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SHORT COMMUNICATION

Phospholipase Dδ regulates pollen tube growth by modulating actin cytoskeleton organization in *Arabidopsis*

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ABSTRACT

The actin cytoskeleton plays pivotal roles in pollen tube growth by regulating organelle movement, cytoplasmic streaming, and vesicle trafficking. Previous studies have reported that plasma membrane-localized phospholipase Dδ (PLDδ) binds to cortical microtubules and negatively regulates plant stress tolerance. However, it remains unknown whether or how PLDδ regulates microfilament organization. In this study, we found that loss of *PLDδ* function led to a significant increase in pollen tube growth, whereas *PLDδ* overexpression resulted in pollen tube growth inhibition. We also found that wild-type *PLDδ*, rather than Arg 622-mutated *PLDδ*, complemented the *pldδ* phenotype in pollen tubes. *In vitro* biochemical assays demonstrated that PLDδ binds directly to F-actin, and immunofluorescence assays revealed that PLDδ in pollen tubes influences actin organization. Together, these results suggest that PLDδ participates in the development of pollen tube growth by organizing actin filaments.

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Introduction

In flowering plants, double fertilization within the pistil is achieved through growth of the tip of the pollen tube. Many pharmacological and genetic studies have indicated that the actin filament is a major mediator of pollen tube tip growth in plants.[1–5] Polar pollen tube growth is regulated by several cellular and extracellular components including reactive oxygen species and the cytoskeleton, as well as processes such as vesicular trafficking[6–8] for targeted synthesis of new plasma membrane and cell wall components.[9]

The plasma membrane acts as both a biological barrier and a location for the sensation of extracellular stimuli. Phospholipids are the main component of the plasma membrane, and phospholipase D (PLD), which hydrolyzes phospholipids, is involved in important physiological and biochemical processes such as signal transduction, membrane transport, cytoskeletal rearrangement, and membrane degradation.[10–14] The *PLD* gene family of *Arabidopsis thaliana* encodes 12 functional proteins, which are classified into six types: *PLDα* (3), *β* (2), *γ* (3), *δ*, *ε*, and *ζ* (2).[15] Among these, *PLDδ* exhibits high expression in the pollen tube.[16] PLDδ physically interacts with both the microtubule and actin cytoskeleton.[13] Studies of tobacco have shown that PLDβ1 activity can be increased by F-actin and inhibited by G-actin.[17] In *Arabidopsis*, plasma membrane-localized PLDδ has been found to negatively regulate plant heat tolerance by depolymerizing cortical microtubules.[18] Recent studies have suggested that self-incompatibility-induced phospholipid acid (PA) derived from PLDδ in pollen delays actin cytoskeleton depolymerization.[19] Moreover, overexpression of tobacco *PLDδ* causes significant reduction in pollen tube growth.[20] These findings indicate that PLDδ is tightly associated with cytoskeletal organization; therefore, in the present study, we examined the role of PLDδ in mediating pollen tube growth via the actin cytoskeleton.

Results and discussion

Given that *Arabidopsis PLDδ* is highly expressed in the pollen tube, it is reasonable to speculate that PLDδ is involved in pollen tube growth regulation. In this study, we used two homozygous T-DNA insertion lines, termed *pldδ-1* (SALK_092469) and *pldδ-2* (SALK_023247), to assess pollen tube behavior *in vitro*.[18] Both mutants exhibited similar pollen germination rates to wild-type (WT), but with longer pollen tubes (Figure 1a–d). Analysis of these results showed that pollen tubes containing *pldδ* were significantly longer than those of WT plants (Figure 1d), and pollen tube growth rates of *pldδ-1* were higher than those in WT (Figure 1e).

To determine whether pollen tube defects were caused by loss of *PLDδ* function, we performed a complementation experiment. The introduction of *PLDδ* into *pldδ-1* and *pldδ-2* (*pldδ-1-COM* and *pldδ-2-COM*) completely rescued the *pldδ* phenotype, whereas the Arg (R) 622 mutation of *PLDδ*, which resulted in almost complete loss of PLDδ activity,[18,21] failed to restore the phenotypes of pollen tube growth (Figure 1a–d). This result demonstrates that Arg (R) 622 may be the crucial site for PLDδ regulation of pollen tube growth. We also

