiment was performed three times. Values in A and B are means ± SE (n = 5).

**FIGURE 6. Alterations of SPHKs change LCBP content and composition in Arabidopsis leaves**

A, total LCBP content (mol%) in leaves from 4 to 5 week-old WT, plda1, sphkl-1, sphk2-1, and SPHK2-OE5.

B, LCBP composition in leaves from 4 to 5 week-old WT, plda1, sphkl-1, sphk2-1, and SPHK2-OE5.

C, total LCBP content in WT Arabidopsis leaves treated with ABA. 4 to 5 week-old Arabidopsis was sprayed with 100 µM ABA with 0.01% Triton X-100 followed by sphingolipid extraction and MS analyses.

D, LCBP composition in the leaves treated with 50 µM ABA with 0.01% Triton X-100 for 0-15 min. Data were calculated as molar percentage over the total amount of LCB (sphinganine (d18:0), 8-sphingenine (d18:1), phytosphingosine (t18:0) and 4-hydroxy-8-sphingenine (t18:1)) and LCBP (d18:0-P, d18:1-P, t18:0-P and t18:1-P). The experiment was performed twice and the results were consistent. Values are means ± SE for one experiment (n = 5). Asterisks in B indicate that the mean value is significantly different from that of the WT at P < 0.05, based on Student’s t test. Asterisks in C indicate that the mean value is significantly different from that of the 0 min ABA treatment for each
Arabidopsis line at P < 0.05. Asterisks in D indicate that the mean value is significantly different from that of the 0 min ABA treatment for each Arabidopsis line at P < 0.05 based on Student’s t test.

FIGURE 7. Altered ABA sensitivity in SPHK-KO mutants and SPHK2 over-expression Arabidopsis

A, addition of ABA (25 µM) induced stomatal closure in WT and mutants lines. COM1 is a complimented line for sphk1-1 and COM2 is a complimented line for sphk2-1. Values are means ± SE (n=50). Asterisks indicate that the mean value is significantly different from that of the WT treated with ABA at P<0.05 based on Student’s t test.

B, root growth of WT, sphk1-1, sphk2-1, and SPHK2-OE lines (OE2 and OE5) on ½ MS medium with 0 µM, 5 µM or 10 µM ABA. Values are means ± SE (n = 20) for one representative experiment. Asterisks indicate that the mean value is significantly different from that of the WT treated with same concentration of ABA at P<0.05 based on Student’s t test.

C, seed germination rate on ½ MS medium with different concentrations of ABA. Desiccated seeds were germinated on ½ MS with or without ABA and scored 3 days after transfer from 4°C. About 100 seeds per genotype were scored for each experiment. Values are means ± SE (n=3).

D, seed germination and post-germination growth on ½ MS medium without ABA (left) or with 1 µM ABA at day 6.

FIGURE 8. Proposed model for the role of SPHK/phyto-S1P and PLDα1/PA in ABA-mediated stomatal closure signaling pathway

This model depicts only the known targets of PLD/PA in the ABA-mediated stomatal closure and other ABA regulators are not included in this model. ABA activates SPHKs through unknown mechanisms and ABA receptors may be involved. The activation of
SPHKs produces phyto-S1P that activates PLDα1 to produce PA. PA inhibits ABI1 function but promotes NADPH oxidase to promote ABA-mediated stomatal closure. Meanwhile, PLDα1-produced PA stimulates SPHK activity through a positive feedback loop. Arrows with solid lines indicate established links and arrows with dashed lines denote putative links.

**Supplemental Figures:**

**Supplemental Figure 1. Growth of plants in soil**

A. Growth of 5-week-old plants. Photograph was taken at 5 week after transplant in soil.

B. Measurement of diameter of plants. Values are means ± SE (n=12). Asterisk indicates that the mean value is significantly different from that of WT Arabidopsis at P < 0.05 based on Student’s t test. The plants were grown in soil in a growth chamber with cool white light of 160 µmol m⁻² s⁻¹ under 12 h light/ 12 h dark and 23°C/19°C cycles.

**Supplemental Figure 2. Assay of SPHK activity using NBD-sphinogosine**

Representative TLC image of SPHK activity using NBD-sphingosine-labeled protoplasts treated with 50 µM ABA for different times in WT, plda1, and sphk1-1.

**Supplemental Figure 3. PLDα1 activity assay with addition of phyto-S1P**

A. TLC image of PLDα1 activity assay. PLDα1 activity assay was done using total protein extracted from WT and plda1 Arabidopsis leaves. 2.5 µg total protein was incubated with 20 µg NBD-PC as substrate under PLDα1 reaction condition (25 mM Ca²⁺, 100 mM MES, PH 6.0, 0.5 mM SDS). The assay was incubated at 30°C with shaking for 20 min. Knockout of PLD α1 resulted in more than 95% decrease of PA production, indicating PLDα1 is responsible for the PLD activity under this assay condition. PLDα1 activity was determined with addition of 5 µM or 25 µM phyto-S1P. There were three replicates for each condition.
B. quantification of lipids isolated from sFigure 2A. The level of PA was calculated as the percentage of NBD-PA over the total NBD-labeled lipids. Values are means ± SE (n=3).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Supplemental 1
Supplemental 3
Chapter 4. Cytosolic Glyceraldehyde-3-Phosphate Dehydrogenases
Interact with Phospholipase Dδ to Transduce Hydrogen Peroxide
Signals in Stress Response in Arabidopsis

This chapter is written as a manuscript which is submitted and under review

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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; L.G. and X.W analyzed the data and wrote the manuscript.

ABSTRACT

Reactive oxygen species (ROS) are produced in plants under various stress conditions and serve as important chemical signals in mediating plant responses to stresses. Here we show that the cytosolic glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenases (GAPC) interact with the plasma membrane-associated phospholipase D PLDδ to transduce the ROS hydrogen peroxide (H$_2$O$_2$) signal in Arabidopsis. Genetic ablation of PLDδ impeded stomatal response to abscisic acid (ABA) and H$_2$O$_2$, placing PLDδ downstream of H$_2$O$_2$ in mediating ABA-induced stomatal closure. A search of PLDδ-interacting proteins led to the identification of PLDδ binding to GAPC1 and 2. The PLDδ and GAPC interaction was demonstrated by co-precipitation using proteins expressed in E. coli and yeast, surface plasmon resonance, and bimolecular fluorescence complementation. H$_2$O$_2$ inhibited the GAPC activity but promoted the GAPC-PLDδ interaction and PLDδ activity. Knockout of GAPCs decreased ABA- and H$_2$O$_2$-induced activation of PLD and stomatal sensitivity to ABA. The loss of GAPCs or PLDδ rendered plants less responsive to water deficit than wild-type. The results indicate that the H$_2$O$_2$-promoted interaction of GAPCs and PLDδ may provide a direct connection between membrane lipid-based signaling, energy metabolism and growth control in plant response.
to water stress.

INTRODUCTION

Reactive oxygen species (ROS) are produced in plant response to a wide variety of biotic and abiotic stresses including ultraviolet irradiation, high light, wounding, ozone, low and high temperatures, drought, and pathogens (1, 2). ROS were originally viewed as by-products of metabolic pathways and a high concentration of ROS is toxic to the cells (1, 3, 4). It has now been well-documented that ROS are generated as chemical signals that alter various cellular and physiological processes in plant growth and development (1, 2, 5, 6). H$_2$O$_2$ is the major and most stable species of ROS and plays a signaling role in plant response to stresses, such as mediating abscissic acid (ABA)-regulated stomatal closure (7, 8). H$_2$O$_2$ is thought to affect target protein activities through modification of thiol groups of cysteine residues (9). However, it is unclear how such oxidative modifications affect 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 (10-14). The Arabidopsis genome contains 12 PLDs, PLD$\alpha$(3), $\beta$(2), $\gamma$(3), $\delta$, $\varepsilon$, and $\zeta$(2), and they exhibit distinguishable biochemical properties and cellular functions. Knockout of PLD$\alpha$I decreases the production of ROS, and addition of PA induces the recovery of the level of ROS in the pld$\alpha$I mutant (10). PA interacts with NADPH oxidase and increases its activity and ROS production (14). PLD and PA play a role in promoting generation of ROS in suspension rice and tomato cells (11, 12). On the other hand, H$_2$O$_2$ induced the activation of PLD which is required for elicitor-induced biosynthesis of phytoalexins in rice cells (11). PLD$\delta$ is activated by H$_2$O$_2$ and ablation of it rendered Arabidopsis cells more sensitive to H$_2$O$_2$-promoted programmed cell death than wild type (WT) (14-17). These results raise the possibility that specific PLDs occupy different steps in stress and ROS signaling pathways: PLD$\alpha$I promotes the ROS production whereas PLD$\delta$ mediates plant responses to ROS.
To determine how \( \text{H}_2\text{O}_2 \) activates PLD\( \delta \) and whether the unique roles of different PLDs function in the same physiological response involving the ROS signaling, we investigated the activation and role of PLD\( \delta \) in *Arabidopsis* response to ABA. A search of PLD\( \delta \)-interacting proteins led to the identification of PLD\( \delta \) binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate in the glycolytic pathway which is important for the energy production and carbon supply for cellular metabolism (18). The *Arabidopsis* genome contains seven phosphorylating GAPDHs, four of which are located in plastids whereas GAPC1, GAPC2, and a non-phosphorylating NP-GAPDH are in the cytosol (19, 20). GAPDHs have been implicated in embryo development, pollen development, root growth and ABA signal transduction (19-22). The catalytic cysteine residues of GAPDH can be oxidized by oxidants such as H\( _2 \)O\( _2 \), leading to fully or partially reversible inactivation of GAPDH (9, 23, 24). GAPC1 has been suggested to be a direct target of H\( _2 \)O\( _2 \), potentially involved in mediating ROS response in *Arabidopsis* (9, 24). Here we show that PLD\( \delta \) binds to GAPC1 and GAPC2, that H\( _2 \)O\( _2 \) promotes the PLD\( \delta \)-GAPC interaction, and that the interaction increases PLD\( \delta \) activity and mediates the ROS signal in plant response to ABA and water deficits.

**RESULTS**

**Ablation of PLD\( \delta \) Compromises ABA and H\( _2 \)O\( _2 \)-induced Stomatal Closure, but not ABA-Promoted H\( _2 \)O\( _2 \) Production**

To determine if PLD\( \delta \) is activated by ABA, we isolated *pld\( \alpha \)1pld\( \delta \)\* (Fig. S1) and assayed the PLD activity in response to ABA in wild type (WT), *pld\( \alpha \)*1, *pld\( \delta \)*, and *pld\( \alpha \)*1pld\( \delta \) using fluorescent-phosphatidylycholine (NBD-PC)-labeled protoplasts. The *PLD\( \alpha \)*1 knockout (KO) mutant was used because PLD\( \alpha \)1 was reported to be responsible for a majority of PA produced in response to ABA (13, 25). PA production was increased two fold after WT protoplasts were incubated with ABA for 20 min (Fig. 1A). The ABA-induced PA production in *pld\( \alpha \)*1 and *pld\( \delta \)* was approximately 62% and 28% lower, respectively, than in WT. No significant PA production increase was observed in response to ABA in
pdl1pdlδ double KO cells (Fig. 1A). The results indicate that PLDδ is activated by ABA and that PLDα1 and PLDδ together account for virtually all ABA-induced PLD activity, with PLDα1 providing the twice as much hydrolysis as PLDδ in Arabidopsis.

