Published in final edited form as:
New Phytol. 2013 July ; 199(1): 228–240. doi:10.1111/nph.12256.

Arabidopsis phospholipase Dβ1 modulates defense responses to bacterial and fungal pathogens

Jian Zhao[1,3,*], **Shivakumar P. Devaiah**[1], **Cunxi Wang**[1], **Maoyin Li**[1,4], **Ruth Welti**[2], and **Xuemin Wang**[1,4,*]

[1]Department of Biochemistry, Kansas State University, Manhattan, KS, 66506 USA

[2]Division of Biology, Kansas State University, Manhattan, KS, 66506 USA

[3]College of Plant Science and Technology, National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, China

[4]Department of Biology, University of Missouri, St. Louis, MO 63121 and Danforth Plant Science Center, St. Louis, MO 63132 USA

Summary

- Pathogen infection of higher plants often induces a rapid production of phosphatic acid (PA) and changes in lipid profiles, but the enzymatic basis and the function of the lipid change in pathogen-plant interactions are not well understood.
- Infection of *PLDβ1*-deficient plants by *Pseudomonas syringae* pv. DC3000 resulted in less bacterial growth than in wild-type plants, and the effect was more profound in virulent *Pst* DC3000 than avirulent *Pst* DC3000 (*avrRpt2*) infection. The expression levels of salicylic acid (SA)-inducible genes were higher, but those inducible by jasmonic acid (JA) were lower in *PLDβ1* mutants than in wild-type plants.
- However, *PLDβ1*-deficient plants were more susceptible than wild-type plants to the fungus *Botrytis cinerea*. The *PLDβ1*-deficient plants had lower levels of PA, JA and JA-related defense gene expression after *B. cinerea* inoculation.
- PLDβ1 plays a positive role in pathogen-induced JA production and plant resistance to necrotrophic fungal pathogen *B. cinerea*, but a negative role in the SA-dependent signaling pathway and plant tolerance to the infection of biotrophic *Pst* DC3000. PLDβ1 is responsible for the major part of PA increased in response to necrotrophic *B. cinerea* and virulent *Pst* DC3000 infection, but contributes less to the avirulent *Pst* DC3000 (*avrRpt2*)-induced PA production.

Keywords

Botrytis cinerea; *Pseudomonas syringae*; *Arabidopsis thaliana*; phospholipase Dβ1; pathogeneses; phosphatidic acid; lysophospholipids; lipid signaling

[*]Corresponding author swang@danforthcenter.org & jianzhao@mail.hzau.edu.cn.

Introduction

Plants develop different but inter-related defense systems to battle against invasion of pathogenic microorganisms (Durrant & Dong, 2004; Abramovitch et al., 2006; Jones & Dangl, 2006). One system recognizes and responds to molecules common to many classes of microbes, including non-pathogens. This defense response is initiated by recognition of a microbe or pathogen-associated molecular pattern (MAMP/PAMP) by the corresponding pattern recognition receptor, which is typically an integral plasma membrane protein. Another system of defense responds to pathogen virulence effectors, either directly or through their effects on host targets. The effector-triggered defense response involves resistance (R) proteins, usually polymorphic NB-LRR proteins that specifically recognize particular pathogen effectors and launch specific defense response, such as the Arabidopsis R protein RPS2 being recognized as the *Pseudomonas syringae* effector AvrRpt2 (Glazebrook, 2005; Jones & Dangl, 2006). The PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) are closely related and interact in an array of defense responses, such as hypersensitive response, biosynthesis of signaling molecules salicylic acid (SA), jasmonate (JA), and ethylene, and the production of pathogenesis-related proteins and phytoalexins (Durrant & Dong, 2004; Abramovitch et al., 2006;). Several parallel, yet cross-talking signaling pathways and defense strategies exist, such as SA-dependent systemic acquired resistance in avirulent bacterial infections and JA/ethylene-dependent inducible systemic resistance during necrotrophic fungal pathogen infections (Glazebrook 2005; Spoel et al., 2003, Truman et al., 2007). However, the biochemical pathways mediating the cross-talk of different defense responses are not well understood (Glazebrook, 2005; Jones & Dangl, 2006).

Different aspects of lipid metabolism and signaling play important roles in disease resistance and susceptibility. For example, suppression of α-DOX1, a 16- and 18-C fatty acid-dioxygenase, confers enhanced susceptibility to *P. syringae* (De León et al., 2002). Mutation of DIR1, a putative lipid transfer protein, renders plant deficient in systematic acquired resistance (SAR; Maldonado et al., 2002). Mutation of a plastidic stearoyl acyl-carrier protein desaturase, SSI2, results in an increased SA-mediated SAR (Nandi et al., 2004). Mutation of a phospholipase A (PLA) SOBER1 (**S**uppressor **o**f Avr**B**sT **E**licite**d** **R**esistance1) increases the resistance of Arabidopsis ecotype Pi-0 against *Ps*t DC3000 (*avrBst*). This study also indicates a potential crosstalk between the lysophospholipid-producing PLA and the phosphatidic acid (PA)-producing phospholipase D (PLD) pathways in plant bacterial pathogen interaction (Kirik & Mudgett, 2009).

