Endoplasmic Reticulum– and Golgi-Localized Phospholipase A2 Plays Critical Roles in Arabidopsis Pollen Development and Germination

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The phospholipase A2 (PLA2) superfamily of lipolytic enzymes is involved in a number of essential biological processes, such as inflammation, development, host defense, and signal transduction. Despite the proven involvement of plant PLA2s in many biological functions, including senescence, wounding, elicitor and stress responses, and pathogen defense, relatively little is known about plant PLA2s, and their genes essentially remain uncharacterized. We characterized three of four Arabidopsis thaliana PLA2 paralogs (PLA2a–b, PLA2γ, and PLA2δ) and found that they (1) are expressed during pollen development, (2) localize to the endoplasmic reticulum and/or Golgi, and (3) play critical roles in pollen development and germination and tube growth. The suppression of PLA2 using the RNA interference approach resulted in pollen lethality. The inhibition of pollen germination by pharmacological PLA2 inhibitors was rescued by a lipid signal molecule, lysophosphatidyl ethanolamine. Based on these results, we propose that plant reproduction, in particular, male gametophyte development, requires the activities of the lipid-modifying PLA2s that are conserved in other organisms.

INTRODUCTION

Phospholipase A2 (PLA2) hydrolyzes the phospholipid molecule at the sn-2 position to produce lysophospholipid and a free fatty acid, both of which are precursors for second messengers of signal transduction pathways and also function as signaling molecules per se. The PLA2 superfamily is generally categorized into five principal families of lipolytic enzymes based on their functional, structural, and catalytic properties, namely, the secreted PLA2s, the cytosolic PLA2s, the Ca2+-independent PLA2s, the platelet-activating factor acetylhydrolases, and the lysosomal PLA2s. To date, only two of these PLA2 families have been reported in plants: the low molecular weight secreted PLA2s and the patatin-like PLA2s (similar to the Ca2+-dependent PLA2s but showing both PLA1 and PLA2 activity) (Schalske and Dennis, 2006). The plant PLA2s are involved in such important physiological processes as senescence, wounding, stress responses, pathogen defense, and the induction of secondary metabolite accumulation (Wang, 2001, 2004; Ryu, 2004; Lee et al., 2005; Mansfeld, 2009; Scherer, 2010). Most of the physiological data reported to date have been obtained from studies on the effects of either PLA2 inhibitors or PLA2 products (Scherer and Arnold, 1997; Paul et al., 1998; Suh et al., 1998) or are limited to nonspecific lipid acyl hydrolases, such as the patatin-related PLA (Holk et al., 2002; Viehweger et al., 2002; Rietz et al., 2004; Scherer, 2010). The involvement of PLA2 in phospholipid signaling in plant microtubule organization was reported by Gardiner et al. (2008), who observed that root tips treated with PLA2 inhibitors show anisotropic growth and disorganization of the microtubule arrays. Arabidopsis thaliana PLA2b has also been shown to function in light-induced stomatal opening as well as shoot gravitropism and cell elongation (Lee et al., 2003; Seo et al., 2008). More recently, Lee et al. (2010) demonstrated that PLA2α modulates PIN-FORMED protein trafficking to the plasma membrane (PM) in the Arabidopsis root, thereby revealing that PLA2 also plays a role in intracellular membrane trafficking in plants.

A cell has many curved membrane systems, such as the endoplasmic reticulum (ER), Golgi, endosome, and multivesicular bodies. Membrane deformation, as a process of vesicle trafficking, budding, and fusion, is regulated by the interplay between lipids and proteins, which act like wedges in the membrane. Changes in lipid composition, including modification of the membrane via, for example, phospholipid acylation and/or deacylation, is also a potential mechanism of membrane deformation (McMahon and Gallop, 2005). Brown et al. (2003) proposed that PLA2 hydrolyzes the phospholipids on one side of a membrane, thereby creating a localized concentration of inverted cone–shaped lysophospholipids that in turn drives the formation of positive
PLA2 Roles in Pollen Development

membrane curvature, a process considered to be the first step in membrane tubule formation. This proposal was based on results showing that PLA2 regulates the Golgi complex and membrane tubulation of the trans-Golgi network (de Figueiredo et al., 1998) and modulates membrane-protein trafficking (Choukroun et al., 2000). Staneva et al. (2004) reported a direct role of PLA2 in the vesiculation processes associated with the intermembrane transport through budding and the fission of giant liposomes. More recently, Gubern et al. (2008) reported that the biogenesis of lipid droplets, which function as storage organelles for energy generation and membrane regeneration, is inhibited by treatment with cPLA2 inhibitors or by cPLA2 suppression with small interfering RNA (siRNA). This steadily accumulating body of data suggests the existence of a relationship between PLA2 and the membrane network. However, most of the information currently available on the function of PLA2 is based on studies of mammalian cells, and little is known on its role in the plant membrane network.

The intracellular membrane network in the developing pollen grain is characterized by the extensive proliferation of the ER and surface-linked membrane vesicles that serve as lipid precursors to increase the surface area of the PM during pollen tube germination (Piffanelli et al., 1998). Pollen development is also typified by the diverse transformation of vacuoles and the biogenesis and movement of ER and Golgi bodies. Observations of ultrastructural changes in vacuoles, ER, and Golgi suggest that these organelles are linked to the accumulation of metabolites necessary for pollen development, pollen germination, and tube growth (Hesse, 1990; Bedinger, 1992; McCormick, 1993; Yamamoto et al., 2003). H+ -ATPases have been reported to be activated by the mobilization of the H+ pool and to play key roles in Golgi organization during pollen development (Dettmer et al., 2005). They have also been found to be regulated by lysophosphatidyl choline (LPC), which is generated by PLA2, in suspension-cultured cells of California poppy (Eschscholzia californica) (Viehweger et al., 2002). It has been reported that phosphatidylinositol 4,5-bisphosphate, inositol 1,4,5-triphosphate, and phosphatidic acid (PA), as second messengers produced from membrane phospholipids by phospholipase C (PLC) or phospholipase D (PLD), promote pollen tube growth (Malhó, 1998; Monteiro et al., 2005; Dowd et al., 2006). However, the possible roles of the structurally different PLA2s with PLC and PLD in pollen tube growth remain to be elucidated.

The aim of this study was to establish the roles of members of the Arabidopsis PLA2 gene family in Arabidopsis. We present evidence that three PLA2 paralogs (β, γ, and δ) reside in the ER and/or Golgi, albeit their structural similarity with animal sPLA2s, and that the suppression of PLA2γ, as evidenced by RNA interference (RNAi), induces pollen degeneration. We also provide biochemical evidence that lysophosphatidyl ethanolamine (LPE), a PL2 product, plays a key role in pollen germination and pollen tube growth.

RESULTS

Expression of PLA2-β, -γ, and -δ Increases with Pollen Maturation

Using RT-PCR to compare the expression profiles of all members of the Arabidopsis PLA2 gene family in detail, we detected PLA2-α and PLA2-β transcripts in all of the sporophytic tissues tested. Unlike PLA2-α, PLA2-β was strongly expressed in pollen. The PLA2-δ transcript was detected exclusively in pollen, while the PLA2-γ transcript was expressed in floral tissues, such as the bud, open flower, and pollen (Figure 1A). However, because the bud and open flower samples contained pollen grains inside their anther sac or on the surface of the stigma, we considered the PCR amplifications of PLA2-γ to originate from pollen in the bud and open flower. Due to its lower expression level, the same number of PCR cycles used to detect PLA2-γ did not produce enough amplified product of PLA2-δ to be visible on electrophoresis. However, PLA2-δ was also detected when more PCR cycles were performed. As shown in the promoter:β-glucuronidase (GUS) analysis (Figures 1C and 1E), PLA2-γ was expressed only in pollen grains, as was PLA2-δ. In a subsequent RT-PCR analysis (Figure 1B), we used cDNAs enriched for three different pollen developmental stages (microspore, bicellular, and tricellular stages) to determine the precise pollen stages at which PLA2 genes are expressed. PLA2-α expression was not detected in pollen at any of these developmental stages, while PLA2-β was expressed continuously during all stages. PLA2-γ expression was slightly at the microspore and bicellular stages, but strongly at the tricellular stage and in mature pollen. PLA2-δ expression was found to be initiated in the tricellular stage (Figure 1B). These results indicate that the expression of PLA2-δ is relatively low during the early stages of pollen development and subsequently increased considerably at the tricellular stage. There was no difference in the expression pattern of the PLA2-δ in dehydrated and hydrated pollen grains (see Supplemental Figure 1 online).