To determine the role of PLDδ in ABA response, we investigated whether the loss of PLDδ alters ABA-promoted stomatal closure and H$_2$O$_2$ production in guard cells. pdlδ1-1 was insensitive to ABA-promoted stomatal closure similar to pdlα1 (Fig. 1B). However, unlike pdl1, H$_2$O$_2$ induced stomatal closure in pdl1 and WT but not in pdlδ (Fig. 1A). Introduction of PLDδ-driven by its own promoter into the pdlδ (PLDδ-COM) restored the phenotype for both ABA and H$_2$O$_2$-induced stomatal closure, indicating that loss of PLDδ is responsible for the ABA and H$_2$O$_2$ response phenotype (Fig. 1B). In addition, unlike pdl1 that decreased ABA-promoted H$_2$O$_2$ production (13), knockout of PLDδ did not affect the ABA-induced H$_2$O$_2$ production. The basal level of ROS in pdlδ and WT cells were also similar, as revealed by the fluorescent dye 2',7'-dichlorofluorescin diacetate (H$_2$DCFDA) intensity (Fig. 1C and D). These results indicate that PLDδ is not required for ABA-induced ROS production, but is required for stomatal response to ABA and H$_2$O$_2$, and PLDδ acts downstream of H$_2$O$_2$ in signaling ABA-induced stomatal closure.

Direct Interaction between GAPC and PLDδ

PLDδ activity was induced within 20 min of ABA treatment, but the transcript level of PLDδ was not increased until 40 min of ABA treatment (Fig. S2). The results indicate that ABA-induced activation of PLDδ in the early phase is not mediated by increased PLDδ expression. To determine how PLDδ is involved in H$_2$O$_2$ response, we screened proteins potentially interacting with PLDδ by immunoprecipitation of Arabidopsis leaf proteins using PLDδ antibodies. Analyses of proteins co-isolated with PLDδ by mass spectrometry identified GAPC1, a cytosolic GAPDH potentially interacting with PLDδ. To verify the interaction, we cloned GAPC1 and GAPC2 cDNAs and expressed them in E. coli. Purified His-tagged GAPC1 and GAPC2 proteins (Fig. S3A) were used for reciprocal pulldown with purified GST-PLDδ. Both GAPC1 and GAPC2 pulled down PLDδ. PLDδ also pulled down GAPC1 and GAPC2, as indicated by immunoblotting with anti-His or GST antibodies (Fig. 2A). In addition, the association of GAPCs and
PLDδ was increased in the presence of H₂O₂ but decreased in the presence of the reducing reagent dithiothreitol (DTT; Fig. 2A). To further validate the interaction, we co-expressed GAPC and PLDδ in yeast (Fig. S3B) and grew the yeast cells with or without H₂O₂. GAPC1 and GAPC2 were detected in the complex with PLDδ when PLDδ was precipitated by GST beads. PLDδ also associated with GAPC1 and GAPC2 when GAPCs were isolated. The presence of H₂O₂ promoted the interaction between GAPC and PLDδ (Fig. 2B). These results indicate that the GAPC-PLDδ interaction was enhanced in an oxidative, but weakened in a reducing environment.

To quantify the interaction between GAPC1 and PLDδ, we used surface plasmon resonance (SPR) to determine the binding kinetics. Purified GAPC1 was immobilized on an NTA chip followed by injection of purified GST or GST-PLDδ. The representative sensorgram showed an increase in the response unit (RU) when GST-PLDδ, but not GST, was injected, indicating that PLDδ interacts with GAPC1 specifically (Fig. 2C). When H₂O₂-treated GAPC1 was used, the GAPC1-PLDδ interaction was enhanced as the RU was much higher than when GAPC1 was not incubated with H₂O₂ (Fig. 2C). H₂O₂-treated or un-treated GAPC1 displayed comparable association rate constants (Kₐ=8.19×10⁴ M⁻¹s⁻¹ vs. 8.33×10⁴ M⁻¹s⁻¹). However, the dissociation rate constant was lower when GAPC1 was exposed to H₂O₂ (Kₐ=5.52×10⁻⁴ s⁻¹ vs. 3.23×10⁻³ s⁻¹). The maximum specific binding is 1564 RU for H₂O₂-treated GAPC1 and 286 RU for GAPC1 without H₂O₂ treatment (Fig. 2C). The equilibrium binding constant Kᵢ is 6.62×10⁻⁹ M for GAPC1-PLDδ interaction in the presence of H₂O₂ and 3.94×10⁻⁸ M for GAPC1-PLDδ interaction without H₂O₂. The results indicate that the interaction GAPC1-PLDδ interaction is significantly enhanced by H₂O₂ and that H₂O₂ stabilizes the interaction by decreasing dissociation between GAPC1 and PLDδ.

To visualize the GAPC-PLDδ interaction in plant cells, we used bimolecular fluorescence complementation (BiFC) that brings together two YFP fragments fused to two interacting proteins (26). GAPC1 or GAPC2 were fused to the N-terminal of YFP (GAPC1-YFPN or GAPC2-YFPN) and PLDδ was fused to the C-terminal of YFP (PLDδ-YFPC). These constructs were co-introduced into in tobacco leaves. No fluorescence was
observed when empty vectors YFP\textsuperscript{N}/YFP\textsuperscript{C} were co-expressed (Fig. S4). In the positive control bZIP63-YFP\textsuperscript{N} and bZIP63-YFP\textsuperscript{C}, the transcription factor forms dimers and brings YFP\textsuperscript{N} and YFP\textsuperscript{C} together to generate fluorescence in the nucleus (Fig. S4). GAPC1-YFP\textsuperscript{N} or GAPC2-YFP\textsuperscript{N} co-expressed with PLD\(\delta\)-YFP\textsuperscript{C} produced fluorescence in the cell, indicating that both GAPCs interacted with PLD\(\delta\) (Fig. 2D).

**GAPCs Promote the Activity of PLD\(\delta\) under an Oxidative Condition**

To determine the function of GAPC interaction with PLD\(\delta\), we first tested the sensitivity of GAPC1 and GAPC2 purified from *E. coli* to \(\text{H}_2\text{O}_2\). \(\text{H}_2\text{O}_2\) inhibited GAPC activity in a dose-dependent manner and virtually all GAPC1 or GAPC2 activity was inhibited at 500 \(\mu\text{M}\) of \(\text{H}_2\text{O}_2\) (Fig. 3A). When different concentrations of DTT were added to GAPCs first, followed by addition of 500 \(\mu\text{M}\) of \(\text{H}_2\text{O}_2\), the loss of GAPC activity was small, showing that \(\text{H}_2\text{O}_2\)-oxidation of GAPCs can be protected by DTT reduction (Fig. 3B). After incubation with 500 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\), partial GAPC activity could be recovered by addition of DTT (Fig. 3C).

Purified PLD\(\delta\) was then incubated with GAPCs treated with and without \(\text{H}_2\text{O}_2\) to determine the effect of \(\text{H}_2\text{O}_2\) and GAPC on PLD\(\delta\) activity. Without GAPC, addition of 100 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) did not affect PLD\(\delta\) activity, verifying that \(\text{H}_2\text{O}_2\) has no direct effect on PLD\(\delta\) activity. Incubation of PLD\(\delta\) with GAPC1 and GAPC2 increased PLD\(\delta\) activity by 34.3\% and 11.5\%, respectively (Fig. 3D). However, pre-treatment of GAPC1 and GAPC2 with 100 \(\mu\text{M}\) \(\text{H}_2\text{O}_2\) increased PLD\(\delta\) activity by 82.1\% and 58.9\%, respectively (Fig. 3D). The data indicate that \(\text{H}_2\text{O}_2\) inactivates GAPC, but promotes the GAPC binding to PLD\(\delta\), and the binding increased PLD\(\delta\) activity.

**GAPC Mediates the \(\text{H}_2\text{O}_2\) 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, pld\(\delta\)*, and WT protoplasts as affected by \(\text{H}_2\text{O}_2\). 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) (Fig. S5). The *GAPC1* transcript was lost in 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 (Fig. 4A). We then generated two double KO lines (gapc1-1gapc2-1 and gapc1-1gapc2-2) by crossing the single mutants. Two lines of triple KO mutants (gapc1-1gapc2-1pldδ and gapc1-1gapc2-2pldδ) were also isolated by crossing the GAPC double KO with pldδ. 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 the four GAPC single mutants (Fig. 4B). GAPC double KO plants gapc1-1gapc2-1 and gapc1-1gapc2-2 had approximately 45% decrease in GAPDH activity (Fig. 4B). The results indicate that GAPC1 and 2 contribute almost equally to the activity and together they account for 45% of NAD-dependent GAPDH activity in Arabidopsis leaves.

To determine if knockout of both GAPCs affects PLD activation by H2O2, protoplasts of WT, pldδ, and GAPC double mutants were labeled with NBD-PC and treated with H2O2. We first examined how GAPDH activity in protoplasts responded to H2O2. Protoplasts from GAPC double KOs had significantly lower GAPDH activity than WT or pldδ (Fig. 4C). H2O2 treatments for 20 min had no significant effect on GAPDH activity the GAPC-double KO, but decreased GAPDH activity WT and pldδ by 15%. Significant decreases in GAPDH activity occurred in all genotypes after 40 min of H2O2 treatment (Fig. 4C). The results indicate that H2O2 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 H2O2 inhibition of GAPCs.

Without addition of H2O2, the PLD activity, as measured by the formation of PA, in gapc1-1gapc2-1 and gapc1-1gapc2-2 was comparable to that of WT (Fig. 4D). The H2O2 treatment increased PA production nearly two fold in WT whereas it increased PA production only 30% in pldδ. The gapc1gapc2 double KOs and gapc1gapc2pldδ triple KOs exhibited similar attenuated PA increase as pldδ in response to H2O2 (Fig. 4D). The results indicate that PLDδ is the main PLD responsible for the H2O2 activation of PLD, and that GAPCs mediates the H2O2–induced increase of PLDδ activity.

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

The molecular species of PA in response to ABA at 10 min were analyzed for WT, *pld\(\delta\), gapc1-1gapc2-1* and *gapc1-1gapc2-2*. 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 in WT *Arabidopsis* leaves (15). 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 (Fig. 5B). In comparison, the levels of PA species including 34:2, 34:3, 36:2, 36:4 and 36:5 PA were significantly higher in WT than in *gapc1-1gapc2-1* and *gapc1-1gapc2-2* after 10 min of ABA treatment (Fig. 5B). The change of PA profile in *gapc1-1gapc2-1* and *gapc1-1gapc2-2* was similar as in *pld\(\delta\)* after ABA treatment. 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 (Fig. 4D).

**H\(_2\)O\(_2\) Mediates ABA-induced Stomatal Closure via GAPC-PLD\(\delta\) Interaction**

To determine if GAPC-PLD\(\delta\) interaction was involved in the process of mediating plant response to ROS, we measured stomatal closure in response to ABA and H\(_2\)O\(_2\) in GAPC double mutants and *GAPC1GAPC2PLD\(\delta\)* triple mutants. Stomata were closed in WT after incubation with ABA or H\(_2\)O\(_2\) (Fig. 6A). In contrast, *gapc1-1gapc2-1* and *gapc1-1gapc2-2* were less insensitive to ABA or H\(_2\)O\(_2\)-induced stomatal closure. Like *pld\(\delta\)*, two triple mutants (*gapc1-1gapc2-1pld\(\delta\)* and *gapc1-1gapc2-2pld\(\delta\)*) were significantly less sensitive to ABA and H\(_2\)O\(_2\)-promoted stomatal closure.