PLD, which hydrolyzes membrane phospholipids to generate PA and a free-head group, is involved in cellular processes including reactive oxygen species (ROS) generation (Sang et al., 2001; Park et al., 2004); hormone signaling (Zhang et al., 2004), and disease resistance (den Hartog et al., 2003;Bargmann et al., 2006; Andersson et al., 2006; Yamaguchi et al., 2009). Plant PLD is composed of a family of heterogeneous enzymes with distinguishable catalytic and regulatory properties (Wang et al., 2006). PLDβ1 from Arabidopsis binds to Ca^{2+} and phosphatidylinositol-4,5-bisphosphate (PIP_2) and hydrolyzes phosphatidylethanolamine (PE) preferentially over phosphatidylcholine (PC) (Pappan et al., 1997). The expression of Arabidopsis *PLDβ1* was induced by both bacterial and fungal

pathogen infections (Zabela et al., 2002). Tomato *LePLDβ1* was induced by fungal elicitors and RNAi knockdown of *LePLDβ1* resulted in an increased defense response in response to fungal elicitors (Laxalt et al., 2001; Bargmann et al., 2006). Knockdown of PLDß1 in rice increased resistance against *Pyricularia grisea* and *Xanthomonas oryzae pv oryzae*. These results indicate that *PLDβ1* plays an important role in plant pathogen interactions and also raise further questions. As a phospholipid-hydrolyzing enzyme, what effect would PLDß1 have on membrane glycerolipid species without and with pathogen infection? How would a gene-knockout of PLDß1 affect SA- and JA-mediated defense response? In this study, we used *PLDβ1*-knockout and RNAi-suppressed Arabidopsis plants to investigate the effect of *PLDβ1* on Arabidopsis interaction with the bacterial pathogen *Pseudomonas syringae* and fungal pathogen *Botrytis cinerea*. The results indicate that *PLD*β*1* plays a role in promoting PA production after pathogen infection. *PLD*β1 plays a negative role in plant resistance to bacterial pathogens but a positive role in plant response to the necrotrophic fungal pathogen *B. cinerea*.

Materials and Methods

PLDβ1 T-DNA-Insertion Knockout Isolation and Complementation

A T-DNA insertion mutant in *PLDβ1* (At2g42010), designated *pldβ1-1*, was identified from the Salk *Arabidopsis thaliana* (L.) Heynh T-DNA knockout collection (Salk_079133) and seeds were obtained from the ABRC at Ohio State University**.** A *PLDβ1* homozygous T-DNA insert mutant was isolated by PCR screening using *PLDβ1*gene specific primers, 5′-ATA CCC TCC ACC TGA AAC TAA ACC GCA-3′ (forward primer) and 5′-TGTGGCTTATTGTGGAATGCATTACGA-3′ (reverse primer) and the T-DNA left border primer, 5′-GCG TGG ACCGCT TGC TGCAACT-3′. The positive T-DNA insertion lines were confirmed by sequencing. The F3 generation shows a homozygous mutation, and deficiency of *PLDβ1* transcripts was confirmed by northern blotting. *pldβ1-1* showed co-segregation with kanamycin resistance in a 3:1 ratio, indicating that the mutant had a single T-DNA insertion. For complementation of the *PLDβ1* knockout mutant, the native *PLDβ1* gene, including its own promoter region, was amplified from 1000 bp upstream of the start codon and 300 bp after the stop codon and then was cloned into the pEC291 vector. The primers for *PLDβ1* complementation were 5′-ATGGCGCGCCAGATTCTCGTCCACTGAGGA-3′ (forward) and 5′-ATGGCGCGCCTAGAGATGGGCTCTGGAGAT-3′ (reverse). The construct, pEC291-PLDgγ3, was transformed into *Agrobacterium tumafaciens* strain GV3101 via electroporation. Homozygous *pld*β*1-1* plants were transformed (Clough and Bent, 1998), the seeds were collected, and transformants were selected on medium containing 1/2MS media, 50 $\mu g^{-1} mL^{-1}$ kanamycin, and 1% agar.

PLDβ1 RNAi Construct and Generation of RNAi Suppression Lines

The sense cDNA exon (492 bp, the last exon plus part of 3′-UTR, from 2784 to 3276 bp in PLDβ1 mRNA, GeneBank accession number U84568) followed by an intron (101 bp, the last intron, from 29898-29999 bp in Arobidopsis BAC clone T6D20, GeneBank accession number U9292382) was amplified by using PCR with forward primer BIR51: 5′-CCCAAGCTTATTTAGAGTGATAATATATC-3′ (*Hind* III site underlined) and reverse

primer BIR31: 5′-CCGGAATTCAGATCTATGGATACAG AAT-3′ (*EcoR*I site underlined). Antisense cDNA (492 bp) (the last exon plus 3′-UTR from 2784 to 3276 bp in PLDβ1 mRNA, GeneBank accession number U84568) was amplified with a forward primer BIR52: 5′-CCGGAATTCTGGTCAGGTAAATCCCGCAAAC-3′(*EcoR*1 site underlined), and the reverse primer BIR32: 5′-CCGCTCGAGATTTAGAGTGATAATATATC-3′ (*Xho*I site underlined). Two PCR products were digested with *EcoR*I and then ligated into a fragment of 1100 bp containing two inverse cDNA repeats separated by an intron. This fragment was further digested with *Xho*I and *Hand* III restriction enzymes, and the resulting fragment was purified and then ligated into pKYLX71-35S^2 vector in *Xho*I and *Hand* III sites. The resulting RNAi vector was confirmed by complete sequencing and transformed into Arabidopsis by the floral dipping method. F1 and F2 seeds were screened using both kanamycin plates and PCR. Two homozygous lines *RNAi1* and *RNAi2* were finally obtained with dramatically decreased *PLDβ1* transcripts in leaves as checked by northern blotting.