Lee et al. (2003) reported that the PLA2-β promoter drives widespread GUS expression in all plant tissues. We found that while the PLA2-γ and -δ promoters drove GUS expression exclusively in the pollen (Figures 1C and 1E), the GUS expression patterns of all three promoters during flower development were similar in that expression was initially weak at the early developmental stages and gradually increased as the floral organs matured. Staining with 4',6-diamidino-2-phenylindole confirmed that the expression of PLA2-β, -γ, and -δ was low at the early stages of pollen development and increased as the pollen matured (Figure 1G). GUS signals remained strong in the germinated pollen and pollen tube (Figures 1D and 1F).

Suppression of PLA2s in Pollen

If PLA2s are essential for pollen development and pollen germination and tube growth, normal pollen development should be impeded by any alteration in their expression. To test this hypothesis, we searched for T-DNA–tagged knockout (KO) mutants and found SALK_022347.48.40 (designated as pla2-γ-1) and SALK_03172.37.00 (designated as pla2-γ-2) for PLA2-γ but none for PLA2-β and -δ. The pla2-γ-1 and the pla2-γ-2 mutants contain a T-DNA in the second exon and third intron of the PLA2-γ, respectively. RT-PCR using PLA2-γ–specific primers confirmed that the pla2-γ-1 and pla2-γ-2 homozygous lines are null mutants (see Supplemental Figure 2 online) and that the other PLA2 isoforms are expressed normally. In addition, the pollen grains of these KO plants did not show any phenotypic
differences compared with the wild type. Given their high degrees of similarity at the amino acid level and their strong expression in pollen, we presume that PLA2-β, -γ, and -δ have a functional redundancy during pollen development, even though they individually may play different roles at several pollen developmental stages.

To suppress the expression of all PLA2 genes in pollen simultaneously, we used the RNAi approach with two different pollen-specific promoters, LATE (Twel et al., 1989) and PLA2-δ. A 576-bp full open reading frame (ORF) from ATG to TAG of PLA2-δ was used as a trigger of RNAi (E-values of estimating similarities: 1e−120, 5e−19, and 4e−04 with PLA2-γ, PLA2-β, and
PLAγ-α, respectively) and cloned into the pHANNIBAL vector in both the sense and antisense orientations under the control of the Lat52 promoter (designated as LPRNAi) and the PLAγ-δ promoter (designated as DPRNAi), and then subcloned into the pART27 vector (Figure 2A). These constructs were transformed into wild-type and plaγ-γ-1 homozygous plants. The normal vegetative tissues and defective male gametophyte of LPRNAi-or DPRNAi-introduced wild-type plants were not phenotypically different from those of plaγ-γ-1 containing LPRNAi or DPRNAi constructs. Consequently, the DPRNAi lines were used in subsequent analyses. Of the 60 DPRNAi lines generated, six lines showing a severely sterile phenotype with highly reduced numbers of seeds in shorter siliques compared with the wild type were chosen for study (Figures 2B and 2C). The anthers of the

Figure 2. Phenotypic Analysis of the RNAi Mutants.

(A) The structure of RNAi expression cassettes.
(B) to (E) The sterile phenotype of the RNAi mutant. Short silique (C) and reduced seed setting phenotype (D) and (E) of the RNAi mutant. Data show means ± SD (n = 60) in (E). WT, wild type. Bars = 5 mm.
(F) RNA gel blot analysis of siRNA lines. Samples from DPRNAi transformants of the T3 generation and plaγ/DPRNAi transformants of the T2 generation are listed at the top. The wild type was used as a negative control. Bottom panel shows the rRNA stained with ethidium bromide as a loading control.
(G) Relative amount of transcripts of PLAγ-β, -γ, and -δ in wild-type and RNAi plants. Three independent real-time PCR reactions were performed per sample. Data show means ± SD (n = 3).
RNAi plants dehisced normally, but most siliques failed to set any seeds, although partially filled siliques with <10 seeds were occasionally produced (Figures 2D and 2E). To confirm whether this sterility was caused by the suppression of PLA2s in pollen by the RNAi approach, we performed RNA gel blot analysis to detect siRNA using total RNAs isolated from flowers. siRNA was detected in both DPRNAi and pla2γ/DPRNAi transgenic plants but not in wild-type plants (Figure 2F). The real-time PCR analysis using RNAs extracted from pollen also showed that the transcript levels of the three PLA2 genes, PLA2γ, β, and ε, were reduced (Figure 2G).

According to Xing and Zachgo (2007), however, at least 10% of RNAi transgenic Arabidopsis plants, regardless of the construct types, produce 20 to ~50% nonviable pollen in an anther as a side effect. We obtained 60 T1 lines from LPRNAi and DPRNAi transgenic plants that survived on kanamycin (kanamycin resistance conferred by the RNAi vector), of which six (10% of transgenic population) showed severe sterility, as shown in Figure 2. Around 80% of the seed sterility in these six RNAi mutants possibly originated from the direct effect of RNAi (50%) combined with the side effects of RNAi on pollen (20 to ~50%). Approximately 50% of 54 transgenic lines were fertile but with half of their pollen grains collapsed. To verify that the observed pollen disorder was caused by RNAi, we performed RNAi silencing of PLA2s in the quartet mutant, in which pollen grains remain in permanent tetrads (Preuss et al., 1994), and also observed that 50% of the pollen grains were aborted (Figures 3A and 3B). We produced and analyzed 10 T1 RNAi transgenic lines in the quartet background. Of these, two produced normal pollen and eight produced defective pollen. Among the eight lines with the defective pollen phenotype, 50% of the pollen was aborted in four lines, 75% in two lines, and <30% in two lines. Although the RNAi transgenic lines in the quartet background were not analyzed as extensively as the RNAi transgenic lines in the wild-type (or pla2γ) background, the defective pollen ratio was consistent, demonstrating that the pollen was aborted due to the suppression of PLA2s in the pollen grain. To ascertain whether or not pollen abortion causes a reduction of PLA2 expression in the RNAi lines, we performed quantitative RT-PCR in the RNAi lines using primers of other pollen-specific genes, such as VAN-GUARD1 (VGD1) (Jiang et al., 2005) and sugar transporter (STP11) (Schneideret et al., 2005). As shown in Supplemental Figure 3 online, these two genes were not downregulated in the RNAi lines. Taken together, these results demonstrate that RNAi suppression of all PLA2 members in the pollen was successful.

Abnormal Pollen Development Is Caused by Disordered Cellular Membranes

As pollen-specific promoters (Lat52 and PLA2γ-δ) were used for RNAi construction, the siRNAs are likely to be produced during pollen development; consequently, vegetative growth would be expected to be normal. This expectation is supported by the normal morphologies found in the RNAi plants. To determine the effect of RNAi suppression of all Arabidopsis PLA2 genes on pollen, we first compared the mature pollen grains of the wild-type and RNAi lines using a scanning electron microscope.

Figure 3. Scanning Electron Microscopy and Semithin Section Images of RNAi Pollen.

