Arabidopsis Phospholipase Dδ Is Involved in Basal Defense and Nonhost Resistance to Powdery Mildew Fungi[1][W]

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Plants have evolved a complex array of defensive responses against pathogenic microorganisms. Recognition of microbes initiates signaling cascades that activate plant defenses. The membrane lipid phosphatidic acid, produced by phospholipase D (PLD), has been shown to take part in both abiotic and biotic stress signaling. In this study, the involvement of PLD in the interaction between Arabidopsis (*Arabidopsis thaliana*) and the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) was investigated. This nonadapted pathogen is normally resisted by a cell wall-based defense, which stops the fungal hyphae from penetrating the epidermal cell wall. Chemical inhibition of phosphatidic acid production by PLD increased the penetration rate of *Bgh* spores on wild-type leaves. The analysis of transfer DNA knockout lines for all Arabidopsis *PLD* genes revealed that PLDδ is involved in penetration resistance against *Bgh*, and chemical inhibition of PLDs in plants mutated in *PLDδ* indicated that this isoform alone is involved in *Bgh* resistance. In addition, we confirmed the involvement of PLDδ in penetration resistance against another nonadapted pea powdery mildew fungus, *Erysiphe pisi*. A green fluorescent protein fusion of PLDδ localized to the plasma membrane at the *Bgh* attack site, where it surrounded the cell wall reinforcement. Furthermore, in the *pldδ* mutant, transcriptional up-regulation of early microbe-associated molecular pattern response genes was delayed after chitin stimulation. In conclusion, we propose that PLD is involved in defense signaling in nonhost resistance against powdery mildew fungi and put PLDδ forward as the main isoform participating in this process.

A wide range of potentially pathogenic microbes are found in the environment of plants. Nevertheless, plants are able to resist the great majority of microbes they encounter and are susceptible to only a small number of specifically adapted pathogens. This capacity hinges primarily on a broad-range form of immunity called "nonhost resistance" (NHR), which is defined as the immunity displayed by an entire plant species against all genetic variants of a pathogen (Thordal-Christensen, 2003). NHR against pathogens of distantly related host species is believed to be due to basal defense activated after the recognition of microbe-associated molecular patterns (MAMPs; Schulze-Lefert and Panstruga, 2011). While adapted

pathogens have evolved effector proteins that target distinct plant mechanisms to, for instance, suppress plant defenses (Jones and Dangl, 2006), the effectors of nonadapted pathogens are not able to sufficiently suppress plant defenses. In this way, NHR is provided by multiple genes, which makes it effective and genetically robust (Schulze-Lefert and Panstruga, 2011). This form of resistance has thus gained particular interest for its agroeconomic potential.

A well-studied model system for this type of NHR is the interaction between Arabidopsis (*Arabidopsis thaliana*) and nonadapted powdery mildew fungi (Micali et al., 2008). After germination on a leaf, fungal spores develop a specialized hyphal structure, called the "appressorium," which attaches to the epidermal cell wall. Subsequently, the fungus attempts to penetrate the cell from the attachment site. Here, plants are thought to recognize fungal MAMPs and induce localized defense responses at the site of interaction. Fungal penetration is usually stopped by the formation of a multilayered cell wall reinforcement known as a papilla. The papilla is rich in callose and other antimicrobial substances, which provides both a physical and a chemical barrier against the invading pathogen (Hardham et al., 2007; Hückelhoven, 2007). When powdery mildew fungi manage to evade this defense, they form a haustorium, a feeding structure that invades the host cell. Successfully penetrating spores

are stopped either by a second level of defense, confinement of the haustorium within a callose encasement, or a third level of defense, a hypersensitive cell death response to stop further fungal development (Lipka et al., 2008). Thus, NHR against nonadapted powdery mildews and other biotrophic fungi can be conceptually divided into pre- and postinvasion defenses.

It is well established that cell wall-based pathogen recognition in plants is mediated by transmembrane pattern recognition receptors (PRRs) that recognize highly conserved MAMPs. Examples of fungal MAMPs are xylanase and chitin (Boller and Felix, 2009; Monaghan and Zipfel, 2012). The corresponding PRRs, ETHYLENE INDUCING-XYLANASE2 in tomato (*Solanum lycopersicum*; Ron and Avni, 2004), CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) in Arabidopsis (Miya et al., 2007), and CHITIN ELICITOR-BINDING PROTEIN in rice (*Oryza sativa*; Shimizu et al., 2010), have been identified. Notably, the chitin receptor CERK1 has been found to be essential for resistance against a powdery mildew fungus in Arabidopsis (Wan et al., 2008). Several molecular and physiological changes are initially brought about by MAMP perception. These include fluxes of various ions including Ca^{2+} over the plasma membrane, protein phosphorylation, generation of reactive oxygen species and nitric oxide, activation of mitogen-activated protein kinase, Ca^{2+}-dependent protein kinase cascades, and, finally, transcriptional changes in defense genes (Hückelhoven, 2007; Schwessinger and Zipfel, 2008; Boller and Felix, 2009). These early responses to MAMP detection are well studied and known to depend on one another (Ogasawara et al., 2008; Ma et al., 2009). However, thorough insight into the regulation and the genetic basis of these events remains fragmented.

Three major components of preinvasion defense were identified in forward genetic screens for Arabidopsis mutants with enhanced penetration frequencies of the nonadapted powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*; Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). These correspond to the *PENETRATION1* (*PEN1*), *PEN2*, and *PEN3* genes, which encode a plasma membrane-localized syntaxin, a glycoside hydrolase, and a pleiotropic drug resistance ATP-binding cassette transporter, respectively. The current model predicts the existence of a PEN1-defined pathway where this syntaxin is involved in polar secretion of material at sites of attempted fungal ingress, together with an ADP ribosylation factor, GTPase, and the corresponding GTP exchange factor, GNOM (Böhlenius et al., 2010; Nielsen et al., 2012). In a second pathway, PEN2 is required for the production of an antifungal glucosinolate product, secreted into the apoplast by PEN3 (Bednarek et al., 2009). The three PEN proteins accumulate at the incipient fungal entry site: PEN1 in exosomes in the papilla body (Meyer et al., 2009; Nielsen et al., 2012), PEN2 in peroxisomes in the cytoplasmic area surrounding the papilla (Lipka et al.,

2005), and PEN3 at the plasma membrane (Stein et al., 2006).