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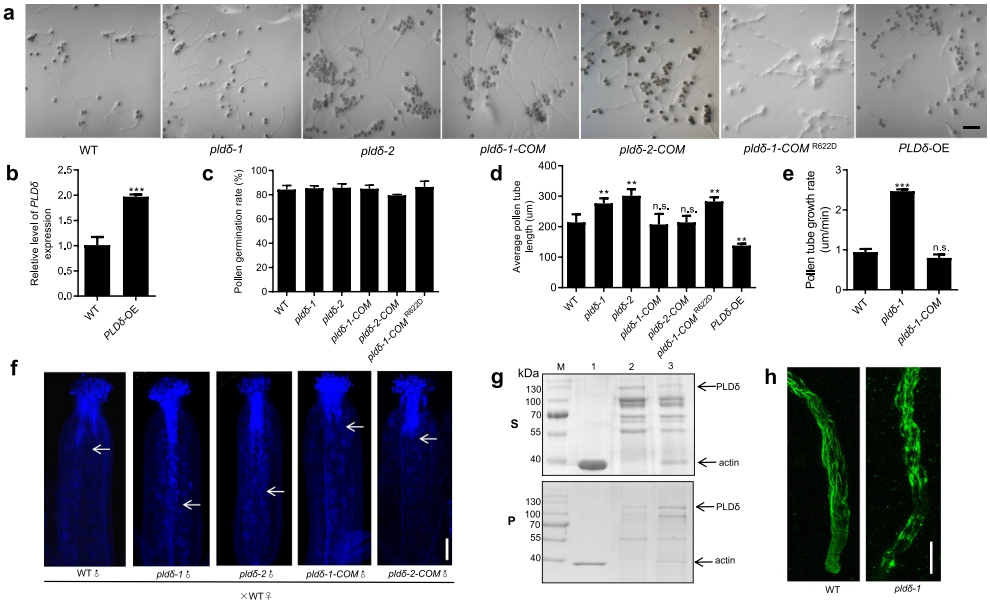

Figure 1. PLDδ interacts with actin filaments and regulates pollen tube growth in *Arabidopsis*. (a) *In vitro*-germinated pollen tubes from WT, *pldδ-1, pldδ-2, pldδ-1-COM, pldδ-2-COM, pldδ-1-COM* ^{R622D}, and *PLDδ*-OE pollen at 4 h after germination. Images were captured under a Zeiss microscope equipped with differential interference contrast optics. Bar = 100 μm. (b) The expression levels of *PLDδ* in the WT and *PLDδ*-OE pollen detected by reverse transcription quantitative PCR (RT-qPCR). *UBQ5* was used as internal control. Asterisk indicates significant differences from WT pollen (***P < .001). Error bars represent ± standard deviation (SD; n = 3) of three replicates. (c) Pollen germination rate after 4 h of *in vitro* culture. Pollen tubes from *pldδ* pollen were consistently longer than those of WT pollen (~200 pollen tubes per data set). (d) Average pollen tube lengths of WT, *pldδ-1, pldδ-2, pldδ-1-COM, pldδ-2-COM, pldδ-1-COM* ^{R622D}, and *PLDδ*-OE. Asterisk indicates significant differences from WT pollen tube (**P < .01). Error bars represent ± standard deviation (SD; n > 120) of three replicates. (e) Pollen tube growth rates of WT, *pldδ-1*, and *pldδ-1-COM*. Asterisk indicates significant differences from WT pollen tube (***P < .001). Error bars represent ± standard deviation (SD; n > 25) of three replicates. (f) Aniline blue staining of pollen germinated *in vivo* for 2 h. Pollen grains from WT, *pldδ-1, pldδ-2, pldδ-1-COM*, and *pldδ-2-COM* were used to pollinate WT stigmata. Pollen grains were then incubated for 2 h before fixation and aniline blue staining. Pollen tubes from each line penetrated the stigmatic tissue similarly and reached the top of the transmitting tracts. White arrows indicate pollen tubes in transmitting tracts. Bar = 100 μm. (g) A co-sedimentation assay was performed to investigate whether PLDδ binds directly to F-actin. Lane 1, F-actin; lane 2, PLDδ; lane 3, F-actin and PLDδ. P, pellet; S, supernatant. (h) An *in situ* immunofluorescence assay was performed to compare F-actin between WT and *pldδ-1*. Actin filaments are fragmented in the *pldδ-1* mutant. Bar = 10 μm.

generated a *PLDδ*-overexpressing line under the control of pollen-specific promoter *Lat52*. Reverse transcription quantitative PCR (RT-qPCR) analyses showed that *PLDδ* transcript level was increased approximately twofold in pollen compared to WT (Figure 1b). Pollen tube elongation was sharply inhibited in the overexpression line (Figure 1a–d). These results demonstrate that PLDδ negatively regulates pollen tube growth.