(A) and (B) Bright-field tetrad analysis showing qrt/qrt (A) and qrt/qrt transformed with DPRNAi (B) tetrads. Bars = 20 μm. (C) and (D) Scanning electron microscopy images of wild-type (C) and RNAi (D) pollen grains. The arrow indicates the partially shrunk pollen grain, and the arrowheads indicate the completely collapsed pollen grains. Bars = 10 μm. (E) to (J) Semithin section images of wild-type [(E) to (G)] and RNAi [(H) to (J)] anthers at different developmental stages. Obvious phenotypic alterations were not detected at anther stage 8 [(E) and (H)] or anther stage 9 [(F) and (I)]. At anther stage 12 [(G) and (J)], completely collapsed pollen grains were observed in the RNAi anthers. Arrowheads indicate completely collapsed pollen grains. The developmental stage of the anther is according to Sanders et al. (1999). Bars = 10 μm.

Compared with the normal pollen of the wild type (Figure 3C), the pollen of the DPRNAi 4-4 line with a 66% pollen abortion rate was partially shrunk (Figure 3D, arrow) or completely collapsed (Figure 3D, arrowhead). An examination of semithin transverse sections of anthers at different developmental stages (Figures 3E
to 3J) revealed that pollen grains of the RNAi lines at the early microspore stage (i.e., anther stage 8; Sanders et al., 1999) were not phenotypically different (Figure 3H) from those of the wild type (Figure 3E). The first signs of abnormal pollen development in the DPRNAi 1-1 line, which had a pollen abortion rate of 67%, such as the detachment of the PM from the cell wall and shrinking cytoplasm, appeared at the vacuolated late uninucleate microspore stage (i.e., anther stage 9; Sanders et al., 1999; Figures 3F and 3I). By the mature pollen stage (i.e., anther stage 12; Sanders et al., 1999), RNAi pollen grains had collapsed completely and were devoid of cytoplasmic content (Figures 3G and 3J), although the exine layer and all other structures, both inside and outside the anthers, were not different from those in wild-type pollen. As depicted in Table 1, aborted pollen ratios were very similar at the bicellular and tricellular pollen stages (52 to ~72%).

The ultrastructure of the pollen grains was observed by transmission electron microscopy (TEM) to determine the underlying factors causing pollen degeneration. In accordance with the observations of Yamamoto et al. (2003), we observed that the wild-type microspores contained a round nucleus, a distinct nucleolus and nuclear membrane, and ribosome binding rough ER (Figures 4A, 4C, and 4E). By contrast, the nucleus of the pollen grain of DPRNAi 4-4 line was irregular in shape with an indistinct membrane, and the ribosomes were scattered around the nucleus membrane (Figures 4B and 4D, arrowheads). Moreover, the RNAi pollen grains failed to form a defined endomembrane system; this was particularly evident for the ER, which had an irregular shape and a fragmented membrane (Figures 4D, arrows). The ribosomes in RNAi pollen were observed to be scattered throughout the shrinking cytoplasm, possibly due to the lack of a defined ER structure and ER fragmentation. While typical Golgi stacks, consisting of five to eight flat cisternae, were observed in the wild-type pollen (Figure 4E), irregular Golgi stacks with a decreased number of cisternae were observed in the RNAi pollen (Figure 4F). Also in the RNAi pollen, a number of small vesicles were observed in the region surrounding Golgi stacks (Figure 4F, asterisks), which suggested that ONO (2-[p-amylicinamoyl]amino-4-chlorobenzoic acid), a PLA2 inhibitor, had induced some degree of disintegration of the Golgi stacks and the formation of numerous vesicles in the trans-Golgi face (Lee et al., 2010). Finally, the PM in the RNAi pollen was detached from the cell wall, and the electron density of the contents of the shrinking cytoplasm was higher than that of the wild type (Figures 4A and 4B).

Table 1. Aborted Pollen Percentages in RNAi Lines

| Line               | Bicellular | Tricellular |
|--------------------|------------|-------------|
| DPRNAi (1-1)       | 72% (n = 110) | 67% (n = 130) |
| DPRNAi (4-4)       | 69% (n = 105) | 66% (n = 123) |
| pla2-γ-1/DPRNAi (2-2) | 54% (n = 118) | 58% (n = 238) |
| pla2-γ-1/DPRNAi (4-4) | 57% (n = 95) | 52% (n = 98) |

Completely collapsed pollen grains were counted. n indicates total counted pollen grains.

Figure 4. TEM of RNAi Pollen.
(A), (C), and (E) Wild-type pollen at the vacuolated microspore stage.
(B), (D), and (F) DPRNAi 4-4 line pollen at the vacuolated microspore stage.
The arrowheads indicate the nucleus membrane. Arrows indicate the ER membrane. Asterisks in (F) indicate large vesicles. N, nucleus; Nu, nucleolus; V, vacuole; M, mitochondrion; P, plastid; G, Golgi apparatus. Bars = 1 μm in (A), 2 μm in (B), 500 nm in (C) and (D), and 200 nm in (E) and (F).

PLA2-γ Localizes to the ER and Golgi, and PLA2-δ to the ER

Our results to this point suggest the possibility that the pollen-expressing PLA2-β, -γ, and -δ play a critical role in pollen development. Seo et al. (2008) reported that PLA2-β localizes to the ER in guard cells and is involved in stomatal opening. Based on our results, as shown in Figure 4, it is possible to conjecture that PLA2-γ and -δ also localize to the ER and/or Golgi and act on the function of membrane deformation and trafficking of these organelles. However, earlier experiments involving the transient expression of green fluorescent protein (GFP)–tagged Arabidopsis PLA2 proteins in onion epidermal cells demonstrated that PLA2-β and -γ are secreted to the cell wall/
During our study, we fused PLA2-γ and -δ with YFP under the control of the PLA2 promoter and then smRS-GFP (Davis and Vierstra, 1998) under the control of ProLat52:PLA2-γ-GFP in Arabidopsis thaliana (Figure 7A). In comparison, the GFP signal of the pollen and pollen tube (Nicotiana tabacum) leaf epidermal cells by Agrobacterium tumefaciens infiltration. Transient expression of the fluorescence of each fusion protein was observed by confocal laser scanning microscopy. STtmd: cyan fluorescent protein (CFP) (a Golgi marker; Figures 5A and 6A), ERD2:GFP (an ER and Golgi marker; Figures 5B and 6B), and GFP-HDEL (an ER marker; Figures 5C and 6C) (Boevink et al., 1998; Sparkes et al., 2006) were used to confirm the localization of PLA2-γ and -δ. Incorporation of the PLA2-γ:YFP construct into the tobacco leaf epidermal cells produced fluorescent signals in the ER network as well as small punctate signals that completely matched with the signal of the Golgi (Figures 5A to 5C), while the PLA2-δ:YFP-expressing cells produced fluorescence in the ER network alone (Figures 6A to 6C). To determine whether colocalization had occurred, we quantitatively analyzed the extent of signal intensity from each fluorophore detected in shared pixels (Figures 5D and 6D). Seventy-two percent of the STtmd:CFP signal overlapped with that of PLA2-γ:YFP, and 16% of the PLA2-γ:YFP signal overlapped with that of STtmd:CFP in the full image, but the signals were found to significantly overlap each other (100 and 86%, respectively) in the region containing small punctate signals (region 1 in Figure 5A). This result indicates that PLA2-γ localized to the trans-Golgi. ERD2:GFP and PLA2-γ:YFP signals overlapped with each other (92 and 75%, respectively) in full image and overlapped (92 and 90%, respectively) in the region containing small punctate signals and networks (region 2 in Figure 5B), demonstrating that PLA2-γ localized to cis-Golgi and ER. GFP-HDEL and PLA2-γ:YFP signals overlapped with each other (87 and 66%, respectively) in the full image, but their degree of overlap (1.2 and 2.3%, respectively) was less in the region containing small punctate signals (region 3 in Figure 5C). This result can be considered to be reliable as GFP-HDEL is only visible as white or bleached red signals for CFP/YFP and as orange-yellow signals for GFP/YFP, respectively. Bars = 10 μm.

Figure 5. Subcellular Localization of PLA2-γ in the Tobacco Epidermal Cell.