To determine how the GAPCs and PLD\(\delta\) interaction in ABA and H\(_2\)O\(_2\) signaling
impacted plant response to water deficits, we evaluated the effect of GAPCs and PLDδ knockouts 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 severe drought stress, respectively (Fig. S6). At 100% FC, pldδ, gapc1-1gapc2-1 and gapc1-1gapc2-2 did not show significant difference from WT in cumulative water transpiration, and photosynthetic rate, but gapc1-1gapc2-1 and gapc1-1gapc2-2 had higher stomatal conductance than WT (Fig. 6B). At 60% FC, pldδ, gapc1-1gapc2-1 and gapc1-1gapc2-2 displayed higher stomatal conductance, higher cumulative water transpiration, and higher photosynthetic rate than WT plants (Fig. 6B). At the severe water deficit (30% FC), stomatal conductance was very low in all genotypes, but pldδ, gapc1-1gapc2-1 and gapc1-1gapc2-2 mutant lines still exhibited the tendency to have more cumulative water transpiration than WT (Fig. 6B).

As the FCs decreased, WT and pldδ, gapc1-1gapc2-1 and gapc1-1gapc2-2 mutants all accumulated less biomass, as that plant growth was inhibited in response to water deficit. pldδ, gapc1-1gapc2-1 and gapc1-1gapc2-2 accumulated more biomass than WT under both mild and severe drought conditions. At 60% FC, the three mutants accumulated approximately 30% more dry matter than WT. The greater biomass in the mutants than WT was consistent with higher stomatal conductance and photosynthetic rate. However, the mutants lost much more water and had lower instant water use efficiency than WT (Fig. S7). 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.

DISCUSSION

The present study demonstrates that PLDδ plays a role in mediating ABA-induced stomatal closure, but it occupies a distinctively different position from PLDα1 in the ABA signaling pathway (Fig. 6C). PLDα1 promotes NADPH oxidase activity and H$_2$O$_2$ production (13), whereas PLDδ mediates H$_2$O$_2$ response but not H$_2$O$_2$ production. Both PLDα1 and PLDδ are activated in response to ABA to generate PA. PA has been shown
to bind to ABI1, NADPH oxidase, and SPHK; all these proteins are involved in the ABA-mediated stomatal closure (13, 25, 27; Fig. 6C). In addition, MAPK might be a target regulated by PA involved in PLDδ-mediated stomatal closure. MAPK has been implicated as a target of PA in various cellular processes such as PCD-induced by H₂O₂ and stomatal closure in response to ABA (15, 28-30). This raises a question of whether PA generated by PLDα1 and PLDδ targets the same or different proteins. The present analyses of PLDδ- and PLDα1-deficient mutants show that PLDα1 produces twice as much PA as does PLDδ in response to ABA, and that PLDδ is the main PLD responsible for H₂O₂-stimulated PA production. Also, temporal comparisons of PA formation in these mutants indicate that PLDα1 is activated earlier than PLDδ. In addition, PLDα1 and PLDδ have different subcellular locations and different substrate preferences. PLDα1 is localized in cytosol and also associates with membrane while PLDδ is localized on plasma membrane (17). It is conceivable that the different magnitude, timing, and location of PA production as affected by PLDα1 and PLDδ impact their product PA interaction with target proteins.

The analyses of GAPC and PLDδ interaction further augment the role of PLDδ and PA in mediating ROS response. The direct interaction between PLDδ and GAPCs has been demonstrated qualitatively and quantitatively using different approaches. H₂O₂ inhibits GAPC activity by oxidizing the catalytic cysteine residues in the enzyme (9). The present results indicate that H₂O₂ promotes the GAPC interaction with PLDδ by decreasing the dissociation constant. Knockouts of GAPCs attenuated the ABA- or H₂O₂-promoted production of PA in the cell, providing in vivo support for the role of GAPCs in the H₂O₂-activation of PLDδ. Plants deficient in GAPCs or PLDδ were less sensitive to ABA-promoted stomatal closure, and had higher transpirational water loss than WT. Without either GAPC or PLDδ, plants are less sensitive to drought-induced growth inhibition. These results provide further support that GAPC-PLDδ interaction increases plant responsiveness to water deficits.

Under the drought conditions tested, the GAPC-deficient or PLDδ-deficient plants actually accumulated more biomass than WT. The data are consistent with the observation that GAPC- or PLDδ-deficient plants have more open stomata and a higher
rate of photosynthesis probably due to more CO₂ uptake than WT. However, the increase in biomass accumulation was at the expense of lower water use efficiency. Decreasing growth under water deficits is one of the key plant strategies for survival. The results indicate that the loss of GAPC or PLDδ compromises plant ability to sense the water stress and to adjust cellular and physiological response accordingly. Indeed, earlier studies showed that knockout of PLDδ decreased plant tolerance to severe stresses such as freezing, UV irradiation, and salt tolerance in Arabidopsis (31-33). Likewise, GAPC-deficit plants decreased plant tolerance to water deficits.

Moreover, the identification of GAPC interaction with PLDδ unveils a new regulatory function of the carbon metabolic enzymes GAPCs and a molecular link between stress signaling and metabolic alteration. Recent studies have shown that some “classical” metabolic enzymes can have crucial regulatory roles in the cell. For example, hexokinase has been found in the nucleus to form a protein complex mediating glucose signaling in yeast and plants (34, 35). In animal cells, GAPDH is involved in non-metabolic processes including gene transcription, DNA replication, nuclear RNA export, endocytosis, microtubule bundling, and oncogenesis (23, 36-38). Oxidized GAPDH is thought to be translocated to the nucleus or interacting with other proteins to regulate cell functions (23, 37). The present study shows that the cytosolic GAPCs interact with the plasma membrane-bound PLDδ and the interaction is promoted by ROS. It has been well documented that ROS are produced in plants under various stress conditions (1, 2). PLD and PA are involved in membrane-based signaling in various plant growth responses (17) whereas GAPDH is a family of glycolytic enzymes involved in providing substrates for energy production and carbons for anabolism (18, 21). We propose that the GAPC-PLDδ interaction in response to ROS provides a molecular link between stress signaling and the alteration of cellular metabolism and growth. 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.

EXPERIMENTAL PROCEDURES
Isolation of Knockouts and \textit{pld\delta} Complementation

\textit{Arabidopsis thaliana} (Col-0) WT and T-DNA insertion lines were obtained from ABRC at Ohio State University. \textit{pld\alpha}1 (SALK_053785) was isolated and confirmed previously (27). Homozygous line of \textit{pld\delta} (SALK_023247) was confirmed by PCR. Homozygous line of \textit{pld\alpha}1\textit{pld\delta} was generated by crossing \textit{pld\alpha}1 and \textit{pld\delta} following by PCR screening (Fig. S1). Four T-DNA lines (\textit{gapc1}, CS328689, SALK_129091; \textit{gapc2}, SALK_016539 and SALK_070902) for GAPC1 and GAPC2 were screened and the homozygous lines were verified by PCR (Fig. S5). The open reading frames (ORF) of \textit{GAPC1} and \textit{GAPC2} share 89.7\% identity while the 3` UTR of both genes are not conserved. Thus, primers in the 3` UTR of \textit{GAPC1} and \textit{GAPC2} were used to distinguish the \textit{GAPC1} and \textit{GAPC2} transcripts. The primers for PCR screening are listed in Table S1.

Plant Growth Conditions and Physiological Analyses

Plants were grown in soil in a growth chamber with cool white light of 160 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) under 12 h light/ 12 h dark and 23\(^{\circ}\)C/19\(^{\circ}\)C cycles. Drought stress was created by a gravimetric approach (39, 40). 10 days old \textit{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 calculated 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 (absolute control) and the other two sets were maintained at 60% (mild stress) and 30% (severe 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 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 first four days after the onset of required stress. At the end of the stress, the shoot was harvested, dried and the weight to recorded to arrive at shoot dry weight.

Stomatal Aperture Measurements
Stomatal aperture was measured according to a described procedure (27). Briefly, epidermal peels were floated in incubation buffer (10 mM KCl, 0.2 mM CaCl₂, 0.1 mM EGTA, 10 mM Mes-KOH, pH 6.15) for 2.5 h under cool white light at 23°C to induce stomatal opening. ABA or H₂O₂ 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 a H₂O₂ dye, 2,7-dichlorofluorescin diacetate (H₂DCF-DA, Sigma-Aldrich) (13). Epidermal peels were floated in incubation buffer for 2 h and then loaded with 50 µM H₂DCF-DA (50 mM stock in DMSO) for 10 min, following with 20 min washing in incubation buffer. 50 µM ABA was added for desired time of treatment. Epidermal peels were observed with a confocal microscope (Zeiss LSM 510) (green fluorescence: excitation 488 nm, emission 525 nm).

**GAPDH Cloning, Protein Purification, and Activity Assay**

The cDNA of GAPC1 and GAPC2 were amplified and ligated to pET-28a-c(+) vector to produce GAPC1 and GAPC2 with 6 histidine residues at the N terminus. The recombinant plasmids were transformed into E. coli BL21(DE3)pLysS. Induction and purification of protein was as described (25). Purified proteins were dialyzed in TBS 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 blue staining. The prepared proteins were used for activity assay or kept in 50% glycerol at -80°C. GAPDH activity assay was done with modification according to the method described previously (19). Fresh leaves were ground to into powder under liquid nitrogen. The powdered material was homogenized with 600 uL of buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM PMSF and 40 mM 2-mercaptoethanol. The homogenate was centrifuged at 12,000g for 20 min at 4°C, and the supernatant was collected. All the enzymes were
assayed spectrophotometrically at 340 nm and room temperature. GAPC activity was measured by following the reduction of NAD\(^+\). The medium contained 50 mM triethanolamine-HCl (pH 8.5), 4 mM NAD\(^+\), 1.2 mM fructose-1,6-biphosphate, 10 mM Na\(_3\)AsO\(_4\), 3 mM DTT and 1 U/ml of aldolase from rabbit muscle. Reactions were initiated by the addition of fructose-1,6-biphosphate, and the rate of increase in the absorbance was linear for at least 3-5 min. Activity increased linearly with increasing enzyme concentration. One unit (U) is defined as the amount of enzyme that catalyzes the formation or consumption of 1 umol min\(^{-1}\) NADPH or NADH under each specified assay condition.

**Protein Co-Pull-down Assays**

GST-PLD\(\delta\) construct and expression of PLD\(\delta\) were described previously (41). To pull down GAPC, purified GST-PLD\(\delta\) agarose beads (~15 µg purified proteins) were incubated with total protein extracted from *E. coli* expressing GAPC1 or GAPC2 at 4°C for 3 h with gentle rotation (42). To pull down PLD\(\delta\), purified GAPC-agarose beads (~10 µg purified proteins) were incubated with total protein extracted from *E. coli* expressing GST-PLD\(\delta\) at 4°C for 3 h with gentle rotation. The beads were collected and washed 3 times and subjected to 10% SDS-PAGE followed by immunoblotting. To coexpress PLD\(\delta\) and GAPC in yeast, PLD\(\delta\) and GAPC1 or GAPC2 were cloned into pESC-HIS vector and transformed into YPH yeast strain (Stratagene). Primers used for cloning were listed in Table S1. PLD\(\delta\) and GAPC1 or GAPC2 were co-expressed in yeast after induction by galactose. Total protein was extracted from harvested yeast and used for co-precipitation analyses.