Pathogen Growth and Inoculation

Arabidopsis thaliana Col-0, *pldβ1-1*, RNAi mutants, and *pldβ1-1* complemented with *PLDβ1* (*PLD β1* complementation) plants were grown in soil in growth chambers at 23/21°C and 60-80% relative humidity under 8/16 h day/night photoperiods (85 μmol·m^{-2}·s^{-1}) for pathogen inoculation, or 16/8 h day/night photoperiods (120 μmol·m^{-2}·s^{-1}) for physiological and genetic analysis. The virulent strain *Pseudomonas syringae* tomato *(Pst)* DC3000 and an avirulent strain *Pst* DC3000 carrying the avirulence gene *avrRpt2, Pst* DC3000 (*avrRpt2*) were grown in King's medium B at 28°C with appropriate antibiotics (50 mg/l of rifampicin for *Pst* DC3000 and 25 mg/l of rifampicin plus 50 mg/L of kanamycin for *Pst* DC3000 (*avrRpt2*). Five week-old, soil-grown plants were inoculated with *Pst* DC3000 and *Pst* DC3000 (*avrRpt2*). Bacterial inoculations were performed as previously described (Nandi et al., 2004). The fully expanded leaves of each plant were infiltrated with a suspension containing 10^7 colony-forming units (cfu) per milliliter of *Pst* DC3000 or *Pst* DC3000 (*avrRpt2*). In parallel, plants similarly infiltrated with 10 mM $MgCl_2$ served as the controls. At 0, 3, and 5 days post inoculation, six infected leaves were collected per genotype to measure the growth of the pathogen. Bacterial counts are expressed as colony-forming units per leaf disc. The rate of the lesion area is according to methods described before (Nandi et al., 2004). Each data point represents three replicates, with three leaf discs per replicate. Approximately 50 plants of each genotype and more than 300 leaves were treated.

Botrytis cinerea IMI169558 was cultivated on in potato dextrose broth medium at 22°C in a growth chamber. Spores were harvested and infections performed as described previously (Nandi et al., 2004). Briefly, a 10 μl drop of freshly harvested *B. cinerea* conidial spore suspension (5×10^7 spores/ml) was placed on three leaves of 4 week-old soil-grown plants pricked with a 23-gauge needle. The inoculum was allowed to air dry; plants were covered with a transparent plastic dome and cultivated at 18°C (80-100% humidity) in a growth chamber programmed for a 14 h/10 h light-dark cycle. Control plants were inoculated with potato dextrose broth medium alone. The number of leaves showing different levels of lesion was scored at 5 days post inoculation (Nandi et al., 2004). Approximately 50 plants of each genotype and more than 300 leaves were tested.

Extraction and Quantification of SA

About 0.3 g fresh leaves were harvested and quickly frozen in liquid N_2. SA extraction and determination were performed according to a method described by Nandi et al. (2004). Briefly, frozen samples were ground and extracted once with 3 mL of 90% methanol and once with 3 mL of 100% methanol. The combined extracts were dried under N_2 gas and suspended in 2.5 mL of 5% trichloroacetic acid. The samples were acid hydrolyzed by adding 200 μL of HCl and incubating at 95°C for 30 min. SA was extracted with 5 mL of a mixture containing cyclohexane: ethylacetate: isopropanol (50:50:1). The sample was dried under N_2 gas and dissolved in 0.5 mL ofthe mobile phase (69:27:4 mix of water: methanol: glacial acetic acid). Samples were filtered through a 0.22-μm filter, and 7.5 to 20 μL were used for high performance liquid chromatography. Samples were passed over a 4.6 × 250-mm C18 reverse-phase column, and SA was eluted with the mobile phase at a flow rate of 0.8 ml/min. Absorbance of the eluted samples was recorded at 310 nm, and SA concentrations were determined by comparison with SA standards.

RNA Blotting and Immunoblotting

Five week-old Arabidopsis seedlings that were treated with pathogens or a control solution (water or 10 mM $MgSO_4$) were used for gene expression analysis. Whole plants were taken for extraction of RNA (Zhao et al., 2011). Twenty micrograms of RNA was loaded and separated by formaldehyde-agrose gel electrophoresis and transferred to a hybridization membrane. ^{32}P-labeled DNA probes for pathogenesis-related genes, *PR-1* (At2g14610) and *PDF1.2* (At5g44420), *PLDα1* (At3g15730), *PLDβ1* (At2g42010), *LOX2* (At3g45140), *AOS* (At5g42650), *PR-5*(At1g75040), were generated using full-length cDNA of these genes. The immunoblotting for *PLDβ1* was performed as described previously (Zheng et al., 2002). Proteins were extracted from Arabidopsis leaves and equal amounts of proteins were subjected to 8% SDS-PAGE, followed by immunoblotting using antibodies against PLDβ1 and PLDα1. The PLD antibodies were raised against the C-terminal 13-amino acid residue peptide (Zheng et al., 2002).

Real-Time qRT-PCR

Rosette leaves of indicated age were inoculated with pathogens. Leaves were harvested before infection and mock treatments (time point 0) and at the indicated time points after treatment. Total RNA was isolated with the RNeasy plant mini kit (Qiagen, USA). RNA was reverse transcribed using a M-MLV Reverse Transcriptase kit (Life Technologies, Invitrogen) according to the manufacturer's instructions. Primer pairs listed in Supplemental Table 1 (Table S1) online were used for real-time quantification by an iQ®5 multicolor real-time PCR detection system (Bio-Rad). Individual PCR reaction mixtures contained 1 μL of diluted cDNA, 10 μL of SYBR Green Mastermix (Thermo Scientific), and 250 μM of each primer in a final volume of 10 μL. In all experiments, three biological replicates of each sample and two technical (PCR) replicates were performed. Data were analyzed with iQ®5 Optical System Software by the comparative ΔΔCT method. The amount of target genes was normalized over the abundance of the constitutive UBQ5 and Tubulin8 genes. Three biological replicates were analyzed, each consisting of 8 individually infected leaves.