Confocal laser scanning microscopy images of single planes. Left panels indicate CFP (A) and GFP (B) and (C); middle panels indicate YFP (A) to (C); right panels are merged signals (A) to (C). Colocalizations are visible as white or bleached red signals for CFP/YFP and as orange-yellow signals for GFP/YFP, respectively. Bars = 10 μm.

(A) STtmd:CFP and PLA2-γ:YFP.
(B) ERD2:GFP and PLA2-γ:YFP.
(C) GFP-HDEL and PLA2-γ:YFP.
(D) Quantitative analysis of the distribution of PLA2-γ relative to Golgi and/or ER markers. The percentage of signal intensity present in the shared pixels (middle; white) relative to the total signal is measured for both the gray (top; PLA2-γ:YFP) and black (bottom; STtmd:CFP, ERD2:GFP, and GFP-HDEL) channels. Gray and black values are denoted as the difference between 100% and the overlap measured for the corresponding channel. The extent of colocalization signal intensity is quantified in the full image as well as in region 1～3 marked in (A) to (C), respectively.

To further confirm the localization of PLA2-γ and -δ in pollen, we fused PLA2-γ and -δ with a soluble-modified red-shifted GFP (smRS-GFP) (Davis and Vierstra, 1998) under the control of Lat52 and with YFP under the control of the PLA2 promoter and then transformed these four constructs into tobacco and Arabidopsis. The GFP signal of ProLat52:smRS-GFP (control) was detected evenly throughout the cytosol and nuclei in the tobacco pollen grain and tube (Figure 7A). In comparison, the GFP signal of the ProLat52:PLA2-γ:smRS-GFP tobacco pollen and pollen tube was observed in a spindle-shape membrane boundary structure, which is considered to be the ER fragment (Cheung et al., 2002),
and in small punctate structures, namely, the Golgi (Figure 7B, arrows). The GFP signal of ProLat52:PLA2-d:smRS-GFP pollen was similar to that of ProLat52:PLA2-y:smRS-GFP pollen, but there was no fluorescence in the Golgi in the former (Figure 7C). These results were identical when the PLA2-y and PLA2-d promoters were used in Arabidopsis (Figures 7D and 7E), leading to the conclusion that PLA2-d localizes to the ER and PLA2-y to both the Golgi and ER. These results, together with those of Seo et al. (2008), which show that PLA2-b, -y, and -d have specific roles in the ER and/or Golgi.

**Pollen Germination Is Inhibited by Treatment with PLA2 Inhibitors**

The patterns of GUS expression driven by the promoters of PLA2s in the germinating pollen and pollen tube (Figures 1D and 1F) suggest that PLA2 enzymes are active during pollen germination and tube growth. This led to the question of whether the observed incomplete pollen germination and/or tube growth could ultimately result in the sterility observed in the RNAi lines. However, direct testing of the involvement of PLA2 enzymes in pollen germination and tube growth in the RNAi pollen was not possible due to (1) RNAi suppression of PLA2s being initiated in the unicellular stage, and (2) complete collapse of the pollen grains before the mature pollen stage. Adopting an alternative approach, we treated wild-type Arabidopsis and tobacco pollen grains with seven different PLA2 inhibitors, manoalide, 4-bromo-phenacetyl bromide (BPB), aristolochic acid (AA), trifluoromethyl ketone (AACOCF3), palmitoyl trifluoromethyl ketone (PACOCF3), bromoenol lactone (BEL), and ONO, and observed their effects on pollen germination. Because various conditions, such as pH, temperature, pollen density, plant age, and flower stage, can greatly affect in vitro pollen germination in Arabidopsis, the bicellular-type tobacco pollen, which is known to be more consistent in terms of in vitro germination (Brewbaker, 1967; Boavida and McCormick, 2007), was used in an initial test to select the most effective PLA2 inhibitors. Manoalide was found to be the most effective inhibitor, with AACOCF3, PACOCF3, and AA having no inhibitory effect at all on tobacco pollen germination. BEL, ONO, and BPB also had no inhibitory effect on pollen germination at concentrations of <10 μM and only a slight inhibitory effect at higher concentrations (see Supplemental Figure 4 online). We treated the recombinant PLA2-b, -y, and -d proteins with manoalide to test for an in vitro enzyme inhibition effect. Manoalide effectively inhibited PLA2-y and -d activities and slightly inhibited PLA2-b activity (Figure 8A). AA has been reported to have an inhibitory effect at the biochemical level on recombinant PLA2-b activity as well as a physiological effect on stomatal opening and root growth (Gardiner et al., 2008; Seo et al., 2008); however, it did not have an inhibitory effect on pollen germination in our experiment. Thus, we focused on analyzing the effect of AA on in vitro PLA2-y, -y, and -d enzyme activities. AA inhibited PLA2-y but not -y and -d (see Supplemental Figure 5 online), which supports our hypothesis that PLA2-y and -d have specific functions in pollen and important roles in pollen germination. The fact that several inhibitors have different effects on the different PLA2 isoforms also suggests that these PLA2s have different mechanisms at the cellular level. Manoalide was chosen
The inhibitory effect of manoalide on the activity of recombinant PLA2-β, -γ, and -δ protein. The enzymatic activity was measured using radiolabeled 1-palmitoyl-2-linoleoyl-14C-phosphatidylethanolamine as substrate in the presence or absence of manoalide (10 μM). Data are means ± SD (n = 4).

(B) The inhibitory effect of manoalide on in vitro pollen germination. Tobacco pollen grains were treated with 3, 5, and 10 μM manoalide for 3 h. Data are means ± SD (n = 500).

(C) to (F) Pollen germination 12 h after manoalide treatment. (C) No manoalide. (D) Three micromoles of manoalide. Pollen germination was slightly inhibited. (E) Five micromoles of manoalide. Pollen germination was completely inhibited. (F) Ten micromoles of manoalide. Pollen germination was completely inhibited. Bars = 200 μm.
The PL$	ext{A}_2$ Inhibitor Manoalide Interferes with VHA-c4 Localization

It is known that V-ATPases are the key regulators of membrane trafficking as well as energizers of secondary active transport. Based on the results of experiments involving concanamycin A, a specific V-ATPase inhibitor, Dettmer et al. (2006) reported that V-ATPase in the trans-Golgi network is essential for endocytic and secretory trafficking. Concanamycin A affects Golgi morphology, such as the bending of Golgi cisternae, swelling of Golgi ends, fragmentation of Golgi stacks, and accumulation of large vesicles. Furthermore, plant V-ATPase has a critical function in the development of the male gametophyte (Dettmer et al., 2005).

In a yeast two-hybrid experiment in which we screened for interactors of PL$	ext{A}_2$-γ and -δ, we found that PL$	ext{A}_2$-γ and -δ interacted with VHA-c4, a subunit of the V-ATPase complex (see Supplemental Figure 6 online). Because PL$	ext{A}_2$ products are known to activate V-ATPases (Palmgren and Sommarin, 1989; Pedchenko et al., 1990; Viehweger et al., 2002), the binding of these two proteins can provide a clue to the function of both PL$	ext{A}_2$ and VHA-c4.

To obtain more direct and reliable evidence for the link between PL$	ext{A}_2$ and VHA-c4, we designed an experiment to monitor changes in the localization of VHA-c4 in pollen caused by the PL$	ext{A}_2$ inhibitor. We generated transgenic Arabidopsis plants that expressed VHA-c4:smRS-GFP under the control of the Late52 promoter. The shape of VHA-c4:smRS-GFP in the apex of pollen tubes revealed a typical ER morphology, as described previously (Lový-Wheeler et al., 2007), suggesting that VHA-c4 localizes to the ER. To obtain evidence supporting the localization of VHA-c4 to the ER, we performed a transient assay using VHA-c4:YFP with the ER marker in tobacco epidermal cells and generated the Pro35S::VHA-c4:YFP transgenic Arabidopsis lines. We found that VHA-c4:YFP colocalized with ERD2::GFP to the ER network of the tobacco epidermal cells but not to the Golgi vesicles (see Supplemental Figure 7A online). The VHA-c4:YFP signal localized to the ER networks, ER body membrane, and small vesicles (see Supplemental Figure 7C online). The morphology of the organelles was similar to those in the hypocotyl cells of the GFP-HDEL-expressed line (see Supplemental Figure 7B online).