**Surface Plasmon Resonance Analyses**

SPR binding assays were performed as described (25) with some modifications. The purified proteins were dialyzed in the running buffer (0.01 M HEPES, 0.15 M NaCl, 50 µM EDTA, pH 7.4) overnight at 4°C, and then the proteins were centrifuged at 13,000g to remove insoluble protein before use. For each experiment, the running buffer with 500 µM NiCl\(_2\) was injected to saturate the NTA chip with nickel. His-tagged GAPC1 protein (200 nM) was immobilized on Biacore Sensor Chip NTA via Ni\(^{2+}\)/NTA chelation. PLD\(\delta\)-
GAPC1 interaction was monitored as GST-PLDδ (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. Kinetic constants including $B_{\text{max}}$, association ($k_{\text{on}}$) and dissociation rate ($k_{\text{off}}$) were analyzed using the BIAevaluation Software.

**Bimolecular Fluorescence Complementation**

The BiFC vectors were constructed, described and provided by Walter et al. (26). GAPC1 or GAPC2 cDNA was cloned into pSPYNE vector (GAPC-YFP$^N$) and PLDδ cDNA was cloned into pSPYCE vector (PLDδ-YFP$^C$). 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 MgCl$_2$, and 150 mg ml$^{-1}$ acetosyringone. 3-week-old Nicotiana benthamiana leaves were infiltrated with the mixed bacteria (GAPC-YFP$^N$ and PLDδ-YFP$^N$) solutions (43). YFP fluorescence was examined in tobacco leaves using a Zeiss LSM 510 confocal microscope, with a 488 nm excitation mirror and a 505-530 nm filter to record images.

**Protoplast Preparation and Assaying PLD Activity**

Protoplasts prepared from leaves of 4 week-old plants were incubated in 0.5 mg/mL NBD-PC for 80 min on ice. To determine PLD activity, as affected by ABA treatment at different time points in vivo, 100 µM ABA was added to the NBD-PC-labeled protoplasts, and 100 µL aliquots (1.5×10$^5$ for each assay) were transferred to a new tube at the end of each treatment. 0.4 mL hot isopropanol (75°C) was added, and the mixture incubated for 10 min at 75°C to inactivate PLD. Lipids were extracted with 0.5 mL chloroform:methanol:water (5:5:1). The phases were separated and 100 µL chloroform were added to the aqueous phase, vortexed, centrifuged at 15,000 g for 2 min, and the lower chloroform phases were pooled. Each sample was dried under a nitrogen and 20 µL chloroform:methanol:NH$_4$OH (65:35:5) were added. NBD-PC and NBD-PA were separated by TLC developed in chloroform:methanol: NH$_4$OH (65:35:5) and visualized under UV illumination. The regions corresponding to NBD-PC and NBD-PA were marked and
scraped from the plates. The scraped silica gel was placed in 600 µL chloroform:methanol (2:1), vortexed, and centrifuged for 5 min at 15,000 g. The eluted lipids were quantified by fluorescence spectrophotometry (excitation 460 nm, emission 534 nm). PLDδ activity was assayed according to the procedure described previously (41).

**ESI-MS/MS Analyses of Lipid Molecular Species**

Lipids were extracted and PA analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) (44). Expanded leaves of 4 to 5 week-old plants were sprayed with 100 µM 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°C) immediately after sampling. The experiment was repeated 3 times with 5 replicates of each treatment each time.
REFERENCE


FIGURE LEGENDS

Fig. 1. Decreased response of pldδ plants to H2O2 and ABA

(A) ABA-induced PA production in protoplasts isolated from WT, plda1, pldδ, plda1pldδ and pldδ-COM. The level of PA was calculated as the percentage of NBD-PA produced by PLD over the total NBD-labeled lipids. COM indicates rescue line of pldδ. Values are means ± SE (n = 3). Asterisks indicate that the mean value is significantly different from that of WT under the same condition (control or ABA treatment) at p < 0.05 based on Student’s t test.

(B) Changes in stomatal aperture after ABA (25 µM) or H2O2 (100 µM) treatment. Stomatal aperture was recorded and measured after 2.5 h after treatment under light. Values are means ± SE (n = 50).

(C) Representative image of ROS production in guard cell. –ABA indicates no ABA treatment for epidermal peels. +ABA indicates epidermal peels were treated with 50 µM ABA for 5 min. Epidermal peels were loaded with H2DCFDA for 10 min followed by addition of ABA. Images were taken using confocal microscope. Bar = 100 µm.

(D) Quantification of ROS production based on fluorescence intensity (pixel intensity). Values are means ± SE (n = 25).

Fig. 2. Interaction of GAPC with PLDδ

(A) Immunoblotting of proteins after pulldown. i, reciprocal pulldown between PLDδ and GAPC1. ii, reciprocal pulldown between PLDδ and GAPC2. GST beads were used to purify GST-PLDδ followed by western blot using His tag antibody for detection of GAPC1 or GAPC2. Separately, NTA-nickel beads were used to purify GAPC1 or GAPC2. Then the complex was subjected to western blot using GST antibody to detect GST-PLDδ. This interaction assay was done under different conditions as indicated.

(B) Co-IP of GAPC and PLDδ in yeast. i, co-expression and Co-IP between PLDδ and
GAPC1 in yeast. ii, co-expression and Co-IP between PLDδ and GAPC2 in yeast. PLDδ was fused with FLAG tag and GAPC1or GAPC2 was fused with a cMyc tag. GAPC1 or GAPC2 band in the figure indicates immunoblotting with cMyc antibody against the IP sample immunoprecipitated with FLAG antibody-conjugated agarose beads for PLDδ. PLDδ band in the figure indicates immunoblotting with FLAG antibody against the IP sample immunoprecipitated with cMyc antibody for GAPC1 or GAPC2.

(C) Quantitative SPR analyses of PLDδ binding to GAPC1. GAPC1 (no H₂O₂ treatment or pretreated with 100 µM H₂O₂) was first immobilized on the NTA chip followed by injection of GST or GST-PLDδ. An increase of RU indicates interaction between proteins.

(D) Representative confocal image of BiFC. Green color represents YFP fluorescence, indicating interaction of GAPC with PLDδ. PLDδ-YFPC was co-transformed with GAPC1-YFPN or GAPC2-YFPN into tobacco leaves by infiltration. Bar = 50 µm.

Fig. 3. H₂O₂ effects on GAPDH and PLDδ activities in vitro

(A) Inhibition of the activity of GAPC1 and GAPC2 by H₂O₂. The inhibition was H₂O₂ concentration-dependent. Values are means ± SE (n = 3).

(B) Addition of DTT protected GAPC1 and GAPC2 from oxidation. Different concentrations of DTT were added to GAPC followed by addition of 500 µM H₂O₂. Values are means ± SE (n = 3).

(C) Recovery of GAPC activity by DTT after H₂O₂ treatment. GAPC was pretreated with 500 µM H₂O₂ for 10 min followed by addition of different concentration of DTT. -H₂O₂/-DTT indicates GAPC activity without DTT and H₂O₂. Values are means ± SE (n = 3).

(D) GAPC1 and GAPC2 promoted PLDδ activity under oxidative condition. GAPC activity was assayed under different conditions as indicated. Values are means ± SE (n = 3).
Fig. 4. H$_2$O$_2$ Effect on GAPDH and PLD$\delta$ activities

(A) RT-PCR detection of GAPC1 and GAPC2 expression in the mutants. 18S rRNA was used as positive control confirming successful synthesis of cDNA.

(B) Assay of GAPC activity using the total protein extracted from the leaves of mutants. Asterisks indicate that the mean value is significantly different from that of WT at $p < 0.05$ based on Student’s $t$ test.

(C) GAPDH activity assay using protein extracted from protoplast after 1 mM H$_2$O$_2$ treatment.

(D) Quantification of H$_2$O$_2$-promoted PA production in protoplasts. The level of PA was calculated as the percentage of NBD-PA over the total NBD-labeled lipids. Triple1, gapc1-1gapc2-1pld$\delta$; Triple1, gapc1-1gapc2-2pld$\delta$.

Values in (B), (C) and (D) are means ± SE (n = 3).

Fig. 5. PA content of GAPC and PLD$\delta$ mutant leaves in response to ABA

(A) Changes in total PA content in leaves harvested at different times after spraying with ABA (100 µM).

(B) PA molecular species in leaves of WT and mutants treated with ABA for 10 min. Values in A and B are means ± SE (n = 5). Asterisks indicate that the mean value is significantly different from that of WT at $p < 0.05$ based on Student’s $t$ test.

Fig. 6. Response of GAPC and PLD$\delta$ mutants to ABA and water deficits

(A) Changes in stomatal aperture after ABA or H$_2$O$_2$ treatment. Values are means ± SE (n = 50).

(B) Measurement of stomatal conductance, cumulative water transpiration,
photosynthesis, instant water use efficiency (WUE) and dry weight. Values are means ± SE (n = 16). Asterisks indicate that the mean value is significantly different from that of WT under the same growth condition at P < 0.05 based on Student’s *t* test.

(C) A proposed model for the role of PLD/PA in regulating upstream ROS production and downstream ROS responses in ABA-mediated stomatal closure signaling pathway. This model depicts only the known targets of PLD/PA in the ABA-mediated stomatal closure and other ABA regulators are not included in this model. Blue line indicates inhibition of GAPC by \(\text{H}_2\text{O}_2\). Red arrow indicates that GAPC interacts with PLD\(\delta\) and promotes PLD\(\delta\) activity. Solid arrows indicate established links and dashed arrows denote putative links.

**Supplemental figures:**

**Fig. S1.** Confirmation of homozygous T-DNA insertion PLD mutants by PCR

PCR was conducted using genomic DNA extracted from soil-grown plant leaves with a pair of gene specific primers (PLD\(\alpha\)1RP+PLD\(\alpha\)1LP for *PLDa*1 and PLD\(\delta\)RP+PLD\(\delta\)LP for *PLD\(\delta\)*) or a combination of a T-DNA left border primer (LB\(a\)1) and gene specific primers (PLD\(\alpha\)1RP or PLD\(\delta\)RP). The presence of a T-DNA band and lack of a PLD\(\alpha\)1 or PLD\(\delta\) band indicate that *pld\(\delta\)* and *plda1pld\(\delta\)* are homozygous T-DNA mutants. The primers used for PCR are listed in Table S1.