Several lipids and lipid metabolites have been shown to function in the signal transduction pathway leading to the activation of plant defense responses (Laxalt and Munnik, 2002; Kachroo and Kachroo, 2009). A prominent role has been postulated for phosphatidic acid (PA), whose levels increase within minutes in plant cells recognizing different MAMPs (van der Luit et al., 2000) or upon the recognition of pathogen effector proteins (de Jong et al., 2004; Andersson et al., 2006; Kirik and Mudgett, 2009). PA has been implicated in the modulation of mitogen-activated protein kinase activity, Ca^{2+} influx from the apoplast, and oxidative burst during abiotic and biotic stresses (Testerink and Munnik, 2011). In plants, PA can be formed via two main pathways: the direct hydrolysis of phospholipids by phospholipase D (PLD) or the combined action of phospholipase C and diacylglycerol kinase (Wang, 2004). Both pathways are involved in the early signaling events of plant-pathogen interactions and contribute to the activation of plant defenses. Specifically, PLD activity has been found to be required for the hypersensitive response induced by the recognition of bacterial effector proteins (Andersson et al., 2006; Kirik and Mudgett, 2009).

The 12 different PLD isoforms encoded in the Arabidopsis genome are classified into six groups (α, β, γ, δ, ε, and ζ) based on sequence similarity and in vitro activity. Two HxKxxxxD (HKD) motifs that interact with each other are essential for lipase activity in all eukaryotic PLD isoforms (Bargmann and Munnik, 2006). Although an increase in PLD activity upon treatment with various MAMPs has been reported for different cell culture systems (van der Luit et al., 2000; Yamaguchi et al., 2005; Suzuki et al., 2007), genetic evidence for the involvement of specific PLD isoforms in pathogen defense so far is missing. Here, we reveal a role for PLD activity in Arabidopsis cell wall-based defense against powdery mildew fungi and genetically identify PLDδ as the responsible PLD isoform. We also report that this PLD is involved in MAMP signaling at a more general level.

RESULTS

Inhibition of PLD-Generated PA Causes Increased *Bgh* Penetration in Arabidopsis

The development of *Bgh* spores on Arabidopsis leaves is synchronous, and penetration attempts occur in a narrow time frame around 12 h post inoculation (hpi; Assaad et al., 2004). It is thus possible to score and compare fungal penetration on different plants at a certain time point. To test a possible contribution of PLD-generated PA to plant resistance against *Bgh* penetration, we exploited the transphosphatidylation activity of PLD, which uses primary alcohols as substrates to form an artificial phosphatidyl alcohol. The preferential formation of this compound impairs PA production (Munnik

et al., 1995). Thus, we employed *n*-butanol to inhibit PA formation by PLD. Leaves were infiltrated with different *n*- or *tert*-butanol concentrations 3 h before inoculation, and penetration was scored at 3 d post inoculation (dpi). Increasing *n*-butanol concentrations caused progressively higher *Bgh* penetration rates, from 16% (SD 1.0) for a water control to 38% (SD 7.5) for leaves treated with 0.6% *n*-butanol (Fig. 1A). In contrast, the penetration rate for leaves infiltrated with *tert*-butanol, an alcohol with no inhibitory effects on PA formation, was not significantly different compared with that of the water control. These results thus indicate the involvement of PLD and PA in cell wall-based defense against *Bgh*. We next attempted to measure PLD activity and PA accumulation during the defense response. To this end, leaf explants were labeled with $^{33}PO_4$ overnight before inoculation with *Bgh* spores. However,

no increase in PA label could be measured at 4, 8, 12, and 24 hpi (Supplemental Fig. S1).

PLDδ Is the Only PLD Isoform Involved in Penetration Resistance against *Bgh*

We set out to identify which, if any, of the 12 known Arabidopsis PLD isoforms contributes to penetration resistance against *Bgh*. For this purpose, we identified at least one transfer DNA (T-DNA) insertion line for each *PLD* gene. The insertion site of each T-DNA line was verified by sequencing (Supplemental Table S1; Supplemental Fig. S2). Homozygous plants for T-DNA insertion lines not previously published were tested by reverse transcription (RT)-PCR for the presence of the corresponding *PLD* transcript using primers flanking the insertion site (Supplemental Fig. S3; Supplemental Table S2). Absence of transcripts confirmed that the T-DNA lines indeed are knockout (KO) mutants of the *PLD* genes. Absence of transcript and/or protein in the *pldα1*, *pldα3*, *pldζ1*, and *pldζ2* mutants was described in previous publications (Zhang et al., 2004; Li et al., 2006; Hong et al., 2008). The different *PLD* single KO lines and two *PLD* double KO lines (*pldβ1-2 pldβ2* and *pldζ1 pldζ2*) were scored for *Bgh* penetration resistance by counting interaction events in leaves stained by trypan blue 2 d after inoculation with *Bgh* spores. Among all, only the *PLDδ* KO line (*pldδ*) displayed a significantly higher *Bgh* penetration rate compared with the wild type (Fig. 1B). The penetration rate in this mutant was approximately doubled compared with the wild type, representing an increase from 12% (SD 0.8) penetration in the wild type to over 23% (SD 2.3) in *pldδ*. None of the *pld* mutants investigated was affected in the cell death response to successfully penetrating spores (Supplemental Fig. S4). Furthermore, none of the lines displayed any visible growth or other phenotype under our cultivation regime (Supplemental Fig. S5).