Based on the interaction of VHA-c4 with PL$	ext{A}_2$-δ in our yeast two-hybrid assay, we concluded that VHA-c4 colocalized with PL$	ext{A}_2$-δ in Arabidopsis. Transgenic lines coexpressing VHA-c4:CFP and PL$	ext{A}_2$-δ:YFP showed that these two signals colocalized to the ER network and ER bodies (see Supplemental Figure 7D online). The VHA-c4:CFP signal was particularly strong at the ER body membrane. Taken together, these results strongly suggest that VHA-c4 mainly localizes to the ER, as do other subunits, such as VHA-c’’ and VHA-e2 (Seidel et al., 2008). Pollen grains obtained from ProLat52::VHA-c4:smRS-GFP transgenic Arabidopsis were germinated for 1 h, and the pollen tubes were subsequently stained with the ER tracker as an ER marker or FM4-64 as an endocytic tracer. In the pollen tube that was not subjected to manoalide treatment, the VHA-c4:smRS-GFP fusion protein colocalized with the ER tracker but not with FM4-64 (Figure 10A; see Supplemental Figures 8A and 8B online). Even although VHA-c4:smRS GFP did not colocalize with FM4-64, we did not rule out the possibility that VHA-c4:smRS GFP can localize to endosomal structures. We believed that this phenomenon is very similar to the previous report on ARA6, which is broadly used as a marker for endosomes. When pollen tubes were incubated with BFA, ARA6 was not correlated with the aggregation of FM4-64 because of the heterogeneity of the endosomal populations (Zhang et al., 2010).

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One hour after treatment with manoalide, VHA-c4:smRS-GFP signals and FM4-64 signals were significantly aggregated in the cytosol, and the structural changes were visible using normal light microscopy. FM4-64 signals in the PM faded 1 h after treatment with manoalide (Figure 10C), similarly to the previous reports (Liao et al., 2010; Zhang et al., 2010). This phenomenon may be explained by two possibilities. One is that the synthesis of the PM is inhibited in the growing pollen tube, due to blocking of both endosome-to-PM recycling and ER-Golgi-vesicle membrane trafficking by the PLA2 inhibitor. This possibility is supported by a previous report that PLA2-α is required for PIN protein trafficking of the PM in the Arabidopsis root (Lee et al., 2010). Another possibility is that the blocking of membrane trafficking may provoke cell degeneration and eventually induce the breakdown of PM. This is supported by our data that show that in a TEM image of RNAi at the bicellular stage, the bilayer structure was indistinguishable and appeared to be disassembled (see Supplemental Figure 9 online) and that pollen grains of the DPRNAi 1-1 line were completely collapsed and devoid of cytoplasmic content at the tricellular stage (Figure 3J).

To confirm the specificity of the PLA2 inhibitor manoalide on VHA-c4 trafficking, we exposed growing pollen tubes to two well-characterized pharmacological inhibitors, U-73122 and 1-butanol. The PLC inhibitor U-73122 inhibits and depolarizes pollen tube growth (Helling et al., 2006), and the PLD inhibitor 1-butanol blocks both pollen tube growth and germination (Potocký et al., 2003). In our experiment, pollen tubes treated with 1-butanol stopped growing, but the VHA-c4:smRS-GFP signal was not different between pollen tubes treated or not with 1-butanol (see Supplemental Figure 10A online), indicating that VHA-c4 trafficking is not influenced by PLD. The appearance of VHA-c4:smRS-GFP in pollen tubes treated with U-73122 was quite different from that in pollen tubes treated with manoalide. The blocking of endocytic membrane recycling by U-73122, as reported by Helling et al. (2006), resulted in the FM4-64 signal remaining only in the PM and not accumulating in the apical region; in addition, the VHA-c4:smRS-GFP signal was spotted and scattered (see Supplemental Figure 10B online). Because PLC activity is required for membrane deformation, including the endocytic and exocytic pathway (Goni and Alonso, 2000; De Matteis and Godi, 2004; McMahon and Gallop, 2005; Helling et al., 2006), it is possible that U-73122 caused disordered VHA-c4 localization. This perturbation in the localization of VHA-c4 by the PLA2 inhibitor possibly indicates that PLA2s have roles in ER membrane deformation or VHA-c4 trafficking, even although the link between VHA-c4 and PLC remains to be determined.

**DISCUSSION**

We demonstrated that Arabidopsis PLA2-β, -γ, and -δ are expressed differentially during pollen development. RNAi resulted in pollen lethality, and treatment with PLA2 inhibitors inhibited pollen germination. We also demonstrated that PLA2 paralogs localize to the ER and/or Golgi, where they play critical roles in pollen development, most likely by modulating membrane deformation and thereby enabling membrane trafficking. PLA2s also play critical roles in pollen germination and tube growth by providing LPE. Our results therefore enlarge the body of knowledge on the roles of PLA2s in plant development and reproduction.

### PLA2s Function in Pollen Development by Regulating Intracellular Trafficking and Membrane Deformation Events

The promotion of membrane deformation by PLA2 has been shown in giant liposomes, where PLA2 treatment induced budding and fission events (Stanava et al., 2004). Recent studies in animal cells have shown that phospholipid remodeling enzymes have an important function in the organization of the Golgi

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**Figure 10.** Aggregation of VHA-c4:smRS-GFP by Treatment with PLA2 Inhibitor in Arabidopsis Pollen.

Single confocal section shows the distribution of VHA-c4:smRS-GFP and FM4-64 in living Arabidopsis pollen tube. From top images to bottom, images were taken with the VHA-c4:smRS-GFP, FM4-64, merged, and bright images, respectively. Bars = 5 μm.

- **A:** One percent ethanol treatment as control for 1 h.
- **B:** Ten micromoles of manoalide treatment for 10 min.
- **C:** Ten micromoles of manoalide treatment for 1 h.
complex. The functional Golgi organization is mediated by various proteins that interact with specific lipid components to form membrane curvature for vesicle formation and tubule fission (Bard and Malhotra, 2006; De Matteis and Luini, 2008; Jackson, 2009). Phosphoinositides recruit proteins for regulating diverse trafficking events, and lysophospholipids, PA, and diacylglycerol may facilitate vesicle or tubule formation by changing the composition of the membrane (van Meer and Sprong, 2004; Gallop and McMahon, 2005; Frost et al., 2009). Diacylglycerol and PA, which are produced by PLC and PLD, respectively, have functional roles in Golgi membrane fission (Yang et al., 2008; Asp et al., 2009). Lysophospholipid, which is generated by PLA2, may be involved in Golgi retrograde trafficking and Golgi cisternal structure by modifying membrane tubule formation (de Figueiredo et al., 1998; Brown et al., 2003). LPA acyltransferase (LPAAT), which converts LPA into PA, directly regulates mammalian Golgi complex structure and function (Schmidt and Brown, 2009).

As in animal systems, the Golgi apparatus in plants is of major importance in the intracellular trafficking of proteins, which is affected by V-ATPase (Matsuoka et al., 1997). Membrane fusion and formation events, such as the invagination of vacuoles and the fusion of small vacuoles and the PM, as well as intracellular trafficking by the Golgi apparatus are active processes during successful pollen production (Yamamoto et al., 2003). Dettmer et al. (2005) reported that V-ATPase is essential for Golgi organization and that its KO causes complete pollen lethality; consequently, severe morphological changes of the Golgi stacks and Golgi vesicles in developing pollen would be the first indication of cell degeneration. However, it remains to be addressed how the lack of V-ATPase activity brings about these morphological changes.