Detection of O_2^- and Measurement of H_2O_2

After inoculation with bacterial or fungal pathogens for the indicated time, inoculated leaves were detached and infiltrated with nitroblue tetrazolium (NBT) for 2 h. The purple formazan precipitate indicates the location and extent of $O^{\cdot -}_2$ accumulation. For quantitative measurement of H_2O_2, frozen leaves (about 0.2 g) were ground to a powder under liquid nitrogen and homogenized with 0.5 ml of 0.2 M $HClO_4$ in a pre-cooled mortar and pestle. The extract was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was collected and neutralized to pH 7.0 to 8.0 with 0.8 M NH_4OH, and briefly centrifuged at 3000 × *g* for 5 min to sediment the insoluble material. After 8-fold dilution, H_2O_2 concentration was measured with an Amplex Red hydrogen peroxide / peroxidase assay kit (Molecular Probes, Cat. No. A22188, Eugene, OR).

Lipid Profiling and JA Measurement

The process of lipid extraction, analysis, and quantification was performed as described (Welti et al., 2002). Briefly, both bacterial and fungal inoculated leaves were collected at the sampling time and immersed immediately into 3 mL 75°C isopropanol with 0.01% butylated hydroxytoluene (BHT) for 15 min to inhibit lipolytic activities. This was followed by the addition of 1.5 mL of chloroform and 0.6 mL of water. After shaking for 1 h, the extracting solvent was transferred to a clean tube. The tissues were extracted with chloroform-methanol five times with 30-min agitation each time. The extracts were combined and washed with 1 M KCl, followed by another wash with water. The solvent was evaporated with a stream of nitrogen. The remaining plant tissues were dried in an oven at 105°C overnight and then weighed for “dry weight”, which here refers to the dry weight minus the lipid content. Lipid samples were analyzed with an electrospray ionization triple quadruple mass spectrometer (API 4000, Applied Biosystems). The molecular species of each lipid class were quantified in comparison to two internal standards as previously described (Welti et al., 2002; Devaiah et al., 2006). Five replicates of each treatment for each phenotype were processed and analyzed. The Q-test for discordant data was done on the replicates of the total lipid. Paired values were subjected to *t*-test to determine statistical significance.

JA analysis in *B. cinerea*-inoculated and control plants was carried out according to the method described previously (Yang et al., 2007; Pan et al., 2008). Leaf samples were harvested at 0, 12 and 24 h post inoculation. About 100 mg of fresh Arabidopsis leaves were sealed in 1.5 mL snap-cap vials and quickly frozen in liquid N_2. The leaf tissues were ground into powder and 500 μL of 1-propanol/H_2O/concentrated HCl (2:1:0.002, vol/vol/vol) with internal standards (50 ng) were added, and the samples were agitated for 30 min at 4°C. One ml of CH_2Cl_2 was added, and the samples were agitated for another 30 min and then centrifuged at 13,000g for 5 min. The lower phase (25 μL) was directly infused into a hybrid triple quadrupole/linear ion trap mass spectrometer (ABI 4000 Q-TRAP®, Applied Biosystems, Foster City, CA) outfitted with an electrospray (ESI) ion source.

Statistical Analysis

For most experimental data, including SA and H_2O_2 measurements, three independent experiments were performed; lipid profiling was performed with 5 repeats. The data were

analyzed using Students *t* test. The differences between two tails of data with the error bars represent 95% confidence limits.

Results

PLDβ1 deletion increased defense response and resistance to Pst DC3000

To characterize the function of *PLDβ1*, we generated RNAi mutants; two lines, *RNAi1* and *RNAi2*, with *PLDβ1* expression being suppressed to different degrees, were isolated and used in this study (Fig. 1A). RNA blotting indicated that *RNAi1* had about 20-25% of the wild-type level of *PLDβ1* transcript, whereas *RNAi2* had less than 10% of the wild-type level of *PLDβ1* transcript (Fig. 1B). Immunoblotting using *PLDβ1* antibody also confirmed that both *RNAi1* and *RNAi2* had a decreased level of PLDβ1 protein (Fig. 1C). We also isolated one knockout line (*pldβ1-1*) with the T-DNA inserted in the first exon of *PLDβ1*, 877 bp downstream of the start codon (Fig. 1D). Elimination of the *PLDβ1* transcript in *pldβ1-1* was confirmed by RNA blotting (Fig. 1D). The three *PLDβ1* mutant lines with varied levels of decreases in *PLDβ1* transcripts were used for further study. Under normal growth conditions, the *PLDβ1*-deficient plants displayed no apparent morphological alterations.

We examined the expression of *PLDβ1* in leaves treated with several compounds associated with stresses to gain insights into its function. *PLDβ1* transcript level was increased in leaves with 0.1 mM methyl jasmonate (MeJA), but decreased with 0.1 mM SA or methyl salicylate (MeSA) (Supplemental data Fig. S1A, B). When leaves were treated with 2 mM H_2O_2, the expression of *PLDβ1* was suppressed in the early phase (3 h) but increased later (20 h). On the other hand, abscisic acid (ABA) treatments induced a transient increase in *PLDβ1* expression at 6 h. When Arabidopsis leaves were inoculated with pathogens, the expression of *PLDβ1* was induced by both virulent (*P*st DC3000) and avirulent [*P*st DC3000 (*avrRpt2*)] bacterial pathogens (Fig. 2A, *left panel*). Fungal pathogen *B. cinerea* infection also markedly induced *PLDβ1* expression (Fig. 2A, *right panel*). In contrast, *PLDβ2* was not induced by the bacterial or fugal pathogens, When Col-0, *RNAi1* and *RNAi2* plants infected with pathogen *B. cinerea*, the *PLDβ1* transcript was increased in Col-0 plants, but only a small amount of *PLDβ1* transcripts were detected in the RNAi lines (Supplemental data Fig. S2).