Absence of the *PLDδ*-specific transcript in *pldδ* was verified with a primer pair annealing downstream of the insertion site in addition to the T-DNA flanking pair (Fig. 2, A and B). To confirm that the penetration phenotype of *pldδ* is caused by the specific loss of the *PLDδ* protein, the mutant was complemented with *PLDδ* coding sequence fused to *GFP* under the transcriptional regulation of a 1.4-kb *PLDδ* promoter region. This restored wild-type levels of *Bgh* penetration (Fig. 2C). In addition, *pldδ* plants were transformed with a mutated version of the *pPLDδ:PLDδ-GFP* transgene, which carried a point mutation turning a His into an Asp residue (H707D) in one of the catalytically active HKD sites of PLDδ. This His-to-Asp mutation was previously shown to abolish the enzymatic activity of a PLD isoform in cabbage (*Brassica oleracea*; Lerchner et al., 2006). Transgenic *pldδ* plants expressing the *pPLDδ:PLDδH707D-GFP* construct displayed *Bgh* penetration levels similar to the untransformed *pldδ* (Fig. 2, B and C), further supporting that PLDδ activity is essential for normal penetration resistance.
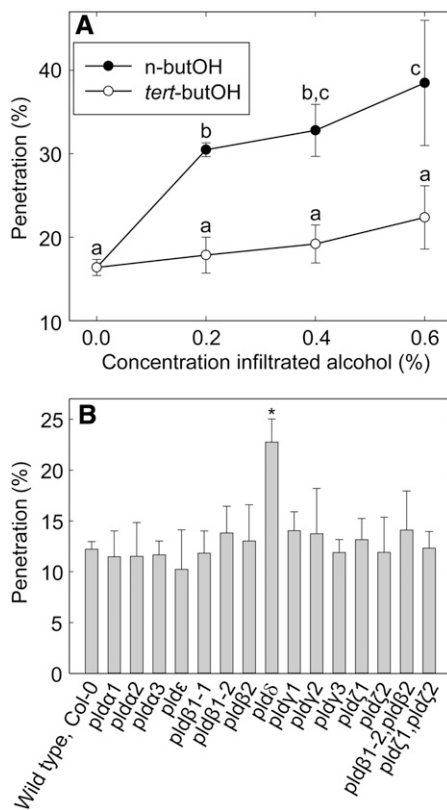
Figure 1. PLDδ-generated PA contributes to Arabidopsis penetration resistance against *Bgh* spores. A, *Bgh* penetration rate (means ± SD; *n* = 3) at 3 dpi on Arabidopsis leaves infiltrated with different *n*-butanol and *tert*-butanol concentrations. Lowercase letters indicate significantly different samples as determined by multiple comparison using one-way ANOVA ($P < 0.02$), with 6 degrees of freedom. B, Screening of single and double Arabidopsis *PLD* T-DNA lines for *Bgh* penetration rate (means ± SD; *n* = 4). Three-week-old plants were inoculated with *Bgh* and observed at 2 dpi. The asterisk indicates a significant difference for *pldδ* compared with the wild type (Col-0) and all other mutant lines as determined by multiple comparison using one-way ANOVA ($P < 0.01$), with 15 degrees of freedom. Three independent experiments were performed with similar results.
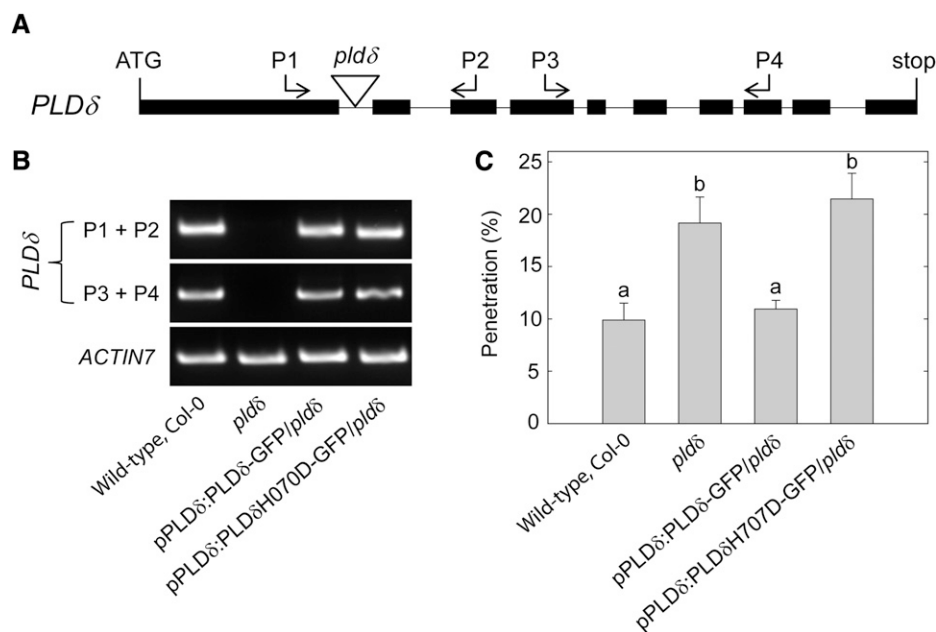
Figure 2. PLDδ enzymatic activity is required for Arabidopsis penetration resistance against *Bgh* spores. A, Schematic representation of *PLDδ* gene structure and the T-DNA insertion site of the *pldδ* mutant. Black bars and lines indicate exons and introns, respectively. Proportions are not represented in scale. B, Semiquantitative RT-PCR analysis of the wild type, the *pldδ* mutant, the PLDδ-GFP fusion line complementing *pldδ*, and the mutated PLDδ-GFP fusion line with *pldδ* background (for description, see text). The relative positions and orientations of the primers employed for the amplification of *PLDδ* cDNA are indicated in A. Amplification of *ACTIN7* cDNA was used as an internal control. C, *Bgh* penetration rate (means ± SD; $n = 4$) at 2 dpi on leaves of the wild type, the *pldδ* mutant, the PLDδ-GFP fusion line complementing *pldδ*, and the mutated PLDδ-GFP fusion line with *pldδ* background. Lowercase letters indicate significant differences between genotypes as determined by multiple comparison using one-way ANOVA ($P < 0.01$), with 3 degrees of freedom. Two independent experiments were performed with similar results.

To verify that PLDδ is the only PLD isoform that plays a role in penetration resistance against *Bgh*, the *pldδ* mutant was treated with *n*-butanol before inoculation with *Bgh*. This treatment caused no further increase in *Bgh* penetration rate (Fig. 3A). The transcripts of *PLDα1* and *PLDδ* are the most abundant among the *PLD* genes in green tissues (Li et al., 2006), while PLDβ1 and PLDβ2 are the two isoforms most closely related to PLDδ (Qin and Wang, 2002). *Bgh* penetration was scored in *pldα1 pldδ* double and *pldβ1-2 pldβ2 pldδ* triple mutants, but none of these displayed higher levels than the *pldδ* single mutant (Fig. 3, B and C). This, together with the *n*-butanol treatment of *pldδ*, verified that PLDδ is the sole PLD isoform involved in penetration resistance. Double mutants combining the *pldδ* mutation with the *pen1-1* or the *pen3-1* mutation did not display higher *Bgh* penetration frequencies than the respective parent *pen* mutants (Fig. 3D).