Based on the results of our experiments on the localization of PLA2-γ and -δ in the ER and/or Golgi in tobacco epidermal cells (Figures 5 and 6) and the detachment of the PM from the cell wall (shrunk cytoplasm; Figures 3i and 4B), the lack of nuclear and ER membrane, ER fragmentation, irregular Golgi stacks, and the formation of vesicles near Golgi in PLA2-suppressed pollen grains (Figures 4B, 4D, and 4F), we suggest that plant PLA2s have functions equivalent to those of their animal counterparts (de Figueiredo et al., 1998; Choukroun et al., 2000; Staneva et al., 2004) in membrane deformation and trafficking. This conjecture is supported by the fact that both the VHA-c4:smRS-GFP signal in the ER and the FM4-64 signal in the endosomes were aggregated by treatment with the PLA2 inhibitor manonoalide (Figure 10).

These physiological and pharmacological results could provide an important clue toward the elucidation of the function of PLA2s in membrane deformation for the following reasons. Membrane deformation is known to occur mainly at a very specific membrane region, called the lipid raft, by budding and fission formation. The lipid raft region is enriched in cholesterol, sphingolipids, and an abundance of membrane proteins. PLA2s are also localized to this raft region, where they trigger membrane curvature by hydrolyzing the sn-2 bond of glycerophospholipids (Lipowsky, 2002; Staneva et al., 2004). This leads to the rather safe assumption that PLA2-γ and -δ interact with VHA-c4 in the lipid raft region, triggering the formation of curvature for membrane deformation and trafficking by hydrolyzing the phospholipid bond. An alternative explanation is that the aggregation of ER in pollen by manonoalide disturbs VHA-c4 trafficking, inhibiting pollen tube growth by an improper membrane trafficking event. The former mechanism is supported by the following experimental results: (1) PLA2 products are known to activate V-ATPases (Palmgren and Sommarin, 1989; Pedchenko et al., 1990; Viehweger et al., 2002), (2) subunits of VHA localize to the ER and trans-Golgi network (Dettmer et al., 2006; Seidel et al., 2008), and (3) VHA-c4:smRS-GFP colocalized with ER tracker but not with FM4-64, which is a well-known endocytic tracer, suggesting that the effect of VHA-c4 is more dominant in the ER than in the other organelles. However, we cannot exclude the possibility that VHA-c4 has an endosome recycling effect because the FM4-64 signal was aggregated following treatment with manonoalide. Recent reports of PLA2-α functioning in root hair development by regulating endocytic recycling of PIN (Lee et al., 2010) and of V-ATPase activity being required for endosome recycling in the root (Dettmer et al., 2008) suggest that PLA2-β, -γ, and -δ may also have some function in the endocytic pathway with VHA-c4. Because PLA2-γ colocalized with STtm-d-CFP as a trans-Golgi marker, the possibility that PLA2 functions in the Golgi as a modulator of the endocytic pathway remains to be determined.

PLA2-γ and -δ Play a Key Role in Pollen Germination by Producing LPE

Several studies have reported that auxin-activated PLA2 increases the concentration of free fatty acids and lysophospholipids, which in turn can activate H+-ATPase to induce apoplastic acidification, leading to cell elongation (Scherer and Arnold, 1997; Paul et al., 1998; Scherer, 2002; Rietz et al., 2004; Ryu, 2004; Lee et al., 2005). Since pollen germination and tube growth are also a specialized type of cell elongation, it is possible that lysophospholipids are active in this process. We therefore examined whether the inhibition of pollen germination by a PLA2 inhibitor could be rescued by different lysophospholipids, namely, 18:1-LPA, 18:1-LPC, and 18:1-LPE. As shown in Figure 9, LPE, which is the major hydrolytic product of PLA2-γ and -δ (Lee et al., 2005), only effectively rescued pollen germination specifically inhibited by manonoalide. This result is consistent with the specific expression of PLA2-γ and -δ in pollen and also indicates that LPE in particular is a key molecule in pollen germination and tube growth.

The most common fatty acids in the sn-1 and sn-2 positions of phosphatidylethanolamine (PE) in the leaf and pollen are 18:2-linoleic acid and 18:3-linolenic acid, and only rarely is 18:1-oleic acid in these positions (Browse et al., 1986; Wolter et al., 1992; Van Aelst et al., 1993). Consequently, our observation that 18:1-LPE can rescue the inhibition of pollen germination caused by manonoalide is most interesting. However, exogenous 18:1-LPE had no effect on pollen germination under normal conditions. These results suggest that only a very minimal amount of 18:1-LPE is needed to regulate pollen germination and tube growth. One possible explanation for this is that PLA2-γ and -δ gene expression level is very low in pollen. Whether 18:1-LPE has a direct function in membrane deformation or works as a lipid signal molecule has not yet been elucidated, and the detailed mechanism of how 18:1-LPE regulates pollen germination and tube growth remains to be solved.
Pollen-Expressed PLA2 Members May Have a Preferential Role in Pollen Development and Germination

All three PLA2s (b, , and d) studied here have primarily the same biochemical function; they catalyze the hydrolysis of phospholipids at the sn-2 position to generate a lysophospholipid and a free fatty acid (Lee et al., 2005). Their role in pollen development, however, appears to be redundant, since no phenotypic differences were found in a single KO mutant. Based on our results and those reported previously, we suggest that PLA2-b, , and d have preferential roles in pollen development and germination. We base this proposal on the following. First, these enzymes were expressed differentially during pollen development. In pollen, PLA2-b was expressed continuously during all stages, while PLA2- and d were expressed predominantly from the tricellular pollen stage onwards, although they were expressed very weakly at earlier stages (Figures 1B and 1G). Second, the subcellular localizations of these three enzymes were slightly different: PLA2-b and d localized to the ER, while PLA2- and d localized to the Golgi and ER (Figures S and S; Seo et al., 2008). Third, all of these enzymes have different acyl group preferences and head-group specificities. PLA2-b shows a preference for palmitoyl acyl chains over linoleoyl acyl chains and slightly prefers PE over phosphatidylcholine (PC), while PLA2- and d have an almost exclusive preference for PE but do not exhibit any significant acyl group preference (Lee et al., 2005). Finally, the extent of enzymatic inhibition by the different PLA2 inhibitors was subtly different: PLA2-b was strongly inhibited by AA, slightly inhibited by manoalide, but not inhibited by ONO, while PLA2- and d were strongly inhibited by manoalide, slightly inhibited by ONO, but not inhibited by AA (Figure 8A; see Supplemental Figure S online; Seo et al., 2008; Lee et al., 2010). We have shown here that different kinds of PLA2 inhibitors had diverse inhibition effects on pollen germination. Similarly, several PLA2 inhibitors have been shown to affect root hair growth in different ways. In PIN-overexpressing plants, root hair growth was conspicuously recovered by ONO, only slightly recovered by AA-COCF3 and PACOCF3, and not recovered at all by HELSS (Lee et al., 2010). Gardiner et al. (2008) reported that AA inhibits root growth by disrupting cortical microtubule arrays in Arabidopsis. In our experiment, however, AA had no inhibitory effect on pollen germination. This difference implies that AA inhibits PLA2- and/or -b but not - and -d. The results of our in vitro inhibition assay of PLA2 by AA confirmed that AA inhibited PLA2- but not - and -d. Such pharmacological evidence supports the belief that each PLA2 isomer has a specific function at the cellular level and is plant tissue dependent.

We therefore infer that PLA2-b may play a more vital role in pollen development than PLA2- and -d, primarily because membrane trafficking and deformation by the ER and Golgi occur as early as the unicellular late microspore stage (Yamamoto et al., 2003) when only PLA2-b was found to be expressed (Figures 1B and 1G). PLA2- and -d were primarily expressed at the pollen maturation stage, suggesting that they may function in processes of pollen germination and tube growth rather than in processes associated with pollen development. This hypothesis is supported by our results showing that pollen germination was inhibited by manoalide and that this inhibition could be rescued by LPE but not by LPC or LPA (Figure 9).