To investigate the role of *PLDβ1*, in plant response to pathogens, we inoculated *PLDβ1* mutant and wild-type Arabidopsis plants with virulent *P*st DC3000 or *P*st DC3000 *(avrRpt2).* Most plants displayed leaf cell collapse in the pathogen-inoculated leaves within 24 h (Fig. 2B). However, the leaf cell collapse was more evident in the two *RNAi* lines and *pldβ1-1* plants than in wild-type plants, with *PLDβ1* mutants displaying both earlier development of cell collapse and larger collapse area (Fig. 2B). The accelerated cell collapse in *PLDβ1* mutants occurred with both *P*st DC3000 and *P*st DC3000 *(avrRpt2)* (Fig. 2B). Genetic complementation of *pldβ1-1* with native *PLDβ1* restored the mutant's collapses to the wild-type phenotype, suggesting that ablation of *PLDβ1* results in an enhanced resistance to biotrophic pathogen *P*st DC3000 (Fig. 2B). No symptoms occurred with control plants treated only with 10 mM $MgSO_4$. A bacterial growth assay showed that

PLDβ1 mutant plants were suppressed in bacterial pathogen growth, and the difference in pathogen growth between *pldβ1-1* and wild-type after virulent *P*st DC3000 infection was greater than that after avirulent *P*st DC3000 *(avrRpt2)* infection (Fig. 2C).

Both *RNAi* mutants and *pldβ1-1* produced higher levels of total SA (free SA and SA glycoside) in response to *P*st DC3000 infections (Fig. 3A) and *P*st DC3000 (*AvrRpt2*) infections (Supplemental data Fig. S3). These differences were evident in both inoculated (local) leaves and neighboring (distal) leaves, suggesting a systematic induction of SA production (Fig. 3B). Both quantitative RT-PCR and RNA blotting were used to evaluate defense-related gene expression in treated/non-treated samples. Consistent with the higher level of SA, the SA-inducible pathogenesis-related (PR) gene *PR1* was up-regulated in *PLDβ1* mutants (Fig. 3C; Supplemental data Fig. S4). However, *PLDβ1* mutants displayed a lower level of expression of JA biosynthetic genes *allene oxide synthase* (*AOS*), *lipoxygenase 2* (*LOX2*), and JA/ethylene-regulated defensive gene *plant defensin 1.2* (*PDF1.2*) than wild-type (Fig. 3C; Supplemental data Fig. S4).

PLDβ1 mutants increased susceptibility to Botrytis cinerea

When plants were inoculated with spores of *B. cinerea*, *PLDβ1* mutants displayed accelerated and larger necrotic lesions than did wild-type plants, as indicated by lesion areas measured at day 5 post inoculation (Fig. 4A). In addition, more infected leaves had necrotic lesions with chlorotic halos (Fig. 4B). qRT-PCR and RNA blotting analyses showed that *PLDβ1* mutants had lower levels of expression of *LOX2*, *AOS*, and *PDF1.2*, but a higher levels of PR1 expression than did wild-type in response to *B. cinerea* (Fig. 4C; Supplemental data Fig. S5).

We then measured endogenous JA and SA levels in *PLDβ1*-KO, wild-type, and *PLDβ1*-complementated plants with or without pathogen inoculation. JA levels in these plants increased greatly 24 h after *B. cinerea* spore inoculation, but the JA level of *pldβ1-1* was significantly lower than that of wild-type and the *PLDβ1*-complemented plants (Fig. 4D). Meanwhile, SA measurement results suggest that *PLDβ1*-KO and knockdown mutants all had slightly higher levels of total SA after 3 days of spores-inoculation (Fig. 4E), which is consistent with higher *PR1* expression. These results suggest that abolishing *PLDβ1* impairs fungal pathogen-induced JA production, rendering plants more sensitive to pathogen.

PLDβ1 mutation increased ROS production in response to pathogens

We examined if there was a difference between *PLDβ1* mutants and wild-type in cellular ROS accumulation during the defense response. When $O^{\cdot -}_2$ reacts with nitro blue tetrazolium (NBT), a precipitate of purple formazan forms (Rao et al., 2000) (Fig. 5A). Pathogen-treated leaves of the *PLDβ1*-deficient mutants displayed more intense blue staining than those of wild-type. The intensity of blue staining was inversely associated with the level of *PLDβ1* transcript in the two RNAi mutants and *pldβ1-1* (Fig. 5A, B). To verify the association of *PLDβ1* deficiency with ROS production, we measured H_2O_2 levels in infected leaves. *PLDβ1* mutants generated more H_2O_2 than wild-type plants in response to both bacterial pathogens, *P*st DC3000 and *P*st DC3000 (AvrRpt2), and fungal (*B. cinerea*) pathogen infections (Fig. 5C, D, Supplemental data Fig.S6). The enhanced ROS production

in *PLDβ1*-deficient plants was correlated with a stronger defensive response and more severe necrotic responses than observed in wild-type plants.

PLDβ1 ablation decreased virulent pathogen-induced production of phosphatidic acid

To determine whether ablation of *PLDβ1* changed the cellular level of PA, we quantitatively profiled phospholipids and galactolipids in Col-0, *pldβ1-1*, and complementation plants with or without pathogen treatments. There was no difference in PA levels between Col-0, *pldβ1-1*, and complementation plants without treatment. Inoculation with *B. cinerea* spores induced significant increases in PA in all three genotypes, but the PA increase in *pldβ1-1* was significantly lower than that in Col-0 and complementation plants (Fig. 6). Levels of the major PA species, 34:2-, 34:3-, 36:4-, 36:5-, and 36:6-PA, in *pldβ1-1* were significantly lower than those in wild-type plants after the fungal pathogen treatment (Supplemental data Fig. S7). .