### PLDδ Contributes to Penetration Resistance against the Nonhost Pea Powdery Mildew

To investigate the conservation of the involvement of PLDδ in penetration resistance against powdery mildew fungi, we used the Arabidopsis nonhost fungal pathogen *Erysiphe pisi* (*Ep*), which is an adapted powdery mildew fungus on pea (*Pisum sativum*) plants. The *Ep* penetration rate on wild-type Arabidopsis leaves was higher than that of *Bgh*, suggesting a higher level of adaptation to Arabidopsis. The *pldδ* mutant displayed a significantly higher penetration rate compared with the wild type for *Ep* (Fig. 4). The penetration success rate increased from 18% (SD 3.1) of the germinated spores on wild-type leaves to 35% (SD 4.0) on the *pldδ* mutant. This clearly demonstrates that the *PLDδ* gene is involved in penetration resistance against this fungus as well.

PLDδ in Chitin-Induced MAMP-Response Gene Activation

Since KO of the *PLDδ* gene caused decreased penetration resistance against two different nonhost powdery mildew fungi, and since the gene appears not specifically linked to either PEN1- or PEN2/PEN3-defined penetration resistance pathways, we hypothesized that the activity of the PLDδ isoform might have a more general role in plant pathogen defense. In particular, it seems reasonable to suggest that the gene might be involved in signaling after recognition of MAMPs. It would be interesting to test

Figure 3. PLDδ is the only PLD iso-form contributing to penetration resistance against *Bgh*. A, *Bgh* penetration rate of *pldδ* leaves infiltrated with water and 0.6% *n*-butanol or *tert*-butanol. No significant difference in penetration was observed for *pldδ* + *n*-butanol, indicating that no other PLD isoform contributes to penetration resistance. B and C, *Bgh* penetration rate at 2 dpi on leaves of different 3-week-old *PLD* mutant plants. KO mutations of *PLDα1* (B) or *PLDβ1* and *PLDβ2* (C) in the *pldδ* background do not cause a further increase in *Bgh* penetration rate. D, *PLDδ* KO in the *pen1* or *pen3* mutant background does not result in any further increase of *Bgh* penetration frequencies. Lowercase letters indicate significant differences between genotypes ($P < 0.05$) as determined by multiple comparison using one-way ANOVA, with 3 (A–C) or 5 (D) degrees of freedom. Means ± sd are shown ($n = 4$). Three independent experiments were performed with similar results.
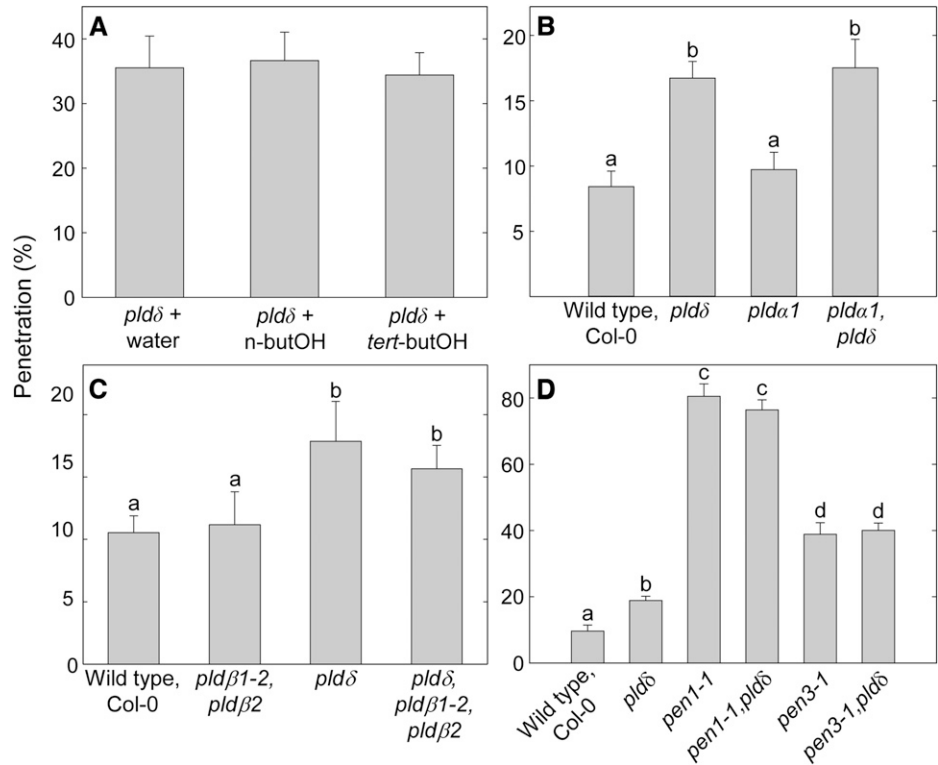

the response of the *pldδ* mutant to MAMPs, such as chitin, since a chitin receptor mutant has been previously found to have reduced powdery mildew resistance (Wan et al., 2008). For this purpose, wild-type and *pldδ* mutant seedlings were treated with a chitin extract, and transcriptional changes of the early MAMP-induced genes, *FLG22-INDUCED RECEPTOR-LIKE KINASE1* (*FRK1*), *NDR1/HIN1-LIKE10* (*NHL10*) and *PHOSPHATE-INDUCIBLE1* (*PHI-1*; Boudsocq et al., 2010; Kwaaitaal et al., 2011), were evaluated by quantitative RT-PCR (Fig. 5). The transcript levels of all three genes rapidly increased following treatment with chitin in both wild-type and mutant plants. *PHI1* and *NHL10* both reached a peak of transcript accumulation 30 min after treatment, whereas *FRK1* transcript increased throughout the experiment. However, the *pldδ* mutant showed a trend for an approximately 50% reduction in transcript accumulation after 30 min for *PHI1* and *NHL10*. For all three genes, a trend for a delayed chitin-induced accumulation of transcripts was observed. Observations for *NHL10* were consistent for all biological replicates. Results for *PHI-1* and *FRK1* demonstrated more variability between experiments and, in some instances, revealed a lower amount of transcript at the zero time point in the *pldδ* mutant. These observations suggest that *PLDδ* is needed for rapid and timely activation of CERK1-controlled defense signaling cascades.