Plant Low Molecular Weight PLA2s Structurally Resemble Animal sPLA2s but Are Functionally Similar to Animal cPLA2

The low molecular weight PLA2s in plants have been classified as secreted PLA2s based entirely on their structural features, such as a large number of disulfide bonds, two antiparallel central helices, a catalytic His-Asp dyad, a hydrogen binding network between the interfacial binding site and the catalytic site, and a calcium binding loop (Berg et al., 2001; Lee et al., 2005; Mansfeld et al., 2006; Mansfeld et al., 2009). The results of a computer-based homology modeling analysis suggest that these plant sPLA2s are similar to those of sPLA2s from bovine pancreas and bee venom in terms of their tertiary structures of the active site and calcium binding loop (Mansfeld et al., 2006). In addition, a transient expression analysis of GFP on onion epidermal cells showed that PLA2-b and - are secreted to the cell wall/extracellular space (Bahn et al., 2003; Lee et al., 2003).

However, we found several pieces of inconsistent experimental data against a classification based on an analogy of sequence homologies. The first consideration is the subcellular localization of PLA2s. The extracellular space in plants is known to be acidic, with a pH 5 to ~6. However, the optimum pH for activities of several plant PLA2s has been determined to be pH 6.5 to ~10 (Stäni et al., 1998; Lee et al., 2005). The pH in the ER is near neutral, namely, 7.2 to ~7.4 (Kim et al., 1998), and the pH of Golgi is more acidic from cis- to trans-Golgi (~6.2) (Caldwell and Howell, 2008). Our confocal microscopy results demonstrate that PLA2- and -d localized to the ER and/or Golgi, which essentially unravels the inconsistency between the subcellular localization and the optimum pH required. Contrary to the extracellular space, the ER and/or Golgi provide the ideal environments for the PLA2s to carry out proper enzyme activity. Second, the head-group specificities of plant sPLA2s are quite different from those of animal sPLA2s. Most of the animal sPLA2s prefer anionic phospholipids, but plant enzymes have a preference for zwitterionic phospholipids, such as PE and PC (Mansfeld et al., 2006; Mansfeld and Ulbrich-Hofmann, 2007). According to Mansfeld (2009), these differences may be due to the evolutionary adaptation of plant PLA2 specificity to the difference in the natural phospholipid composition between plants and animals. Therefore, our results provide us with a possible explanation of why typical cPLA2s have not been found in plants and provide experimental evidence for classifying these so-called plant sPLA2s as functional cPLA2s.

In conclusion, we suggest that the PLA2-b, , and -d expressed in pollen play critical roles in the physiological processes involved in pollen development most likely by modulating membrane trafficking and deformation as well as by providing the lipid signaling molecules for pollen germination and tube growth. We also suggest that the plant low molecular weight PLA2 is functionally similar to cPLA2 rather than to the sPLA2 found in animals.

METHODS

Vector Constructions

The PLA2- and PLA2-d promoters were isolated from Arabidopsis thaliana genomic DNA. A total of 660 bp of the PLA2-5' flanking region...
(ProPLA2-γ; 252 bp of the 3’ untranslated region [UTR] of the preceding gene + 402 bp of the promoter region + 6 bp of the 5′ UTR) and 550 bp of the PLAt-δ 5′ flanking region (ProPLA2-δ; 224 bp 5′ UTR of the preceding gene + 223 bp of promoter region + 103 bp of 5′ UTR) were PCR amplified. The PCR products were digested with HindIII and BamHI and inserted into the HindIII/BamHI sites of the pB121 vector (Clontech). To make the RNAi constructs, the 576-bp full-length ORF from ATG to TAG inserted into the gene + 402 bp of the promoter region + 6 bp of the 5′ flanking region and antisense (BamHI) orientation under the control of the Lat52 promoter (Twell et al., 1989) (designated as LPRNAi), respectively, and both fusion proteins were then subcloned into the pART27 binary vector (Gleave, 1992) at a NotI restriction site. To identify the subcellular localization of PLAt-γ and -δ in pollen, ProLat52:PLAt-γ::smRS-GFP and the ProLat52:PLAt-δ::smRS-GFP were constructed as follows. First, Lat52 promoter, PLAt-γ ORF, PLAt-δ ORF, smRS-GFP ORF, and NOS terminator were amplified with the specific primer sets (see Supplemental Table 1 online). Second, the amplified Lat52 promoter was digested with HindIII and BamHI and then cloned into the HindIII/BamHI-digested pZP221 (Hajdukiewicz et al., 1994), and the NOS terminator was inserted into pZP221 using Saci/EcoRI. Third, the PCR-amplified smRS-GFP product was digested with BamHI and SacI and cloned into the BamHI/Saci-digested construct. Finally, each amplified PLAt-γ and PLAt-δ ORF was introduced into the BamHI site of this construct. For construction of the Pro35S:PLA2-γ-YFP fusion protein construct, YFP was inserted into the SmaI/BamHI-digested pFAST vector (Clontech), and the full-length PLAt cDNA was subsequently cloned into the KpnI-digested pFAST-YFP vector. For PLAt-YFP expression in Arabidopsis pollen, PLAt promoters (-γ and -δ) were substituted for the cauliflower mosaic virus 35S promoter in the Pro35S:PLA2-γ::YFP vector (designated ProPLA2-γ::PLA2-γ::YFP and ProPLA2-δ::PLA2-δ::YFP, respectively). PCR reactions were performed using Phusion High-Fidelity DNA polymerase (Finnzymes), and all of the constructs above were verified by DNA sequencing. The primers used for the cloning are described in Supplemental Table 1 online.

### Plant Materials, Growth Conditions, and Transformation

*Arabidopsis* (ecotype Columbia-0) plants were grown in soil (Sunshine Mix #5; Sun Gro Horticulture) at 22°C and 50% humidity in a growth chamber under long-day conditions (16/8 h, light/dark). Two T-DNA insertion lines, namely, platγ-1 (SALK_022347.48.40) and platγ-2 (SALK_033172.T3.00), were obtained from the ABRC at Ohio State University. *Nicotiana tabacum* cv Xanthi plants were grown in the greenhouse (16/8-h light/dark photoperiod). All binary plasmid constructions were introduced into *Arabidopsis* plants using Agrobacterium tumefaciens strain GV3101 (Koncz and Schell, 1988) by the floral dip method (Clough and Bent, 1998) with minor modification. Transgenic plants were screened on solid Murashige and Skoog (1962) medium containing either 75 mg/L kanamycin or 50 mg/L gentamycin. At least 20 independent transgenic lines were characterized for each construct.

### In Vitro Pollen Germination Assay

The different pollen grains were harvested on a large scale as described by Johnson-Brousseau and McCormick (2004), with minor modifications, and germinated in vitro as described by Boavida and McCormick (2007) on solid germination medium (0.01% H2BO3, 1 mM MgSO4, 5 mM KCl, 5 mM CaCl2, 10% sucrose, and 1.5% low-melting agarose, pH 7.5) at 22°C and 100% humidity in the dark. The germination conditions for *Arabidopsis* and tobacco pollen were the same. Pollen tube germination rates were computed by dividing the total number of germinated tubes by the number of grains. The PLAt inhibitors manolamide (BioHit), DBI (TCl, AACOCF3 [BioHit], PACCOCF3 [BioHit], bromoendo lactone [BEL, Cayman], and ONO-RS-082 (BioHit) were dissolved in DMSO, and the PLAt inhibitor AA (Biomol) was dissolved in water. Each inhibitor was used at concentrations of 3, 5, 7, 10, 20, and 50 μM. For the complementation assay by lipids, 18:1-LPA, 18:1-LPC, 18:1-LPE, and 18:1-PA dissolved in solvents were purchased from Avanti Polar Lipids. Fresh stock solutions were prepared by evaporating the solvent in nitrogen gas and then dissolving the lipids in water by sonication. The lipid stocks were added to the pollen germination medium to a final concentration of 50 μg/mL.