The PA increase was also observed when the plants were treated with virulent pathogen *P*st DC3000 (Fig. 6). *P*st DC3000 infection significantly increased PA production 24 h post inoculation, but *pldβ1-1* had lower PA levels than wild-type and *PLDβ1*-complemented plants after *Pst* DC3000 infection (Fig. 6). The major PA species such as 34:2-, 34:3-, 36:4-, 36:5- and 36:6-PA were significantly lower in *pldβ1-1* than wild-type and *PLDβ1*-complementation plants treated with virulent bacterial pathogens (Supplemental data Fig. S7). Inoculation with avirulent pathogen *P*st DC3000 (*avrRpt2)* induced more PA production than did *P*st DC3000 and *B. cinerea* infections, but *pldβ1-1* did not show much difference from wild-type or *PLDβ1*-complemented plants in PA levels (Fig. 6). These results suggest that *PLDβ1* plays a different role in virulent and virulent bacterial pathogen-induced PAPA production: whereas it contributes significantly to the virulent pathogen-induced PA production, but less to the avirulent pathogen-induced PA under this assay condition.

The level of total PC, PE, PI, PG, and DGDG, were comparable in wild-type and *pldβ1-1* with or without pathogen infection (Fig. 6). However, infections resulted in different changes between wild-type and *pldβ1-1* when lipid molecular species were analyzed (Supplemental data Fig. S8). Treatment with *B. cinerea* tended to raise levels of 34:3, 36:5, 36:6 PCs and 34:3 PE, and treatment with *P*st DC3000 resulted in higher levels of 36:5 and 36:6 PC in the *PLDβ1* mutant (Supplemental data Fig S8). These higher levels of PC and PE molecules in *PLDβ1* mutants versus wild-type plants after pathogen treatments are associated with the lower levels of PA production in *PLDβ1* than wild-type. These data might suggest that PC and PE are potential *in vivo* substrates of PLDβ1.

PLDβ1-KO had elevated lysophospholipid levels in virulent pathogen-infected leaves

In contrast to the lesser increase in PA in *pldβ1-1*, higher levels of total LPC, LPE, and LPG levels were found r in *pldβ1-1* than in wild-type and complemented plants after *B. cinerea* infections, whereas without infection, *pldβ1-1* and wild-type displayed no significant difference (Fig. 7, top panel). After virulent *P*st DC3000 infection, *pldβ1-1* plants also had higher levels of LPE and LPG that wild-type (Fig. 7, middle panel). However, after the avirulent pathogen *P*st DC3000 (*avrRpt2*) infection, only LPG was significantly higher in

pldβ1-1 than wild-type, and the difference in LPG between wild-type and *pldβ1-1* was also smaller than that of virulent *Pst* DC3000-infected plants (Fig. 7, bottom panel). In addition, the avirulent *Pst* DC3000 (*avrRpt2*) infection showed an increase in lysophospholipid levels in wild-type, *pldβ1-1*, and complementation plants (Fig. 7, bottom panel). However, no such increase was observed in wild-type and complementation plants after virulent *Pst* DC3000 or *B. cinerea* infections (Fig. 7, top and middle panels).

The major LPC and LPE species were 18:3-, 18:2-, and 16:0-species (Supplemental data Fig. S9A, B), whereas 18:3-, 16:1-, and 16:0-were the major LPG species (Fig. 7D). At 24 h post inoculation with *Pst* DC3000, both 18:3-LPC and 18:2-LPC were higher in *pldβ1-1* than in wild-type and complemented plants (Supplemental data Fig. S9C). In the *pldβ1-1* plants, the formation of 16:1-LPG after pathogen inoculation was particularly apparent (Supplemental data Fig. S9C).

Discussion

Our results show that PLDβ1 is a negative regulator of the SA-dependent resistance to *Pseudomonas syringae, Pst* DC3000, but a positive regulator of the JA-dependent pathway and resistance to *B. cinerea.* The negative role of PLDβ1 was indicated by the increased production of SA, SA-inducible genes, and decreased damage of virulent bacterial infection. The positive effect of PLDβ1 on the JA-dependent signaling pathway was indicated by the decreased expression in JA-biosynthetic and responsive genes, decreased JA production, and increased damage in response to *B. cinerea* inoculation. *B. cinerea* inoculation has been reported to induce a substantial increase in JA (Truman et al., 2007, Yang et al., 2007). On the other hand, the expression of *PLDβ1* is induced by JA and also by wounding (Wang et al 2001). Of the four PLDs (α1, γ1, γ2, and β1) examined, the greatest increase for *PLDβ1* mRNA occurred 30 min after wounding, whereas that for *PLDγ1* and *γ2* transcripts was at 60 min after wounding and for *PLDα* mRNA was at 3 to 6 hr after wounding (Wang et al., 2001). However, the wounding-induced JA production in Arabidopsis peaked one hour after wounding (Wang et al., 2001). These results could mean that PLDβ1 is involved in pathogen- and wounding-induced JA production. The SA-dependent and JA/ethylene-dependent signaling pathways in Arabidopsis contribute to resistance against distinct microbial pathogens (Durrant & Dong, 2004; Spoel et al., 2003; Truman et al., 2007). Yet, a close interplay exists between the two pathways. For example, *NPR1* (*NON-EXPRESSOR OF PR GENES1*) mutant that is impaired in SA signaling produces higher level of JA and shows an increased susceptibility to pathogens (Spoel et al., 2003). The effects of PLDβ1 on plant pathogen interactions indicate that PLDβ1 and associated lipid changes are involved in the SA-dependent and JA/ethylene-dependent plant defenses against bacterial and fungal pathogens.