PLDδ Is Localized to the Plant Plasma Membrane at *Bgh* Attack Sites

The complementation of *pldδ* phenotype by the expression of the *pPLDδ:PLDδ-GFP* transgene indicates that the PLDδ-GFP translational fusion is functional. Although the fluorescent signals of the pPLDδ:
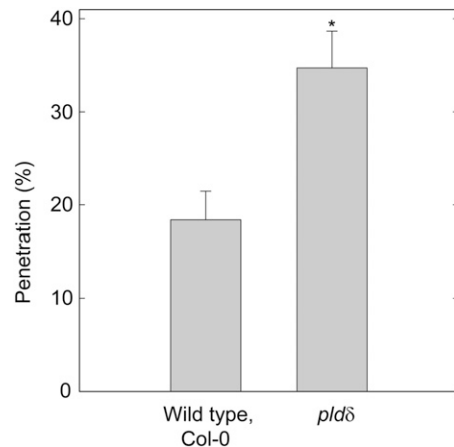
Figure 4. *PLDδ* contributes to penetration resistance against the non-host powdery mildew fungus *Ep*. Scoring of penetration events was carried out at 2 dpi on wild-type and *pldδ* leaves. Means ± sd are represented ($n = 4$; Student's *t* test, $P < 0.01$). Three independent experiments were performed with similar results.
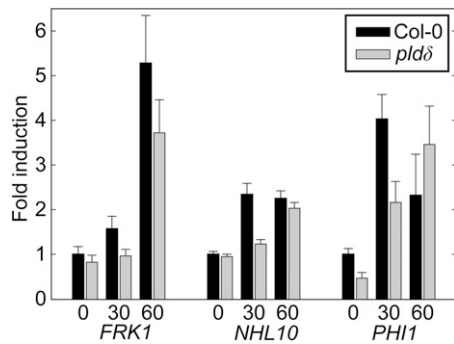
Figure 5. Chitin-induced defense transcript accumulation is delayed in the *pldδ* mutant. Col-0 and *pldδ* seedlings were grown in liquid culture on a microtiter plate. Ten-day-old seedlings were treated with chitin extract, and 0, 30, and 60 min later, the seedlings were harvested. Total RNA of approximately 20 seedlings was extracted per sample for transcript analysis. Transcript levels of the early MAMP-activated defense genes *NHL10*, *FRK1*, and *PHI-1* were determined by quantitative PCR. Relative expression levels compared with Col-0 at the zero time control are presented. The data were normalized using *At4g26410* as a reference gene. The average of three technical replicates is shown, and error bars show SE. At least three independent experiments were performed with similar results.

PLDδ-GFP and pPLDδ:PLDδH707D-GFP lines were clearly visible in leaves observed with an epifluorescence microscope (Supplemental Fig. S6), they were not sufficiently high for confocal imaging. Thus, we placed the coding sequence for the PLDδ-GFP fusion under the transcriptional regulation of the *35S* promoter and transformed this transgene into wild-type plants. The p35S:PLDδ-GFP line proved to be suitable for confocal microscopy, and PLDδ-GFP clearly localized to the plasma membrane of leaf epidermal cells (Fig. 6A). Twenty-four hours after *Bgh* inoculation, PLDδ-GFP was visible in the plasma membrane in general and at the regions of fungal attack where the appressoria attempted penetration. Here, PLDδ-GFP fluorescence labeled the rim of the forming papilla (Fig. 6B). Z-stack observations and three-dimensional visualization of several fungal attack sites displayed an accumulation of PLDδ-GFP at the plasma membrane, which forms a cup-like shape around the papilla (Fig. 6, C and D; Supplemental Movie S1). Occasionally, the accumulation of PLDδ-GFP around *Bgh* interaction sites extended to the surrounding membrane in the shape of a compact disk (Fig. 6E). Three-dimensional reconstruction of these entry sites showed a small plasma membrane invagination corresponding to a small papilla (Supplemental Movie S2). Nontransformed wild-type leaf epidermal cells exhibited low autofluorescence in the GFP channel before (Supplemental Fig. S6A) and 24 h after (Supplemental Fig. S6B) inoculation with *Bgh* spores. The autofluorescence was mainly associated with stomatal guard cell walls and did not coincide with PLDδ-GFP signal.

DISCUSSION

Effective resistance against pathogenic microbes depends on the timely deployment of the appropriate defense responses. A functional signaling pathway ensures that pathogen recognition is promptly communicated to the cellular machinery responsible for the execution of defense. Our results clearly indicate that the enzymatic activity of PLDδ contributes to the accomplishment of penetration resistance against nonadapted powdery mildew fungi in Arabidopsis. This is supported by the observation that the penetration resistance was decreased by the PLD inhibitor *n*-butanol and by KO of the *PLDδ* gene. Finally, introduction of a
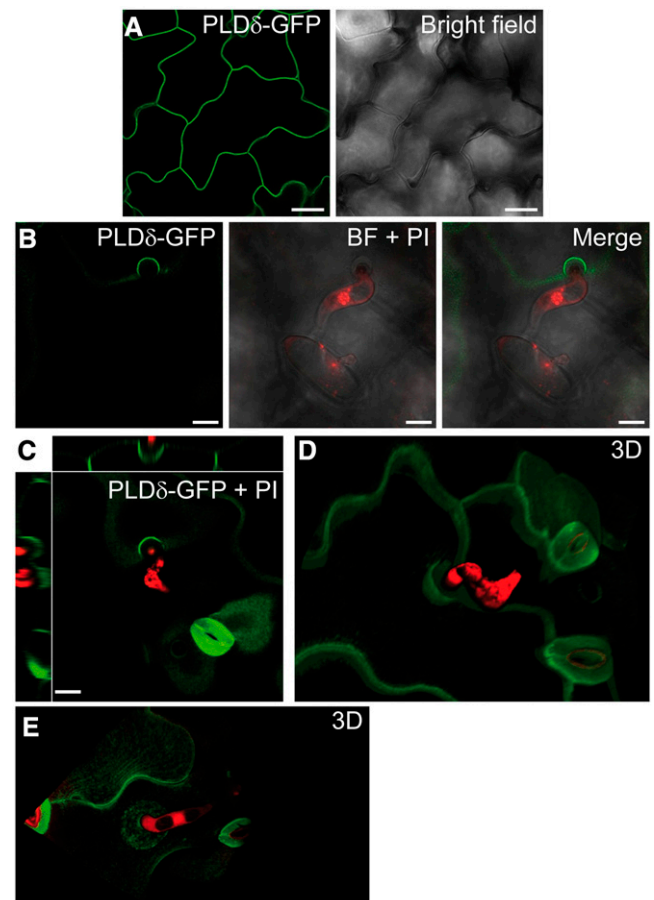
Figure 6. *Bgh* induces the accumulation of PLDδ at the plasma membrane around the site of fungal attack. A, Plasma membrane localization of PLDδ-GFP in leaf epidermal cells of noninoculated p35S::PLDδ-GFP plants. B, Plasma membrane accumulation of PLDδ-GFP at the site of attempted *Bgh* penetration. Fungal structures are stained with propidium iodide (PI). BF, Bright field. C, Image depicts *xy* plane of PLDδ-GFP accumulation at the *Bgh* attack site and side views of it along the *z* axis. D and E, Three-dimensional (3D) reconstruction of the *Bgh* attack site and PLDδ-GFP localization around it (Supplemental Movies S1 and S2). Images were taken at 24 hpi with *Bgh*. Two independent experiments were performed with similar results. Bars = 25 μm (A and B) and 10 μm (C).