**Fig. S2.** Expression level of *PLD\(\delta\)* in response to ABA

RNA was extracted from leaves sprayed with 100 \(\mu\)M ABA with 0.01% Triton X-100. *PLD\(\delta\)* transcript level was measured by real-time PCR normalized to *UBQ10*. The ABA response gene *ABI1* was used as a positive control. The experiment was repeated three times with similar results. Values are means ± SE (n = 3) for one representative experiment. The primers for real-time PCR are listed in Table S1.
Fig. S3. Purification and immunoblotting of PLDδ and GAPCs produced in *E. coli* and yeast

(A) Coomassie blue staining of purified PLDδ and GAPCs. PLDδ was expressed in *E. coli* as a GST fusion. GAPC1 and GAPC2 were fused with His tag and expressed in *E.coli*. PLDδ was purified using GST beads and GAPCs were purified using Ni-NTA agarose. Proteins were separated on a 10% SDS-PAGE gel followed by coomassie blue staining.

(B) Immunoblotting of PLDδ and GAPCs expressed in yeast. PLDδ was co-expressed with GAPC1 or GAPC2 in yeast. Total protein (10 µg) extracted from yeast was loaded on a 10% SDS-PAGE gel. PLDδ was immunoblotted with anti-FLAG antibody conjugated with alkaline phosphatase. GAPC1 and GAPC2 were immunoblotted with anti-cMyc antibody conjugated with alkaline phosphatase.

Fig. S4. Negative and positive control for BiFC.

Empty vectors were used as a negative control and did not show YFP fluorescence. The positive control bZIP63-YFP^N^ was co-expressed with bZIP63-YFP^C^. Green color (spot) in the lower panel represents YFP fluorescence, indicating formation of dimers of bZIP63 in the nucleus of plant cells. The constructs were co-transformed into tobacco leaves by infiltration. Photographs were taken with a Zeiss LSM 510 confocal microscope. Bar=50 µm.

Fig. S5. Isolation of GAPC T-DNA homozygous lines

(A) Diagram showing the gene structure with the sites of T-DNA insertion. Two individual T-DNA insertion lines were isolated for GAPC1 and GAPC2, respectively.

(B) PCR genotyping of mutants. All four lines are homozygous mutants as shown of absence of gene specific bands in mutants (LP+RP). PCR was done using genomic DNA and the primers are listed in Table S1.
Fig. S6. Growth phenotype of WT and GAPC and PLDδ mutants under control and drought conditions

Photos were taken at the end of experiment when plants were 6-week old. Three experiments were performed with similar results. D1 represents gapc1-1gapc2-1 and D2 represents gapc1-1gapc2-2.

Fig. S7. Instant water use efficiency of GAPC and PLDδ under 100% and 60% FC

10 days old Arabidopsis seedlings were transplanted to pots and maintained at 100% FC and 60% FC. Instant WUE was calculated as ratio of photosynthetic rate to stomatal conductance, and the measurements were taken on first four days after the onset of required stress. Values are means ± SE (n = 16). Asterisks indicate that the mean value is significantly different from that of WT under the same growth condition at p < 0.05 based on Student’s t test.
## Table S Primer list

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Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

PA (nmol/mg dry weight) vs. ABA treatment (min).

B

PA (nmol/mg dry weight) vs. PA molecular species (total acyl carbons:double bonds).
Figure 6
Supplemental Figure 1
Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4
Supplemental Figure 5
Supplemental Figure 6
Supplemental Figure 7
Chapter 5. Characterization of *Arabidopsis* lacking Cytosolic Glyceraldehyde-3-Phosphate Dehydrogenases and Phospholipase Ds

**ABSTRACT**

Both GAPDH and PLDs are implicated in stress responses. Here we characterized three PLD mutants, *pldα1*, *pldδ* and *pldα1pldδ*, together with *GAPC* mutants. Overexpression (OE) of *GAPC2* and *PLDδ* inhibited plant growth while knockout of *GAPCs* led to accelerated growth of plants to accumulate more biomass and produce more seeds. Knockout of both *GAPCs* increased the salt sensitivity of plants measured by shorter root length on agar plates containing 150 mM NaCl. Knockout of either *PLDα1* or *PLDδ* did not cause significant increase of salt sensitivity, but the double knockout *pldα1pldδ* showed retarded root growth on salt plates. Decreased freezing tolerance was observed for *GAPC* mutants, especially for the homozygous double knockout mutants. This decreased freezing tolerance was similar to PLD mutants, *pldα1*, *pldδ* and *pldα1pldδ*. Oil analyses suggested that knockout of a single *GAPC* did not impact oil content while knockout of both *GAPCs* led to 4-6% decrease in oil content. Profiling of phospholipid and galactolipid suggested that GAPC deficiency caused alterations in lipid content and composition in *Arabidopsis* leaves. These results suggest that GAPC has a limited effect on plant growth and lipid metabolism under normal growth conditions, but GAPC deficiency renders the plant more sensitive to stresses including salt and freezing.

**INTRODUCTION**

GAPDH is a well-known housekeeping enzyme involved in the glycolytic pathways (1). The biochemical functions of GAPDH involved in energy metabolism have been widely studied and understood (1). There are seven phosphorylating GAPDHs in *Arabidopsis thaliana* (3). Cytosolic GAPC1 and GAPC2 are involved in glycolysis, catalyzing the NAD-dependent conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate (4, 5). Recent studies suggest that GAPDH is involved in regulatory processes in animals.
and plants (6-9). In particular, stimuli-induced oxidative stress has been shown to induce the oxidation and inhibition of GAPDH (6, 9). In animals, oxidized GAPDH relocates into the nucleus and participates in regulation of apoptotic process (6). In plants, the cytosolic GAPDH have been studied and described, but no evidence of involvement of GAPC in regulatory processes has yet been shown yet. Previous study in chapter 4 identified that GAPC interacted with PLDδ in stress response, leading to the understanding of the regulatory functions of GAPDH.

GAPDH is a key enzyme in glycolysis, which provides essential metabolites to allow plants to synthesize other compounds (1). Glycolysis is also the fundamental metabolic pathway required by plants to develop and grow. The physiological functions of two plastidial GAPDH genes (GAPCp1 and GAPCp2) have been intensively studied (3, 10, 11). Knockout of both plastidial GAPDHs delayed the root development and the sugar and amino acid balance was altered in *Arabidopsis* lacking of both plastidial GAPDHs (3). The plastidial GAPDH is also critical for viable pollen development in *Arabidopsis*. Deficiency in plastidial glycolytic GAPDH leads to male sterility in *Arabidopsis* because the pollen from homozygous *gapcp1gapcp2* could not germinate (10). *Arabidopsis* deficient in plastidial GAPDH shows insensitivity to abscisic acid (ABA) in growth, stomatal closure and germination (11). Recently, *Arabidopsis* lacking one of the cytosolic GAPDH genes (*GAPC1*) has been characterized (4). The *gapc1* knockout and RNAi lines show delayed growth, changes in morphology of siliques and low seed number (4). These studies suggest that GAPDH is important for plant development and growth. We isolated 4 *GAPC* single knockout lines, 2 *GAPC* double knockout lines and 2 *GAPC1GAPC2PLDδ* knockout lines in previous study. The present study was undertaken to further characterize the effect of GAPC on the growth and metabolism of *Arabidopsis* and determine if GAPC plays a role in the stress tolerance in *Arabidopsis*.

**RESULTS**

**Sequence Analyses of GAPDH in *Arabidopsis***

There are seven putative phosphorylating GAPDH genes in *Arabidopsis* genome (3). Five
are predicted to be localized in plastids including GAPA1 (At1g12900), GAPA2 (At3g26650), GAPB (At1g42970), GAPCp1 (At1g79530) and GAPCp2 (At1g16300). Two are cytosolic GAPC1 (At3g04120) and GAPC2 (At1g13440) which are involved in glycolysis. GAPA and GAPB are the dominant photosynthetic GAPDH isozyme (NAD(P)-dependent), and GAPCp is NAD-specific GAPDH of plastids (3). The nucleotide and amino acid sequences were obtained from Arabidopsis database (http://www.arabidopsis.org). Phylogenetic tree (NJ-tree) of these seven GAPDHs was constructed using DNAMAN software based on their open reading frame (ORF) sequences (Fig. 1A).

The amino acid sequences of Arabidopsis GAPC1 and GAPC2 were compared and showed 97% similarity (Fig. 1B). These two GAPCs are only different in 9 amino acids and have identical number of amino acids (338 amino acids). Arabidopsis GAPC also contains two cysteines residues (red color) which are critical for GAPDH activity (Fig. 1C). Two cysteines residues in mouse GAPDH have been shown to be oxidized by oxidative reagents, resulting in loss of GAPDH activity (12, 13).

**OE of GAPC and PLDδ Leads inhibits Arabidopsis Growth**

To determine GAPC subcellular localization and explore the GAPC functions in Arabidopsis, we generated Arabidopsis transgenic lines that overexpress YFP-GAPC1 and YFP-GAPC2 under the control of the 35S promoter. Production of YFP-GAPC1 and YFP-GAPC2 proteins in OE plants was confirmed by immunoblotting with antibody against the FLAG tag (Fig. 2A). Four YFP-GAPC OE lines for each GAPC were checked and showed that YFP-GAPC1 (3 positive lines) was expressed at a much lower level compared to YFP-GAPC2 (4 lines are all positive) (Fig. 2B). The GAPDH activity was assayed using total proteins extracted from the leaves of OE lines. YFP-GAPC2 OE lines showed significant increase (ranging from 47% to 94%) of GAPDH activity (Fig. 2B). However, YFP-GAPC1 OE lines did not increase the GAPDH activity significantly, which might be due to the low expression level and high GAPDH background activity in plants. The YFP-GAPC2 OE lines were grown with PLDδ OE lines in soil. The plant size of YFP-GAPC2 OE lines and PLDδ OE lines were smaller compared to WT and PLDδ knockout (Fig 2C).
**GAPC1 and GAPC2 Are Both Localized in the Cytosol**

To determine the subcellular localization of GAPC1 and GAPC2, the overexpression (OE) lines were selected for confocal microscope analyses. The leaves had high background level, so we checked the expression of YFP-GAPC1 and YFP-GAPC2 in the root of OE lines. Both GAPC1 and GAPC2 OE lines showed expression of protein, indicated by fluorescence in the root while WT did not show strong fluorescence (Fig. 3A). It was hard to determine the subcellular localization in the root cells due to the large volume of vacuole. Thus transient expression of YFP-GAPC in tobacco leaves was used. The YFP fluorescence (green) and chloroplasts (red) were observed under a confocal microscope. The results indicate that both GAPCs were primarily localized in the cytosol based on the location of chloroplast in the cell (Fig. 3B).