### RT-PCR and Quantitative Real-Time PCR

Total RNA was extracted using a Nucleospin RNA plant extraction kit (Macherey-Nagel). A total of 5 μg RNA was reverse transcribed using SuperScript III (Invitrogen) with the oligo(dT) primer according to the manufacturer’s instructions. For the RT-PCR analysis, 23 cycles were used for elf4-a1 as an internal control and 30 cycles for the PLAt. For the real-time PCR, the SYBR Green PCR master mix (Roche) and LightCycler 480II (Roche) were used according to the manufacturer’s instructions. The PCR conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. The primers used for RT-PCR and real-time PCR are listed in Supplemental Table 1 online.

### siRNA RNA Gel Blot Analysis

Total RNA was extracted from the buds and the open flowers, and 40 μg of total RNA was fractionated in a 15% polyacrylamide gel. The probe for the detection of siRNA was labeled with 32P-d-dCTP using a Random Primer DNA labeling kit (Takara). DNA oligomers of 23 and 27 nucleotides were used as molecular size markers. Hybridization signals were detected using a BAS-2500 Bioimaging analyzer (FujiFilm).

### Scanning Electron Microscopy and TEM

For scanning electron microscopy, the pollen grains from open flowers were mounted on stubs over double-sided carbon tape and coated with gold particles using a sputter coater (SEM Coating System; Bio-Rad). Specimens were observed with a scanning electron microscope (JEOL5300; Jeol) at an accelerating voltage of 25 kV. The images were transferred to a digital image by Semafone (Jeol), a digital image recording and processing system for scanning electron microscopy. For the semithin sections and TEM, flowers were first immersed in a fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.5 M cacodylate buffer, pH 7.5), and the sepals and petals were then removed to allow the fixative to reach the anther, and the samples were vacuum infiltrated (25 Pa for 30 min). After overnight fixation at 4°C, the samples were rinsed with 0.5 M cacodylate buffer, postfixed with 1% OsO4 in 0.5 M cacodylate buffer, pH 7.5, overnight at 4°C and then washed again with 0.5 M cacodylate buffer. The samples were dehydrated at 4°C stepwise through an ethanol series (10% increments, 20 min per step, 10 to 100%), and then transferred successively to 3:1, 1:1, and 1:3 (w/v) mixtures of ethanol and Spurr’s resin (Ted Pella; 6 h at each step), and then finally transferred to 100% Spurr’s resin and left overnight. Each sample was cured in the flat embedding mold for 2 d at 70°C. Semithin sections (1 μm) were cut on an ultramicrotome model MTX (RMC) using a glass knife and then stained with 1% (w/v) toluidine O. For TEM, ultrathin sections (80-nm thick) were collected on copper grids (200 mesh), stained with 2% (w/v) uranyl acetate and Reynolds’s lead citrate, and analyzed by JEM 1010 (Jeol) at 80 kV.

### Subcellular Localization Using the Confocal Laser Scanning Microscope

To express transient fluorescent proteins in tobacco leaves, we modified published agroinfiltration methods (Bendahmane et al., 2000; Sparkes et al., 2006). The infiltration solution contained 10 mM MgCl2, 10 mM MES, pH 5.7, 0.5% glucose, and 200 mM acetosyringone.
(5′-dimethoxy-4′-hydroxy acetoephone; Sigma-Aldrich). Three or four days after infiltration, the abaxial leaf surface was observed with a confocal laser scanning microscope (LSM510 Meta; Carl Zeiss). For CFP, YFP, and GFP, the excitation wavelengths were 458, 514, and 488 nm, respectively, and the emitted fluorescence was collected with a band-pass filter at 470 to ~500, 530 to ~600, and 505 to ~550 nm, respectively. Imaging colocalization of the GFP and YFP constructs was according to the method described by Brandizzi et al. (2002). The excitation lines of an argon ion laser (GFP, 458 nm; YFP, 514 nm) were used alternately with line switching, using the multitrack facility of the LSM510 Meta confocal microscope. Fluorescence was detected using a 458/514-nm dichroic beam splitter and band-pass filters of 470 to 500, 500 to 530, and 530 to 560 nm for GFP and 560 to 590 nm for YFP. For the quantitative colocalization analysis, spectral bleed-through was eliminated. Threshold values were selected to remove background. FM4-64 and ER-tracker (ER-Tracker Red; Invitrogen) were detected using 488/>615 and 543/>600 excitation/emission filter sets, respectively. The argon ion laser line power was set at 10%. All confocal images were processed with LSM5 software version 4.0 (Carl Zeiss).

**PLA2c** inhibitor assay

PLA2c were cloned into the pET-40b(+) vector and expressed in *Escherichia coli* BL21(DE3)pLysS (Novagen). Recombinant PLA2c was isolated as described previously (Bahn et al., 2003; Lee et al., 2003, 2005), dialyzed overnight at 4°C in 50 mM Tris-HCl (pH 6.5 for PLA2c-β and pH 8.0 for both PLA2c-γ and PLA2c-δ), and digested by enterokinase. The reaction mixture for PLA2c activity contained 0.5 mM Ca2+ and 0.05% Triton X-100 in a final volume of 300 μL (50 mM Tris-HCl; pH 6.5 for PLA2c-β and pH 8.0 for both PLA2c-γ and PLA2c-δ). Before substrates were added to the enzyme reaction, and 10 μM manolide dissolved in ethanol was preincubated with PLA2c at 37°C for 60 min. Unlabeled PE (0.5 μmol) and 35 pmol radiolabeled [3-3H]-ethanolamine-1,2-dioleoyl (55 mCi/mmol; GE Healthcare) were used as substrates for each reaction. The enzyme reaction was performed at 37°C for 30 min. Lipid products were separated by thin layer chromatography (Silica Gel 60; Merck) and developed with chloroform/methanol/acetic acid/water (85:15:12.5:3.5, v/v/v/v). 14C-PE and 14C-clysoPE were detected on BAS-MS Imaging plates (Fujifilm) and quantified using the Bio-Imaging Analyzer (FLA7000; Fujifilm).

**GUS Staining**

Flowers and germinated pollen grains of ProPLA2c-γ:GUS and ProPLA2c-δ:GUS were stained in a solution of 1 mM X-gluc, 100 mM sodium phosphate buffer, pH 7.0. 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 10 mM EDTA, and 0.1% (v/v) Triton X-100. After GUS staining, chlorophyll was removed using 100% ethanol.

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: PLA2c-α (At2g06925), PLA2c-β (At2g19690), PLA2c-γ (At4g29460), PLA2c-δ (At4g29470), VHA-c4 (At1g76530), VGD1 (At2g47040), STP11 (At5g23270), and elf4a-1 (At3g13920).

**Supplemental data**

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

**Supplemental figure 1.** Comparison of expression of PLA2c genes between dehydrated and hydrated pollen grains.

**Supplemental figure 2.** Gene structure of PLA2c-γ and -δ and RT-PCR of the pla2c-γ mutants.

**Supplemental figure 3.** Real-time PCR of pollen-specific expressed genes in RNAi plants.

**Supplemental figure 4.** Analysis of in vitro pollen germination in the presence of various PLA2c inhibitors.

**Supplemental figure 5.** The inhibitory effect of aristolochic acid on the activity of recombinant PLA2c.

**Supplemental figure 6.** Yeast two-hybrid screening of PLA2c-γ and -δ.

**Supplemental figure 7.** Subcellular localization of VHA-c4 in tobacco epidermal cells and Arabidopsis hypocotyl cells.

**Supplemental figure 8.** Localization of VHA-c4:smRS-GFP in the pollen tube.

**Supplemental figure 9.** Transmission electron microscope images of plasma membrane in bicellular stage.

**Supplemental figure 10.** Treatment of ProLat52::VHA-c4:smRS-GFP Arabidopsis pollen with 1-Butanol, PLD Inhibitor, U-73122, and PLC inhibitor.

**Supplemental table 1.** Primers used in this article.

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Endoplasmic Reticulum– and Golgi-Localized Phospholipase A₂ Plays Critical Roles in Arabidopsis Pollen Development and Germination
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