construct encoding a catalytically inactive PLDδ did not complement the loss of the *PLDδ* gene, whereas a functional copy did complement the mutant phenotype. The involvement of PLDδ in cell wall-based defense apparently extends to at least two different nonadapted powdery mildew fungi. However, we failed to detect an increase in PA levels after inoculation of wild-type Arabidopsis plants with *Bgh*. It seems likely that the limited number of leaf epidermal cells interacting with *Bgh* spores is not sufficient to provoke a detectable increase in PA against the background of the whole leaf tissue. The PA signal might also be more restricted in time than what we addressed. Nevertheless, we demonstrated that chemical inhibition of PA production via PLD enhanced the *Bgh* penetration rate and provided clear genetic evidence that specific enzymatic activity of PLDδ is required for the full penetration resistance to nonhost powdery mildew fungi in Arabidopsis.

PLDδ has previously been implicated in the freezing tolerance of Arabidopsis. Loss of or overexpression of PLDδ resulted in reduced and increased PA levels, respectively, but did not alter cold-regulated gene expression (Li et al., 2004). Thus, PLDδ seems to control unique PA-dependent cellular signaling events in response to cold stress. In this study, we cannot determine, based on the penetration resistance phenotype alone, where mechanistically in the defense pathways PLDδ acts. However, we tentatively propose that PLD activity is involved in upstream early signaling events rather than in the execution phase of penetration resistance. Mutations in *PEN1*, *PEN2*, and *PEN3* result in a severalfold increase in the *Bgh* penetration rate (Collins et al., 2003; Lipka et al., 2005; Stein et al., 2006). Based on their cellular and molecular functions, the three PEN proteins are regarded as main components in the execution of preinvasion resistance. The effect of the *pldδ* mutation on *Bgh* penetration resistance was clearly much lower than that caused by the loss of any of the *PEN* genes. In addition, introduction of the *pldδ* mutation into the *pen1* and *pen3* backgrounds did not cause additional effects on the frequency of *Bgh* penetration in any of the combinations. Thus, it appears that PLDδ cannot be directly linked to either the PEN1 or the PEN2/PEN3 pathway. This leaves room for several different interpretations. However, as the forms of NHR studied here are considered to be triggered by fungal MAMPs (Schulze-Lefert and Panstruga, 2011), and as we observe that chitin-triggered defense gene transcript accumulation is delayed in the *pldδ* mutant, we propose a role for PLDδ early in MAMP-triggered penetration resistance. Furthermore, this is in agreement with the documented role of a chitin receptor in powdery mildew resistance (Wan et al., 2008).

Mutants in *PEN1* (Assaad et al., 2004) and its ortholog *ROR2* in barley (*Hordeum vulgare*; Böhlenius et al., 2010) both exhibit delayed callose deposition, which correlates with the increased susceptibility of these mutants. This illustrates the importance of a timely activation of defense to repel invading pathogens. Therefore, delayed activation of defense downstream of MAMP-induced activation of PRR in *pldδ* might have a significant impact on the success of a single cell in defending itself against pathogen invasion. The *pldδ* mutation and *n*-butanol treatment cause only a partial loss of resistance, likely reflecting a large degree of redundancy in the signaling pathway. Such a signaling function of PLDδ in preinvasion defense is in agreement with the role of PA as a second messenger in cellular responses to different biotic stimuli (de Jong et al., 2004; Andersson et al., 2006; Kirik and Mudgett, 2009). An alternative interpretation to the involvement of PLDδ in penetration resistance might be that the PLD activity is involved in controlling the polar secretion of antimicrobial compounds by, for instance, aiding in vesicle fusion to the plasma membrane (Jenkins and Frohman, 2005). This model, however, is less easily reconcilable with the effect of the *pldδ* mutation on MAMP-triggered transcriptional activation of defense transcripts. Alternatively, PLDδ might be involved in regulating the amount and targeting of PRRs to the plasma membrane. Finally, a combination of the three might be a possibility. However, the most consistent model with the data presented in this and previous studies seems to be that PA is generated as a second messenger following MAMP recognition and that PA acts as one part of an otherwise partly redundant signaling network including Ca^{2+} and kinases.

Gene expression studies of the whole Arabidopsis genome in response to *Bgh* inoculation did not reveal any change in *PLDδ* transcription during NHR (Zimmerli et al., 2004). Considering previous studies demonstrating the induction of PA production via PLD within minutes after the elicitation of tomato cells with fungal MAMPs (van der Luit et al., 2000; Raho et al., 2011), it is likely that PLDδ is activated at a posttranslational level upon the recognition of a *Bgh* penetration attempt. Further support for this notion is the fact that the *PLDδ* gene together with PLDα1 is the most highly expressed PLD isoform in leaves (Li et al., 2006). Notably, none of the other PLD isoforms appeared to be involved in preinvasion defense, although they were all expressed in wild-type leaves. This likely reflects the differences among the 12 PLD isoforms in terms of activation requirements, subcellular localization, and substrate preferences (Li et al., 2009). The PLD isoforms were shown to be differentially involved in the plant response to specific stress conditions (Zhang et al., 2003, 2004; Li et al., 2006; Hong et al., 2008). The expression profile, the subcellular localization, and the biochemical properties of PLDδ together make it a candidate PLD isoform responsive to the stimulus of fungal penetration attempts. The plasma membrane localization shown here for a PLDδ-GFP fusion protein confirmed previous reports based on immunochemical assays (Wang and Wang, 2001). PRRs perceive fungal MAMPs at the cell surface and trigger downstream signaling cascades (Monaghan and Zipfel, 2012). Hence, PLDδ activation

might be an early event at the plasma membrane after MAMP recognition, and the subsequently produced PA could stimulate the activation of plant defenses.