To directly observe pollen tube behavior, we pollinated WT stigmas with pollen derived from WT, *pldδ*-mutant, and *PLDδ*-complementation lines. At 8 h after pollination, pollinated pistils were fixed and stained with aniline blue and observed by confocal microscopy. We found that pollen tubes of *pldδ* mutants had penetrated through the style and reached the top of the transmitting tract (Figure 1f). By contrast, pollen tubes from WT and both *PLDδ*-complemented lines had only begun to penetrate the style, suggesting that pollen tube growth in the style and transmitting tract is accelerated in *pldδ* mutants.

The actin cytoskeleton is crucial for pollen tube growth;[4,22–24] it acts as a structural element that supports the turgor pressure required to drive and maintain rapid pollen tube growth. PLD-derived PA interacts directly with capping proteins, leading to their separation from actin, which promotes actin polymerization.[25,26] PA also binds to MAP65-1 and mediates microtubule stabilization,[27] and plasma membrane-associated PLDδ negatively regulates plant thermotolerance via destabilizing cortical microtubules.[18] Moreover, *Arabidopsis* PLDδ decorated subapical plasma membrane in tobacco pollen tube, but no plasma membrane signal was found at the tip.[20] Considering that PLDδ might also function as an actin-binding protein in pollen tubes, we performed a co-sedimentation assay to investigate whether PLDδ binds directly to F-actin. The resulting supernatants and pellets were analyzed separately by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The results showed that PLDδ co-sedimented with F-actin and that higher levels of PLDδ were present in pellets containing F-actin than in those without F-actin (Figure 1g); these results are consistent with those of a previous study that reported PLDδ interaction with actin based on epitope-tagged affinity pull-down assays.[13]

Actin cytoskeleton drives the intracellular transport system, and the dynamic organization of actin cytoskeleton is essential for pollen tube growth.[2,28,29] We next observed F-actin organization in pollen tubes using immunostaining under a confocal microscope. In WT plants, the growing pollen tubes produced large amounts of actin cables that became disorganized near the tip region (Figure 1h). By contrast, clear disorganization of the microfilament bundles was apparent in the *pldδ-1* mutant, which formed fragmented and less axially oriented microfilaments (Figure 1h), indicating that PLD may regulate the organization of actin filaments. It should be noted that both excess stabilization and destabilization of actin filaments will lead to retarded pollen tube growth.[2,28] Together, our findings suggest that PLDδ regulates F-actin dynamics in pollen tubes; therefore, we propose a phospholipid-based mechanism underlying pollen tube

growth regulation. Certainly, the potential role for PLDδ and its detailed mechanisms in regulation of the turnover of actin filaments in pollen tube needs to be examined in the future.

Methods

Plant materials and pollen germination conditions

In this study, we used the *Arabidopsis* (*Arabidopsis thaliana*) ecotype Columbia. The identification and verification of the T-DNA-insertion mutant and complement lines of PLDδ were reported previously.[18] *Arabidopsis* pollen was isolated from newly opened flowers and placed on pollen germination medium [1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.01% (w/v) H_3BO_3, and 18% (w/v) sucrose solidified with 0.8% (w/v) agar, pH 7.0]. The plates were cultured at 27°C under moist conditions. Pollen germination and pollen tubes were observed using a Zeiss fluorescence microscope equipped with differential interference contrast optics. The pollen germination percentage and average pollen tube length were calculated based on at least three experiments, each measuring 50 pollen tubes. The ImageJ software (National Institutes of Health) was used to measure pollen tube length.