**Knockout of GAPC Increases Plant Growth under Normal Growth Conditions**

GAPC single knockouts (GAPC1 KO: gapc1-1, gapc1-2; GAPC2 KO: gapc2-1, gapc2-2), double knockouts (gapc1-1gapc2-1 and gapc1-1gapc2-2) and GAPC1GAPC2PLDδ triple knockouts (gapc1-1gapc2-1pldδ and gapc1-1gapc2-2pldδ) were isolated to characterize the effect of GAPC on plant growth. Three PLD mutants (pldα1, pldδ and pldα1pldδ) were also included in following studies to determine the physiological functions of GAPC-PLDδ interaction. PLDα1 and PLDδ are two most abundant PLDs in Arabidopsis (14, 15). Results from a NBD-PC-based PLD activity assay showed that knockouts of both PLDα1 and PLDδ resulted in almost a complete loss of PA production (Fig. 4). A previous physiological study suggested that knockout of GAPC led to increases in stomata conductance and biomass (Chapter 4). Soil-grown GAPC mutants under normal conditions (12 h light/ 12 h dark or 16 h light/ 8 h dark and 23°C/19°C cycles) consistently showed increases in plant rosette diameters, and dry weight accumulation of the GAPC mutants compared to WT (Fig. 5). Knockout of PLDδ did not significantly alter the plant growth and morphology under a normal growth condition. However, knockout of PLDα1 showed retarded growth. Especially, knockout of both PLDα1 and PLDδ greatly retarded plant growth as indicated by decreased plant size (diameter) and biomass (dry weight) (Fig. 5B and 5C).
When plants were grown under 16 h light/8 h dark and 23°C/19°C cycles, GAPC-KO mutants also did not display any growth defect. Compared to WT, the GAPC double KOs had higher stalks after flowering (Fig. 6A and 6B). The seed production of pldδ and GAPC double KOs were higher than WT while that in GAPC single KOs were not altered significantly (Fig. 6C). The double loss of PLDα1 and PLDδ (pldα1pldδ) resulted in growth defects as indicated by decreased stalk height and seed yield (Fig. 6C). Collectively, these results suggest that the knockout of GAPCs does not cause growth defects in Arabidopsis under normal growth conditions, and on the contrary, the loss of both GAPCs results in an increase in plant growth and biomass accumulation.

**Knockout of GAPC Compromises Plant Growth under Salt Stress**

To test whether knockout of GAPC alters stress response, we grew the mutant seedlings together with WT and measured the root elongation on ½ MS agar plates with 150 mM NaCl or without NaCl. GAPC mutants displayed longer roots than WT without salt, indicating faster growth of GAPC mutants (Fig. 7A). This is consistent with the observation that soil-grown GAPC mutants had a larger size and accumulated more biomass (Fig. 5). PLD mutants, plda1, pldδ and plda1pldδ, showed no apparent difference in growth compared to WT without salt stress (Fig. 7A). However, plda1pldδ, GAPC double and GAPC1GAPC2PLDδ triple knockouts were more sensitive to salt stress than WT. In the presence of 150 mM NaCl, plda1pldδ, GAPC double and GAPC1GAPC2PLDδ triple knockouts all had shorter root length than WT (Fig. 7B). The GAPC single knockouts and PLD single knockouts displayed no significant difference compared to WT (Fig. 7B). These results suggest that PLDα1, PLDδ, and GAPC are required for normal growth under salt stress.

**Knockout of GAPC and PLDs Decreased Freezing Tolerance**

Previous results suggest that GAPC is involved in the regulation of PLDδ under oxidative stress (Chapter 4). Here we show that plda1, pldδ and plda1pldδ were more sensitive to freezing. -8°C and -10°C caused severe damage to the PLD mutants and freezing virtually killed plda1pldδ at -8°C or -10°C (Fig. 8A). Knockout of either GAPC1 or GAPC2 did not alter the freezing tolerance apparently compared to WT (Fig. 8B). GAPC
double knockouts and \textit{GAPC1GAPC2PLD}δ triple knockouts had increased sensitivity to freezing, as -10°C caused extremely retarded growth for \textit{GAPC} double knockouts and \textit{GAPC1GAPC2PLD}δ triple knockouts, suggesting that \textit{GAPC} is involved in freezing tolerance in \textit{Arabidopsis} (Fig. 8C).

**Double Knockout of \textit{GAPCs} Decreases Seed Oil Content**

Glycolysis is a central metabolic pathway that provides substrates for anabolism such as biosynthesis of fatty acids. Knockout of \textit{GAPC} potentially affects the glycolysis pathway, and thus may alter fatty acid biosynthesis. \textit{GAPC} single knockout seeds had a similar level of oil as WT. However, \textit{GAPC} double knockouts and \textit{GAPC1GAPC2PLD}δ triple knockouts had a significant decrease in oil content (Fig. 9A). \textit{gapc1-1gapc2-1} (\textit{D1}) and \textit{gapc1-1gapc2-2} (\textit{D2}) had a 5.6% and 4.8% decrease of oil compared to WT. Fatty acid composition of \textit{GAPC} double knockouts was also altered. \textit{D1} and \textit{D2} had a higher percentage of C18:1 and other 16C and 18C fatty acids are also intended to be higher. But \textit{D1} and \textit{D2} had a significant decrease in the C20:1 level than WT (Fig. 9B).

**Effect of \textit{GAPC} Knockouts on Membrane Glycerolipid Content and Composition**

Total phospholipid and galactolipid composition and levels in WT and \textit{GAPC} double knockout rosette leaves were analyzed by electrospray ionization mass spectrometry (ESI-MS/MS) (22). Phospholipids include PC, PE, PI, PS, PA and PG (lyso phospholipids were not included here) and galactolipids include MGDG and DGDG. Total lipids content was 6.9% lower in \textit{D1} compared to WT (Fig.10B). PC, PE, MGDG and DGDG content remained the same as WT in \textit{D1} and \textit{D2} (Fig.10A). PI and PS were significantly lower in both \textit{D1} and \textit{D2} while PG was higher in \textit{D1} and \textit{D2}. PA was significantly lower in \textit{D2} but remained the same in \textit{D1} (Fig. 10A). The content of different molecular species of phospholipid and galactolipid were analyzed (Fig. 10C). The level of each species of PE and DGDG was not altered. There is no general trend of changes in levels of various lipid species in each lipid class. The level of several PS species was lower compared to WT. PA species including 34:1 PA, 34:2 PA, 36:2 PA, 36:3 PA, 36:4 PA and 36:5 PA were decreased in \textit{D2} but remained the same in \textit{D1} (Fig. 10C).
DISCUSSION

Although primary metabolism is essential for plant development and growth, we did not observe obvious growth defects in \textit{GAPC} mutants even when both \textit{GAPCs} are absent. This might be due to the compensation effect of other GAPDHs. GAPDH activity is very abundant in plant cells (1). Knockout of both \textit{GAPCs} only caused less than 50\% loss of NAD-dependent GAPDH activity, indicating that other GAPDHs are able to use NADH. It is likely that GAPA and GAPB might be able to compensate the effect of loss of \textit{GAPCs}. Another non-phosphorylating GAPDH (NP-GAPDH) might compensate the loss of \textit{GAPCs}. NP-GAPDH is localized in the cytosol in plants, which is involved in the export of NADPH from the chloroplast to the cytosol. Especially it has been shown that loss of NP-GAPDH induced significant increase of \textit{GAPC} transcripts and enzyme activity (23). Although an earlier study suggests that \textit{GAPC1} is essential for normal fertility in \textit{Arabidopsis} and loss of \textit{GAPC1} caused growth defects (4), our \textit{GAPC} mutants are growing even larger under normal condition. The use of different T-DNA lines is only a partial explanation. However, our present study used two different \textit{GAPC1} mutant alleles that give consistent results. In addition, the results from two \textit{GAPC2} mutant alleles also support our observation.

Interestingly, knockout of \textit{GAPC} increased the biomass of the plants grown in soil under unstressed conditions. \textit{GAPC} interacts with PLD\(\delta\) to mediate ABA-induced stomatal closure, and \textit{GAPC} mutants displayed increased stomata conductance and photosynthesis rate (Chapter 4). \textit{GAPC} mutants might have impaired sensitivity to regulate stomatal aperture, and thus stomata are opened wider to allow more \text{CO}_2 to enter the cell, leading to higher photosynthesis to produce more biomass under a normal growth condition.

\textit{GAPC} deficiency leads to a lower oil content in \textit{Arabidopsis} seeds. These results could mean that \textit{GAPCs} play a role in glycolysis in storage lipid production. Furthermore, the long-chain fatty acid C20:1 is significantly decreased in the seed oil of \textit{GAPC} double mutants. It is possible that knockout of \textit{GAPCs} decreases the cytosolic
acetyl-CoA produced by glycolysis, which is used for fatty acid elongation to make C20:1. GAPC is a key enzyme involved in glycolysis to breakdown glucose to provide energy and precursor for anabolism. Knockout of GAPCs altered the seed oil content, it will be of interest to determine whether it is correlated with shifts in the profiles of metabolites, such as pyruvate and acetyl-CoA, which are important intermediates in energy metabolism and lipid biosynthesis.

GAPC mutants and PLD mutants had decreased tolerance to salt and freezing. Both GAPDH and PLD have been implicated to be involved in different stress responses (5, 24). For example, both are induced by salt, cold, and dehydration (20, 24, 25). These stresses also induce the ROS production and oxidative stress is able to alter the function of GAPC. GAPC might act as an oxidative sensor and it might be involved in the regulation of plant stress tolerance. Salt is one of the stresses which induce ROS (16, 17). Both PLDα1 and PLDδ are implicated in salt tolerance (18, 19). PLDδ is involved in freezing tolerance in Arabidopsis, and knockout of PLDδ decreases while OE of PLDδ enhances freezing tolerance (21). It can be hypothesized that GAPDH interacts with and regulates PLDδ to help the plant respond the stresses.

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Treatments

The mutants used in this study were screened and confirmed as described in previous chapters. Plants were grown in soil in a growth chamber with cool white light of 160 µmol m⁻² s⁻¹ under 12 h light/12 h dark or 16 h light/8 h dark and 23°C/19°C cycles. The root elongation assay was performed on agar plates containing ½ Murashige and Skoog (MS) medium. For root elongation measurements, 4 day-old seedlings were transferred to ½ MS medium with or without NaCl and root lengths were recorded daily.

Subcellular Localization of GAPCs

GAPC1 and GAPC2 cDNA were cloned into p35S-FAST/eYFP, which was derived from p35S-FAST by introducing eYFP. The primers used for cloning and construction of p35S-
FAST/eYFP-GAPC1 are: Forward, 5’- GCG TTAATTAA CATGGCTGACAAGAAGATTAGG-3’ (PacI); Reverse, 5’- GCG GTGCAG TTACACCGGGAAGTACCCAGAAGATTAGG-3’ (SalI). The primers used for p35S-FAST/eYFP-GAPC2 are Forward, 5’- GCG TTAATTAA CATGGCTGACAAGAAGATCAGA-3’ (PacI); Reverse, 5’- GCG GTGCAG TTAGGCCCTTATGACATGTA -3’ (SalI). Agro-infiltration for transient protein expression in tobacco leaves was performed as described previously (26). p35S-FAST/eYFP was transformed as control. The eYFP fluorescence was examined in tobacco leaves using a Zeiss LSM 510 confocal/multi-photon microscope, with a 488 nm excitation mirror and a 505-530 nm and 530-560 nm emission filter to record images. The above eYFP-GAPC constructs were transformed into Arabidopsis to obtain transgenic plants.

**NAD-dependent GAPDH Activity Assay**

GAPDH activity assay was described previously in chapter 4.