This is also supported by the effect of the *pldδ* mutation on transcriptional changes of early MAMP-activated defense genes. The observation that *PHI-1* and *NHL10* exhibit an altered chitin-triggered transcriptional up-regulation in the *pldδ* mutant links PLDδ action to CERK1-activated Ca^{2+} signaling (Boudsocq et al., 2010). In parallel, PLDδ activation might contribute to the complex lipid rearrangements occurring at the plasma membrane, which lead to the formation of a lipid raft-like microdomain at sites of fungal penetration attempt (Bhat et al., 2005). In this sense, the presence of PLDδ at fungal interaction sites shown here is significant and further supports the role of PLDδ in penetration resistance. Interestingly, PLD was also shown to cluster in regions of the plasma membrane adjacent to bacterial attack sites in rice leaves resistant to *Xanthomonas oryzae* pv *oryzae* (Young et al., 1996). In vitro PLDδ activity is stimulated by oleic acid and phosphatidylinositol bisphosphate and requires high micromolar to low millimolar Ca^{2+} concentrations (Wang and Wang, 2001; Qin and Wang, 2002). The influx of Ca^{2+} from the extracellular space is a well-established event occurring after the recognition of MAMPs and effector proteins (Ma and Berkowitz, 2007). An increase in cytosolic Ca^{2+} levels was shown to trigger a PLD-dependent accumulation of PA after the recognition of two bacterial effectors in Arabidopsis (Andersson et al., 2006). Stimulation of PLDδ activity by oleic acid was found to be a surface-dilution effect, as Triton X-100 was also shown to stimulate this activity (Qin et al., 2002). However, the levels of free unsaturated fatty acids, including oleate, in plant cells have been shown to increase after treatment with a fungal elicitor (Kirsch et al., 1997).

Recently, *PLDδ* has been implicated in a PA-dependent growth response to the root endophytic fungus *Piriformospora indica* (Camehl et al., 2011). This observation, together with our data, raises the question of whether PLDδ is part of a common signaling mechanism communicating the occurrence of a biotic stimulus at the cell surface. PLDδ activity is also stimulated by hydrogen peroxide (Zhang et al., 2003), and this activation was recently shown to require the association of PLDδ to cytosolic glyceraldehyde-3-phosphate dehydrogenases (Guo et al., 2012). Hydrogen peroxide production is a well-known event occurring early in plant-pathogen interactions and in response to MAMP perception (Torres et al., 2006). It thus seems plausible that a stimulation of PLDδ activity might depend on rapid hydrogen peroxide production during a penetration attempt of a nonhost powdery mildew fungus. Details of how PLDδ is activated, as well as the function and nature of the targets of the produced PA, remain to be determined.

To conclude, we herein demonstrate that the enzymatic activity of PLDδ and thus the membrane lipid PA contributes to cell wall-based defense against nonadapted powdery mildew fungi in Arabidopsis. We further propose that PA produced by this PLD isoform is part of a signaling network activated by the recognition of fungal MAMPs.

MATERIALS AND METHODS

Plant Growth Conditions and Mutant Isolation

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) lines were grown under short-day conditions (8-h day and 16-h night, 180 μmol m^{-2} s^{-1}) at 22°C and 60% relative humidity. For analysis of chitin-induced transcript accumulation, Col-0 and *pldδ* mutant seeds were sterilized and grown on 1× Murashige and Skoog medium for 11 to 12 d with 16 h of light (at 21°C) and 8 h of dark (at 19°C) as described (Kwaaitaal et al., 2011). SALK (Alonso et al., 2003) and GABI-Kat (Kleinboelting et al., 2012) T-DNA lines were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Identifier numbers and gene-specific primers used for the isolation of homozygous lines are listed in Supplemental Table S1. The LBb1.3 (5'-ATTTTGCCGATTTCG-GAAC-3') and GK_8409 (5'-ATATTGACCATCATACTCATTGC-3') oligonucleotides were used as T-DNA-specific primers for genotyping the SALK and GABI-Kat lines, respectively. The *pldζ1-1 pldζ2-1* double mutant line was kindly provided by Prof. Xuemin Wang.

Semiquantitative RT-PCR

Total RNA was extracted from 100 mg of leaf tissue using the GeneJET RNA purification kit (Thermo Scientific) and treated with DNase I (Thermo Scientific) to remove traces of genomic DNA. RT of mRNA was achieved with the RevertAid Premium First Strand cDNA Synthesis kit (Thermo Scientific) using oligo(dT)$_{18}$. Subsequent PCR amplification of *PLD*-specific transcripts was performed with the primers listed in Supplemental Table S2. Amplification of the *ACTIN7* gene (At5g09810) was used as a control.

Fungal Inoculation and Scoring of Fungal Penetration

The nonhost powdery mildew fungi *Blumeria graminis* f. sp. *hordei* (isolate DH14) and *Erysiphe pisi* (isolate CO-01) were propagated on barley (*Hordeum vulgare*) and pea (*Pisum sativum*) plants, respectively. Inoculations were carried out as described previously (Zhang et al., 2008). Leaves were stained with trypan blue as described (Koch and Slusarenko, 1990). Successful penetration and cell death were determined by the presence of a fungal haustorium and trypan blue uptake, respectively. At least 50 interaction sites of two leaves from three or four independent plants were counted for each treatment/mutant line. The average frequency of penetrations from two leaves from each plant was used for multiple comparison with one-way ANOVA with Fisher's LSD using the software package GraphPad Prism 6.0 (Graphpad Software) or paired Student's *t* test using Excel 2010. To study the effect of *Bgh* inoculation on phospholipid metabolism, leaf explants were labeled with $^{33}PO_4$ overnight before *Bgh* inoculations. The lipids were extracted and separated by thin-layer chromatography as described previously (Andersson et al., 2006), and lipid radiolabel was quantified after liquid scintillation of PA-containing regions of the thin-layer plates.