Treatment of plants with *P. syringae* with or without AvrRpm1 or AvrRpt2, rhizobium, fungal pathogen, or elicitors all have been reported to induce PA production (van der Luit et al., 2000; de Jong et al., 2004; Bargmann et al., 2006; Andersson et al., 2006). However, the specific PLD that is responsible for the pathogen-induced PA production was unknown. The current study shows that knockout of *PLDβ1* decreases the PA production induced by *B. cinerea* and virulent *Pst* DC3000 infection, but the loss of *PLDβ1* had no major impact on

the avirulent pathogen *P*st DC3000 (*avrRpt2)*-induced PA production. The results indicate that *PLDβ1* is responsible for a major portion of the PA generated during virulent bacterial and fungal pathogen attack, but it contributed less to the avirulent pathogen-induced PA production under the present assay condition. It is possible that other PLDs, such as PLDα1 and PLDδ, besides PLDβ1, are activated in the plant-avirulent DC3000 (*avrRpt2)* interaction, thus masking the difference in PA production between *pldβ1-1* and wild-type plants. Specifically, PLDδ has been shown to be responsible for most H_2O_2-induced PA production (Zhang et al., 2003). The avirulent infection induced more H_2O_2 production than virulent infection (Fig. S6 vs Fig. 5D), which may result in increased PLDδ activity under the DC3000 (*avrRpt2)* infection. Thus, these data indicate that other PLDs, besides *PLDβ1*, are involved in the pathogen-induced PA production, and under avirulent infection the other PLDs contribute more, thus overwhelming the effect of *PLDβ1* abrogation. However, *pldβ1-1* still displayed increased resistance to avirulent DC3000 (*avrRpt2*) infection even though difference in PA levels was undetected between *pldβ1-1* and wild-type plants. One of the explanations for this is that the PA produced by other PLDs may play a less (or a different) role in heightening disease sensitivity, than does PLDβ1-derived PA. Different PLDs and their derived PA have been shown to play distinguishable roles in plant response to different stresses (Zhang et al., 2003; 2009).

Unlike the attenuated increase in PA, the levels of lysophospholipids, LPC, LPE, and LPG in the pathogen-infected *PLDβ1*-deficient plants were higher than those of wild-type plants. This inverse relationship between PA and lysophospholipids in bacterial infection was also observed with another mutant, SOBER1, which affected Arabidopsis-*P*st DC3000 (*avrB*st) interactions (Kirik & Mudgett, 2009). SOBER1 is a PLA_2 that negatively regulates pathogen-induced PA accumulation. Both SOBER1 and PLDβ1 are negative regulators in *P*st DC3000 (AvrBst) and virulent *P*st DC3000 defense, respectively. The data could mean a cross-talk between PLD and PLA pathways in plant-pathogen interaction. Such an interaction has been also proposed to occur in plant response to wounding and senescence (Ryu & Wang, 1996; Ryu et al., 1997). LPE was reported to inhibit PLDα to decrease PA production and delay senescence (Ryu et al., 1997). It is conceivable that lysophospholipids may inhibit PLDβ and other PLDs to suppress PA production (Kirik & Mudgett, 2009). The present findings suggest that the ablation of PLDβ1 results in an increase in PLA activity. It would be of interest in future studies to determine how PLDβ and its derived PA suppress PLA and the production of lysolipids.

PLD and PA are both involved in ROS production and response (Sang et al., 2001; Park et al., 2004; Andersson et al., 2006). PLDα1-derived PA has been shown to directly interact with NADPH oxidase on the plasma membrane and promote ROS production (Zhang et al., 2009). However, the present study shows that PLDβ1-deficient plants produced a lower level of PA, yet a higher level of ROS in response to virulent bacterial and fungal pathogen. The same trend has also been observed in PLDβ1-deficient rice and tomato (Bargmann et al., 2006; Yamaguchi et al., 2009). These results suggest that PLDβ1 and its derived PA are unlikely to promote ROS production, and instead they may play a role in mediating ROS response, similar to that of PLDδ where ablation of PLDδ rendered plants more sensitive to ROS-promoted cell death (Zhang et al., 2003). *PLDβ1* mutants showed an increased HR

phenotype in response to avirulent or virulent *Pst* DC3000 infections. The effects may result from increased ROS damage in *PLDβ1* mutants compared to wild-type plants. The increased level of ROS in PLDβ1-deficient mutants may increase SA biosynthesis, which in turn enhances ROS level because SA inhibits catalase activity (Durner & Klessig, 1995; Martinez et al., 2000). Rapid biosynthesis of SA is important for plant to establish systematic resistance to *Pst* DC3000 (Durrant & Dong, 2004).

Conclusion

The present data indicate PLDβ1 plays a positive role in pathogen-induced PA production, JA accumulation, JA-dependent defense gene expression, and plant resistance to pathogenesis of necrotrophic fungal pathogen *B. cinerea*. On the other hand, PLDβ1 suppresses SA-dependent signaling pathway and defense gene expression, thus, negatively affecting plant resistance to the infection of biotrophic *Pst* DC3000. In addition, the results suggest that PLDβ1 may suppress the production of lysolipids, implying a cross-talk between the PLDβ1- and PLA-mediated signaling in plant response to bacterial and fungal pathogens. Further study is needed to determine whether the inverse relationship between PA and lysophospholipid production may underlie a chemical mechanism that modulates plant-pathogen interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mary Roth for lipid analysis and Maoyin Li for hormone analysis. The work was supported by grants from National Science Foundation (IOS-0818740; MCB-0922879) and the US Department of Agriculture to XW. Equipment acquisition and method development at the Kansas Lipidomics Research Center were funded by National Science Foundation (EPS 0236913, MCB 0455318 and 0920663, DBI 0521587), Kansas Technology Enterprise Corporation, K-IDeA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20RR16475), and Kansas State University.