**Fluorescence-Based Phospholipase D Activity Assay**

PLD activity assay was performed using NBD-PC as substrate. NBD-PC kept in chloroform was dried under nitrogen gas. Water was added to dried NBD-PC followed by quick sonication to suspend NBD-PC in water. Total proteins were extracted from leaves of 4 week-old plants and protein concentration was determined by Bradford protein assay. PLD activity was assayed under PLDα1 reaction condition or PLDδ reaction condition (hong). Briefly, PLDα1 activity was assayed in the presence of 25 mM Ca²⁺, 100 mM MES, pH 6, 0.5 mM SDS, and 2 mM PC. The PLDδ reaction condition was 100 mM MES, pH 7, 2mM MgCl₂, 80mM KCl, 100 mM CaCl₂, 0.15mM PC, and 0.6 mM oleate. To determine PLD activity, 10 mg total protein was incubated with 50 µg NBD-PC in 30°C water bath with gentle shaking for 15 min. 0.4 mL hot isopropanol (75°C) was added, and the mixture incubated for 10 min at 75°C to inactivate PLD. Lipids were extracted with 0.5 mL chloroform:methanol:water (5:5:1). The phases were separated and 100 µL chloroform were added to the aqueous phase, vortexed, centrifuged at 15,000 g for 2 min, and the lower chloroform phases were pooled. Each sample was dried under a nitrogen and 20 µL chloroform:methanol (95:5) were added. NBD-PC, NBD-PA and
NBD-butanol were separated by TLC developed in chloroform:methanol:NH$_4$OH (65:35:5) and visualized under UV illumination.

**Freezing Tolerance Assay**

Freezing tolerance was tested using soil-grown plants described previously (21). Plants were grown in soil in a growth chamber with cool white light of 160 µmol m$^{-2}$ s$^{-1}$ under 12 h light/12 h dark and 23°C/19°C cycles until 4-week-old. The temperature was set at 4°C in the growth chamber to allow the plants acclimate to the cold for 3 days. After 3 days of cold acclimation, plants were incubated with ice chips at -1°C overnight to allow ice nucleation. The temperature was decreased 1°C/h until reached the desired temperature. Plants were kept at the final temperature for 2 h, then moved to 4°C chamber and kept overnight. Plants were grown under normal condition for 2 weeks after freezing treatment and photographs were recorded.

**Fatty Acid Composition and Oil Content**

Dried *Arabidopsis* seeds (~5 mg/sample) were placed in glass tubes with Teflon-lined screw caps, 1.5 ml 5% (v/v) H$_2$SO$_4$ in MeOH, and 0.2% butylated hydroxyl toluene. The tubes were incubated for 2 h in 90 °C water bath for oil extraction and transmethylation. Fatty acid methyl esters (FAMEs) were extracted with hexane. FAMEs were quantified using gas chromatography supplied with a hydrogen flame ionization detector and a capillary column SUPELCOWAX-10 (30 m; 0.25 mm) with He carrier at 20 ml/min. The oven temperature was maintained at 170°C for 1 min and then increased in steps to 210°C, raising the temperature by 3°C every min. FAMEs from TAG were identified by comparing their retention times with known standards. Heptacanoic acid (17:0) was used as the internal standard to quantify the amounts of individual lipids.

**ESI-MS/MS Analyses of Lipid Content**

Lipids were extracted and analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) (22). Expanded leaves of 4-week-old plants were were excised and immersed in 3 mL of isopropanol with 0.01% butylated hydroxytoluene (preheated to 75°C) immediately after sampling. The experiment was repeated 3 times with 5
replicates.
REFERENCES


FIGURE LEGENDS

FIGURE 1. Sequence Analyses of *Arabidopsis* Phosphorylating GAPDH Genes and Proteins

(A) Phylogenetic tree of the *Arabidopsis* phosphorylating GAPDH genes. There are 7 phosphorylating GAPDH genes in *Arabidopsis*. The tree was built based on the opening reading frame (ORF) sequence. The bootstrap value is indicated on the branches.

(B) Comparison of two GAPC proteins sequence. GAPC1 and GAPC2 share 97% similarity in their deduced amino acid sequences. Both GAPC1 and GAPC2 have 338 amino acids.

(C) Sequence alignment of *Arabidopsis* GAPC1 and mouse GAPDH proteins. Two Cysteine residues are marked red, which are required for GAPDH activity. These two Cysteine residues in mouse GAPDH have been shown to be oxidized by oxidative reagents, leading to inactivation of GAPDH.

FIGURE 2. Effect of Overexpression of GAPC in *Arabidopsis*

(A) Immunoblotting of overexpressed GAPC1 and GAPC2 in *Arabidopsis*. Total proteins were extracted from the leaves of F1 transgenic lines and WT was used as negative control. YFP fluorescent tag and FLAG tag were fused to N-terminus of GAPC1 or GAPC2. 5 µg total protein was separated on 10% SDS-PAGE gel, transferred to membrane and blotted by anti-FLAG antibody.

(B) GAPDH activity assay using total proteins extracted from transgenic lines. Values in are means ± SE (n = 3). Asterisks indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s *t* test.

(C) Morphology of 4-week-old plants grown in soil under 12 h light/12 h dark and 23°C/19°C cycles. Overexpression of GAPC2 and PLDδ leads to slightly smaller plants.
FIGURE 3. Subcellular Localization of GAPC1 and GAPC2

(A) Confocal analyses of the subcellular localization YFP-GAPC1 and YFP-GAPC2 in the roots of overexpression plants (F1). Both GAPC1 and GAPC2 overexpression lines exhibited fluorescence in the root. However, the subcellular localization can’t be concluded. WT plant was used as negative control which had no YFP fluorescence.

(B) Subcellular localization of GAPC1 and GAPC2. YFP was used as positive control. Green color represents YFP fluorescence and red color marks chloroplasts as a reference. The constructs were transiently transformed into tobacco leaves by infiltration.

FIGURE 4. PLD Activity Assay

PLD activity assay using total protein extracted from WT and PLD mutants. NBD-PC was used as substrate which was hydrolyzed to produce NBD-PA and NBD-butanol (if 1-butanol added). The reactions were carried out under PLD$\alpha_1$ and PLD$\delta$ conditions (indicated by $\alpha_1$ and $\delta$). N control indicated that no protein was added in the reaction. Knockout of PLD$\alpha_1$ decreased most PLD activity under both $\alpha_1$ and $\delta$ conditions. Knockout of PLD$\delta$ moderately decreased PLD activity under both conditions. PLD activity was almost completely lost in $pld\alpha_1pld\delta$ double knockout under both conditions.

FIGURE 5. Effects of Knockout of GAPC and PLD on Plant Growth

(A) Knockout of GAPC leads to slightly bigger plants. Plants were grown under 12 h light/12 h dark and 23°C/19°C cycles. Photograph was taken at 6-week.

(B) Measurement of plant size. Values in are means $\pm$ SE ($n = 12$).

(C) Measurement of dry weight of the mutants. The shoots were cut and dried under 105 °C overnight and the dry weight was recorded next morning. Values are means $\pm$ SE ($n = 12$).

Data in B and C were collected when plants were 6-week-old. $D1$ represents homozygous
gapc1-1gapc2-1 double knockout and D2 represents homozygous gapc1-1gapc2-2 double knockout. T1 represents homozygous gapc1-1gapc2-1pldδ triple knockout and T2 represents homozygous gapc1-1gapc2-2pldδ triple knockout. Asterisks in B and C indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s t test.

FIGURE 6. Effects of Knockout of GAPC and PLD on Plant Height and Seed Production

(A) Growth of 8-week-old GAPC knockout plants. Photograph was taken at 8-week.

(B) Measurement of the height of main inflorescence stalks of 8-week-old plants.

(C) Seed production of PLD and GAPC mutants.

Plants were grown under 16 h light/ 8 h dark and 23°C/19°C cycles. The data in B and C were collected when plants were 8-week-old. Values in B and C are means ± SE (n = 12). Asterisks in B and C indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s t test.

FIGURE 7. Effects of Knockout of GAPC and PLD on Salt Tolerance

Root elongation of seedlings grown on ½ MS agar plates without NaCl (A) or with 150 mM NaCl (B). Primary root length was recorded after 10 days after transfer. Knockout of both GAPCs decreased salt tolerance. Values in A and B are means ± SE (n = 15). Asterisks in A and B indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s t test.

FIGURE 8. Decreased Freezing Tolerance in GAPC and PLD Mutants

(A) Decreased freezing tolerance of pldα1, pldδ and pldα1pldδ.

(B) Growth of gapc single knockouts after freezing tolerance. gapc single knockouts
showed slightly decreased freezing tolerance.

(C) Decreased freezing tolerance of GAPC double knockouts and GAPC1GAPC2PLDδ triple knockouts.

4-week-old plants were subjected to freezing treatment at -6°C, -8°C or -10°C. Photographs were taken after plants were grown 2 weeks after freezing under 16 h light/8 h dark and 23°C/19°C cycles.

FIGURE 9. Decreased Oil Content in Double Knockout of GAPCs

(A) Seed oil analyses of GAPC and PLD mutants. Seed oil content was analyzed after seeds were harvested and dried under room temperature for 2 months. Seed oil content was calculated as the percentage of oil over the seed weight. Knockout of one GAPC did not have significant impact on seed oil content while knockout of both GAPC2 significantly decreased the oil content.

(B) Fatty Acid composition of GAPC double knockouts compared to WT. Fatty Acid composition was calculated as mol%.

Values in A and B are means ± SD (n = 3). Asterisks indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s t test.

FIGURE 10. Lipid Profiling of GAPC Double Knockouts

(A) Phospholipid and galactolipid content in leaves of WT and GAPC double knockouts. Phospholipids include PC, PE, PI, PS, PA, PG; galactolipids include MGDG and DGDG. Lipids from leaves of 4 week-old soil-grown plants were quantified by ESI-MS/MS. D1 represents homozygous gapc1-1gapc2-1 double knockout and D2 represents homozygous gapc1-1gapc2-2 double knockout.

(B) Total lipid in WT and GAPC double knockouts leaves. Total lipid represents the total amount of phospholipid and galactolipid.

(C) Lipid species of phospholipid and galactolipid in WT and GAPC double knockouts
leaves.

Values in A, B and C are means ± SE (n = 5). Asterisks indicate that the mean value is significantly different from that of WT at P < 0.05 based on Student’s t test.
Figure 2
Figure 3
1. WT; 2. pldα1; 3. plδ; 4. pldα1plδ

Figure 4
Figure 5

A

WT  gapc1-1  gapc1-1  gapc1-1

gapc1-1  D1  D2

B

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C

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<thead>
<tr>
<th>Treatment</th>
<th>Dry Weight (mg)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
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</tr>
<tr>
<td>plda</td>
<td>0.20 ± 0.03</td>
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<tr>
<td>plda-plda</td>
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<tr>
<td>gapc1-1</td>
<td>0.15 ± 0.02</td>
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<tr>
<td>gapc1-2</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>D1</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>D2</td>
<td>0.13 ± 0.00</td>
</tr>
<tr>
<td>T1</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>T2</td>
<td>0.11 ± 0.00</td>
</tr>
</tbody>
</table>

Figure 5
Figure 6
Figure 7

A

[Graph showing root length (cm) with different conditions and treatments, labeled with WT, pldc-1, pldc-5, pldc-1/pleo, gappc-1-1, gappc-1-2, gappc-2-1, gappc-2-2, D1, D2, T1, T2, and 1/2 MS only.]

B

[Graph showing root length (cm) with 150 mM NaCl conditions, labeled with WT, pldc-1, pldc-5, pldc-1/pleo, gappc-1-1, gappc-1-2, gappc-2-1, gappc-2-2, D1, D2, T1, T2, and 150 mM NaCl.]