Plasmid construction

To generate the PLDδ overexpression line, *PLDδ* was cloned from *Arabidopsis* cDNA by polymerase chain reaction (PCR) and subcloned into the pSUPER 1300 vector between the site of *SalI* and *SpeI*, driven by the pollen-specific promoter *Lat52* for transformation into *Arabidopsis*. Primers used for PCR amplification are as follows: 5′-CGCGGATCCATACTCGACTCAG AAGGTATTGAGG-3′ (BamHI) and 5′-AAAGTCGACTAAT TGGAAATTTTTTTTTTGGTGT-3′ (SalI) for *Lat52* promoter; 5′-ACGCGTCGACATGGCGGAGAAAGTATCGGAGG A-3′ (SalI) and 5′-CGGACTAGTTTACGTGGTTAAAGTG TCA GGAAGA-3′ (BcuI) for *PLDδ*.

RNA extraction and RT-qPCR

Total RNA from pollen grains were collected from open flowers and extracted using RNAiso regent (Takara). *PLDδ* expression level was analyzed by reverse transcription quantitative PCR (RT-qPCR) using SYBR mix. The primers used for *PLDδ* were 5′- ACAACAGGCAAGTCCTAGCTC-3′ and 5′-GCAAATCGTGCCAAGGTTGT −3′. *UBQ5* was used as internal control. The primers used for *UBQ5* were 5′-CGTGAAAACCCTAACGGGGA −3′ and 5′- GACTCGCCA TGAAAGTCCCA −3′.

Aniline blue staining assay

Pre-emasculated mature WT flowers were pollinated with pollen from WT, *pldδ-1, pldδ-2, pldδ-1-COM*, and *pldδ-2-COM*. After 8 h, the pollinated pistils were fixed in fixing solution containing ethanol:acetic acid (3:1) for 2 h at room temperature and then washed with distilled water three times (5 min each); the pistils were then incubated in 8 M NaOH solution overnight. The following day, the pistils were washed in distilled water three times (1 h each). The pistils were then stained in aniline blue solution (0.1% aniline blue in 0.1 M K_2HPO_4-KOH buffer, pH 11.0) for 5 h in the dark and then were observed under the confocal laser scanning microscope (Zeiss 780). The ultraviolet excitation wavelength maximum was 350 nm, and the emission spectra were collected from 440 to 612 nm.

Protein purification and co-sedimentation assay

PLDδ cDNA was amplified from plant total cDNA and linked to the PGEX-4 T-1 vector. The plasmid was transformed into *Escherichia coli* (BL21). Protein expression was induced at 23°C overnight with 1 mM isopropyl-β-d-1-thiogalactopyranoside. To purify the protein, the harvested bacterial cells were resuspended in phosphate-buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, with 1 mM phenylmethylsulfonyl fluoride and 1 mg/mL lysozyme. After incubation on ice for 30 min, samples were subjected to ultrasonic treatment and centrifuged at 10,000 × *g* for 30 min at 4°C. GST-PLDδ was then affinity purified using glutathione resin (GenScript, Nanjing, China) and subjected to SDS-PAGE.

For the co-sedimentation assays, we prepared 700 μL F-actin buffer (630 μL general actin buffer and 70 μL actin polymerization buffer) in a centrifuge tube on ice. Actin was incubated with GST-PLDδ (20 μg) in the F-actin buffer for 30 min. After centrifugation at 40,000 g for 20 min at 25°C, the resulting supernatants and pellets were separated. The pellets and supernatants were combined with Laemmli reducing sample buffer (4% [w/v] SDS, 20% [v/v] glycerol, 125 mm Tris-HCl, pH 6.8, 0.004% [v/v] bromophenol blue) and then analyzed by SDS-PAGE using 10% acrylamide gels.

Immunofluorescence staining

The pollen tubes were stained after growth on solid germination medium at 27°C for 2 h. Pollen tubes were fixed in fixative solution (4% paraformaldehyde, 1 mM $MgCl_2$, 1 mM EGTA, 10% sucrose, and 100 mM PIPES buffer, pH 6.9) for 1.5 h and then washed gently with PBS buffer three times. The fixed pollen tubes were treated with digestion buffer (1% cellulose and 0.5% pectinase in PBS buffer) at room temperature for 45 min and then washed gently with PBS containing 0.1% Nonidet *P*-40 three times. The pollen tubes were incubated in primary antibody at 4°C overnight and then incubated in secondary antibody at 37°C in germination medium for 2 h. The primary antibody was diluted to 1:600 in liquid germination medium containing 5% Gly with mouse anti-F-actin monoclonal antibody (CMCTAG, China). Alexa 488-conjugated goat anti-mouse IgG (diluted to 1:400, Invitrogen) was used as the secondary antibody.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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