Butanol Infiltration

Leaves were infiltrated with alcohol solutions in deionized water using a 1-mL syringe without needle from their abaxial side using gentle pressure. Attention was paid to ensure complete diffusion of the solution to the whole leaf lamina. Plants were subsequently returned to the growth chamber for 3 h before fungal inoculation to allow absorption of the solution and to let the leaves dry.

MAMP Treatment and Quantitative RT-PCR

Experiments were performed as described by Kwaaitaal et al. (2011) with the following modifications. Total seedling RNA was isolated using TRIzol (www.lifetechnologies.com/) according to the protocol provided with the

reagent. Subsequently, 2 μg of total RNA was used to generate complementary DNA (cDNA) using the Advantage RT-for-PCR Kit (www.clontech.com) in a total volume of 20 μL. Finally, 80 μL of double distilled water was added to the reaction mixture. A volume of 2.4 μL of cDNA was used in the quantitative PCRs, with final primer concentrations of 0.5 mM of the forward and reverse primers for the reference gene *At4g26410*, *FRK1*, and *NHL10* and 0.125 mM of the forward and reverse primers for *PHI-1* (Boudsocq et al., 2010). Primer sequences are listed in Supplemental Table S3. The volume was adjusted to 6 μL, and 6 μL of 2× SYBR Green Jumpstart *Taq* ReadyMix (www.sigma.com) was added. The quantitative PCR was run in a Stratagene Mx3000P (Amersham Biosciences, GE Healthcare) thermal cycler, and the results were analyzed using Stratagene MxPro Mx3005P software (Amersham Biosciences, GE Healthcare). The cycling program used was as follows: 10 min at 95°C, then 40 cycles of 30 s at 95°C, 30 s at 62°C, and 25 s at 72°C. The melting curves of the PCR products were recorded between 55°C and 95°C.

Generation of the *PLDδ-GFP* Transgene and Site-Directed Mutagenesis of *PLDδ*

The complete coding sequence of the *PLDδ* gene excluding the final stop codon was amplified from wild-type leaf cDNA (generated as described above) using the *PLDδ_cds* primers (Supplemental Table S4). The obtained product was inserted into the pENTR/D-TOPO vector through directional TOPO cloning (Invitrogen) and subsequently verified by sequencing. To obtain a *PLDδ* 3′ terminal fusion to *GFP*, the *PLDδ* coding sequence was transferred using Gateway technology (Invitrogen) to the pB7FWG2 vector (Karimi et al., 2005), resulting in the *p35S:PLDδ-GFP* T-DNA construct. For generation of the *pPLDδ:PLDδ-GFP* transgene, the pB7FWG2 vector was modified prior to recombination with the *PLDδ* coding sequence as follows. A 1.4-kb *PLDδ* untranslated promoter region upstream of the first ATG was amplified from wild-type genomic DNA using the *pPLDδ* primers (Supplemental Table S4). The amplified fragment was used to replace the *p35S* region in the pB7FWG2 vector (Karimi et al., 2005) through digestion with *Hin*dIII and *Spe*I endonucleases and subsequent ligation. The modified vector was verified by sequencing. The obtained T-DNA plasmids carrying the *p35S:PLDδ-GFP* and *pPLDδ:PLDδ-GFP* transgenes were introduced into *Agrobacterium tumefaciens* strains GV3101 and C58C1, respectively. Transformation of wild-type and *pldδ* plants was achieved through the floral dip method (Clough and Bent, 1998). The pENTR/D-TOPO vector containing the *PLDδ* coding sequence was employed to obtain the *PLDδ*H707D mutant open reading frame (ORF). The C-to-G mutation at position 2,152 of the *PLDδ* ORF was introduced by amplifying the whole plasmid by PCR (26 cycles) with the phosphorylated *PLDδ*H707D ORF primers (Supplemental Table S4). The original template plasmid was digested with *Dpn*I endonuclease (acting on methylated DNA), and the PCR product was subsequently circularized by ligation. The mutant *PLDδ*H707D ORF was verified by sequencing and subsequently transferred to the pB7FWG2 vector (modified as described above) in order to generate the *pPLDδ:PLDδ*H707D-GFP transgene. The obtained plasmid was introduced into *A. tumefaciens* strain C58C1 and transformed into *pldδ* plants.

Fluorescence and Confocal Microscopy

Transgenic PLDδ-GFP lines were scored for GFP fluorescence in leaves using a 20× objective mounted on a Zeiss Axio Scope.A1 epifluorescence microscope. Localization of PLDδ-GFP was investigated with a Zeiss LSM 700 confocal laser scanning microscope using a 40× water-immersion objective (numerical aperture, 1.1). For visualization of fungal spores, inoculated leaves were mounted in a solution of 2.5% mannitol/0.1% (v/v) Silwet/0.02 mg mL^{-1} propidium iodide. Images were analyzed with Volocity (Perkin-Elmer) and ImageJ software.

Sequence data of the genes used in this article can be found in the GenBank/EMBL data libraries. Gene accession numbers are listed in Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. Quantification of radiolabeled PA after *Bgh* inoculation.

Supplemental Figure S2. Schematic representation of the structure of the *PLD* genes and the T-DNA insertion sites.

Supplemental Figure S3. Semiquantitative RT-PCR analysis of *PLD* T-DNA lines.

Supplemental Figure S4. No postpenetration cell death phenotype was observed in *pld* mutants.

Supplemental Figure S5. Growth phenotypes of the *PLD* T-DNA lines used in this study.

Supplemental Figure S6. Expression of *pPLDδ::PLDδ-GFP* and *pPLDδ::PLDδH707D-GFP* transgenes in the *pldδ* mutant background.

Supplemental Figure S7. Control image of the autofluorescence of non-inoculated and *Bgh*-inoculated leaves.

Supplemental Table S1. T-DNA mutant alleles of Arabidopsis *PLD* genes and gene-specific primers used for genotyping.

Supplemental Table S2. Primers used for semiquantitative RT-PCR analysis of *PLD* T-DNA mutant lines.

Supplemental Table S3. Primers used for quantitative RT-PCR analysis of MAMP-responsive genes.

Supplemental Table S4. Primers used for the molecular cloning of *PLDδ* transgenes.

Supplemental Movie S1. Three-dimensional reconstruction of the *Bgh* attack site and PLDδ-GFP localization around it at 24 hpi of p35S::PLDδ-GFP plants.

Supplemental Movie S2. PLDδ-GFP localization around a small papilla at 24 hpi of p35S::PLDδ-GFP plants.

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