Figure 8
Figure 9

A

B

Fatty Acid Composition (mol%)

WT
D1
D2

C16:0  C18:0  C18:1  C18:1  C18:2  C18:3  C20:0  C20:1  C20:2  C22:1

Fatty acid species
Figure 10

![Graph A: Lipid Content (nmol/mg dryweight)]

- PC
- PE
- PI
- PS
- PG
- PA
- DGDG
- MGDG

WT, D1, D2

![Graph B: Total Lipid Content (nmol/mg dryweight)]

WT, D1, D2
Figure 10

C

Lipid content (nmol/mg dry weight)

Lipid molecular species (total acyl carbons:total acyl carbon double bonds)
Chapter 6. Conclusions and Perspectives

The central hypothesis tested in this study was that PA and specific PLDs mediate cell signaling by interacting with other cell signaling components. Specifically, this study characterized the interaction of PLD/PA with: i) sphingosine kinase (SPHK), and ii) cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPC) (Fig. 1). Evidence has been obtained for these interactions and the physiological functions of these interactions have been investigated and determined.

The findings that both PLD/PA and SPHK/phyto-S1P are involved in stomatal closure raise the question whether the two lipid signaling processes interact to mediate plant responses to ABA and stresses. Our study investigated the direct interaction of PA with two Arabidopsis SPHKs. PA binds to both Arabidopsis SPHKs and the interaction stimulates SPHK activity. The identification of SPHK as molecular target of PA indicates that PA may mediate the ABA activation of SPHK in plants (Fig. 1). Indeed, in response to ABA, the LCBP level is lower in plda1. In addition, the application of PA increased the LCBP production in protoplasts. These data are consistent with the hypothesis that SPHK activation by ABA is mediated at least partially by PA. On the other hand, in response to ABA, the PA production in sphk1-1 and sphk2-1 was significantly lower than WT while overexpression of SPHK increased PA production, suggesting that PLDα1 activation depends on SPHK.

To delineate the signaling steps of PLDα1 and SPHKs in the ABA signaling, PA and phyto-S1P were supplemented to the epidermal peels of PLDa1- or SPHK-deficient plants. PA promoted stomatal closure in PLDa1-KO or SPHK-KO leaves, whereas phyto-S1P promoted stomatal closure in SPHK-KO but not in PLDa1-KO. In addition, the addition of 1-butanol, which suppresses PA production by PLD, attenuated the effect of phyto-S1P-caused stomatal closure. These data suggest that phyto-S1P-mediated stomatal closure requires PLDα1, and that SPHK/phyto-S1P acts upstream of PLDα1. Taken together, these results indicate co-dependence of PLD/PA and SPHK/phyto-S1P in the production of PA and phyto-S1P lipid messengers. The interplay between PLDα1 and
SPHK provide a mechanism communicating between the plasma and vacuolar membranes (Fig. 1). The subcellular localization of membrane-based lipid signaling is expected to play an important role in regulation of enzyme activation, generation of lipid messengers and mediation of downstream signals.

The stress hormone ABA is produced under various stresses, such as drought and high salinity. ABA activates PLDα1 to generate PA which binds to NADPH oxidase to produce reactive oxygen species (ROS) (Fig. 1). How ROS mediates downstream effect is not well understood. We identified that GAPCs interacted PLDδ under oxidative stress and this interaction promoted the activity of PLDδ (Fig. 1). Genetic and physiological analyses indicate that both GAPC and PLDδ have positive roles in ABA-mediated signaling pathway. GAPC and PLDδ mutants are less sensitive to ABA-induced stomatal closure. Knockout of both GAPCs abolishes the activation of PLDδ in response to ABA based on enzyme activity assay. Lipid analysis also indicates that PLDδ activation requires GAPCs to produce PA in Arabidopsis. These data suggest that GAPC interacts with PLDδ to mediate ROS signal in response to ABA.

The functional study on GAPC-PLDδ interaction was also carried out and is currently going on using GAPC and PLDδ mutants and overexpression (OE) lines. Although GAPDHs are viewed as key glycolysis enzymes in plants, GAPC mutants are developing normally and do not show growth abnormality. In fact, GAPC mutants grow faster and accumulate more biomass while GAPC OE lines show inhibited growth compared to WT under normal growth condition. Growth of GAPC and PLDδ mutants was tested under different stresses including ABA, salt, freezing, ultraviolet (UV) light and drought. GAPC1GAPC2 double knockouts and GAPC1GAPC2PLDδ triple knockouts had shorter root length compared to WT under salt stress, indicating that these mutants are more sensitive to salt stress. In addition, knockout of both GAPCs renders the plants more sensitive to freezing stress. Freezing caused more damage to PLDδ knockout, GAPC1GAPC2 double knockouts and GAPC1GAPC2PLDδ triple knockouts than WT, suggesting that GAPC might be involved in freezing tolerance mediated by PLDδ. Data in chapter 4 and 5 suggest that GAPC and PLDδ might function together and play
important roles in plant stress responses.

The main conclusions from this research are as follows:

1. Two *SPHK* cDNA sequences are cloned from *Arabidopsis*. Sequence analyses, real-time PCR and mutant analyses suggest that the annotated At4g21540 locus of *Arabidopsis* contains two separate *SPHK* genes. Purified SPHKs from *E.coli* are active and are able to phosphorylate various LCBs.

2. SPHK in *Arabidopsis* is a molecular target of PA. PA binds to SPHK and stimulates SPHK activity. The PA stimulation of SPHK is concentration-dependent, and 50 µM PA gave the best stimulation of SPHK activity.

3. Surface dilution kinetics analyses indicate that PA stimulates SPHK activity by promotion of lipid substrate binding to the catalytic site of the enzyme.

4. *SPHK* KO mutants are less insensitive to ABA in stomatal closure, root elongation and germination, whereas *SPHK* OE lines are more sensitive to ABA. Phyto-S1P promoted stomatal closure in *sphk1-1* and *sphk2-1*, but not in *pldα1*.

5. SPHK/phyto-S1P and PLDα1/PA are co-dependent to mediate ABA signaling. PA is involved in SPHK activation by ABA. Knockout of *PLDα1* decreased the activation of SPHK in response to ABA. Addition of phyto-S1P promotes PLDα1 activity. Knockout of *SPHK* attenuates PLDα1 activation in response to ABA, suggesting that PLDα1 is downstream of SPHK in ABA signaling pathway.

6. PLDα1 and PLDδ function in different steps in the ROS stress pathway. PLDα1 promotes the ROS production, whereas PLDδ mediates plant responses to ROS in *Arabidopsis*. 
7. GAPCs act as a molecular link between ROS and PLDδ activation in stress signaling. GAPCs bind to PLDδ and promote the activity of PLDδ under oxidative stress. This interaction acts as a mediator in ROS signaling pathways. GAPC deficiency mutants displayed decreased sensitivity to ABA-mediated stomatal closure.

8. Knockout of GAPCs does not cause growth defect in *Arabidopsis* under normal growth conditions but the loss of GAPCs lead to decreases in freezing and salt tolerance.

9. The loss of GAPCs results in decreases in seed oil content, changes in fatty acid composition, and alterations in membrane glycerolipid composition, indicating a role of GAPCs in cytosolic lipid metabolism.

**Significance and Perspectives**

Drought stress causes severe damage to crops and leads to yield loss. Water resource is limited to plants and plants need to adapt to the changing environment to cope with drought stress to survive. Guard cells control the water loss and gas exchange in the leaves. Stomata are closed in response to many environment stimuli including water deficiency, light, hormone, and CO₂. Understanding of guard cell signaling pathways could provide solutions for engineering of drought-resistant crops to increase yields. ABA is one of plant hormones that is induced during drought and is essential for induction of stomatal closure to decrease water loss. ABA signaling involves multiple pathways and many regulatory elements. This research was focused on the regulation of ABA signaling by PLD/PA. The study has identified SPHK as a molecular target of PA. SPHK/phyto-S1P and PLD/PA are acting together to regulate ABA-induced stomatal closure (Fig. 1). The results unveil a regulatory process in ABA-mediated stomatal closure, thus providing a new insight for improving plant water usage. ABA-induced activation of PLDα1 requires SPHK, and phyto-S1P produced by SPHK is able to activate PLDα1. How SPHK/phyto-S1P activates PLDα1 remains elusive. Phyto-S1P does not activate PLDα1
directly *in vitro*, indicating that other factors mediate the activation of PLDα1 by phyto-S1P in the cell. There are other LCBP species which have similar structures as phyto-S1P. It should be determined if other LCBPs are also involved in signaling the stomatal closure. While SPHK/phyto-S1P and PLD/PA are important to the signaling ABA action in stomatal closure, it would be of interest to determine if these interactions are involved in regulating other plant processes in growth and stress responses.

In nature, plant growth and development are affected by many other stresses such as salinity, freezing, and high temperature. The molecular mechanisms of how plants respond to different stresses are still not well understood. PLD, which produces PA and a free head group, is involved in plant growth, development, and response to abiotic and biotic stresses. This study identified GAPC as a molecular link of ROS and PLDδ (Fig. 1). The interaction of PLDδ with cytosolic GAPDH is firmly established and this interaction is critical to H2O2 response in *Arabidopsis*. Knockout of GAPC decreased plant’s sensitivity to ABA and also made plant less tolerant to salinity and freezing. It would be worth to investigate if overexpression of GAPC increases plant’s stress tolerance. If so, the information could be applicable to developing crop plants with enhanced stress tolerance and productivity. Further study is also needed to define the specific role of PLDδ involved in mediating stomatal closure. Further characterization of PLD-GAPC interaction will be helpful to explore the possibility that PLD-mediated lipid signaling acts as a molecular link to the oxidative stress and energy metabolism. PLDδ plays important roles in plant tolerance to freezing, drought and salinity. The common feature of these stresses is that they all induce the ROS production. So it is of interest to test if GAPC functions as an oxidative sensor and interacts with PLDδ to facilitate plant to cope with these stresses. GAPC is an energy metabolism enzyme; the effect of the GAPC on energy metabolism and cellular redox status will be further investigated.
Figure 1 Proposed model for the roles of PLDα1, PLDδ, SPHK and GAPC in ABA-mediated signaling pathway. ABA may be perceived by the receptor (PYR/PYL/RCAR) in the cytosol, leading to activation of SPHK to produce phyto-S1P which initiates a cascade to activate PLDα1. PLDα1 hydrolyzes phospholipids to increase PA level in membrane (plasma membrane and tonoplast). PLDα1-deprived PA promotes the ABA effect through three targets: (i) PA binds to ABI1 and tethers ABI1 to the membrane to inhibit its negative effect; (ii) PA stimulates plasma membrane-localized NADPH oxidase to form secondary messenger: ROS; (iii) Increased PA in tonoplast interacts with SPHK and promotes its activity to form a positive loop. Meanwhile, increase of ROS inhibits GAPC, but promotes GAPC binding to PLDδ to activate PLDδ. PLD/PA- and SPHK/phyto-S1P-mediated signaling cascade activates ion channel activity, leading to ion flux in guard cell and finally stomatal closure. Note that this model summarizes the crosstalk between PLDα1/PA and SPHK/phyto-S1P, and the interaction between GAPC and PLDδ in ABA-mediated stomatal closure, not all ABA signaling components are included in this model. Arrow indicates positive regulation, bar indicates repression. Red arrow represents reactions which produce secondary signaling molecules.