Abbreviations

ABA	abscisic acid
HR	hypersensitive response
JA	jasmonic acid
ROS	reactive oxygen species
PA	phosphatidic acid
PLD	phospholipase D
PR	pathogenesis-related protein
SA	salicylic acid
SAR	systemic acquired resistance

ISR	induced systemic resistance
Pst	*Pseudomonas syringae* pv. tomato
***Pst* (*avrRpt2*)**	*Pseudomonas syringae* pv. tomato carrying the *avrRpt2* gene
MAMP/PAMP	microbe or pathogen-associated molecular pattern
PTI	PAMP-triggered immunity
ETI	effector-triggered immunity

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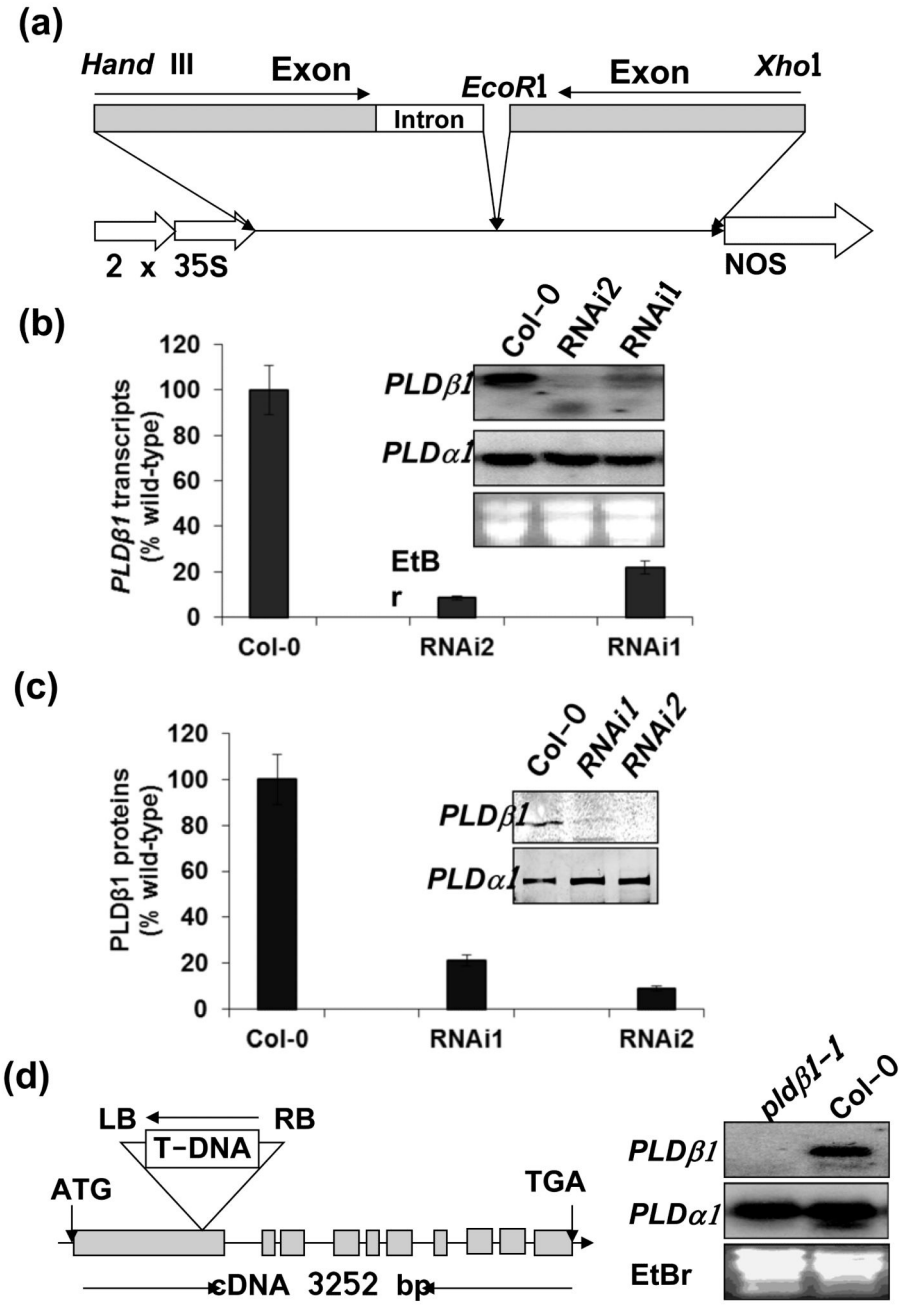

Figure 1. Production and Confirmation of *PLDβ1* RNAi Knockdown and T-DNA Insertion Knockout Arabidopsis Lines

(**a**) Construction of *PLDβ* RNAi vector. Inverted repeats of exons (gray boxes) and an intron (empty boxes) with restriction sites were cloned in tandem, and their expression was driven by double CaMV 35S promoters and terminated by an NOS terminator.

(**b**) RNA blotting of *PLDβ1* and *PLDα1* expression in *RNAi* lines. Equal amounts of total RNA from Arabidopsis leaves were loaded and rRNA detected with ethidium bromide (EtBr) was used as loading control. PLDα1 protein levels were also used as loading

controls. The density of the *PLDβ1* RNA band was quantified from three experiments. Data bars represent the mean (±SD) of three repeats.

(c) Immunoblotting of PLDβ1 and PLDα1 protein levels in *RNAi* lines. Equal amounts of Arabidopsis leaf proteins were subjected to SDS-PAGE, followed by immunoblotting using antibodies against PLDβ1 and PLDα1. The band density of PLDβ1 was quantified from three experiments. Data bars represent the mean (±SD) of three repeats.

(**d**) T-DNA insertional knockout of *PLDβ1*. Left panel, T-DNA insertion position in *PLDβ1* with exons (gray boxes) and introns (bars between exons). Right panel, northern blot showing the absence of the *PLDβ1* transcript in T-DNA insertion knockout. *PLDα1* expression and 28S RNA (detected with ethidium bromide) were used as loading controls.

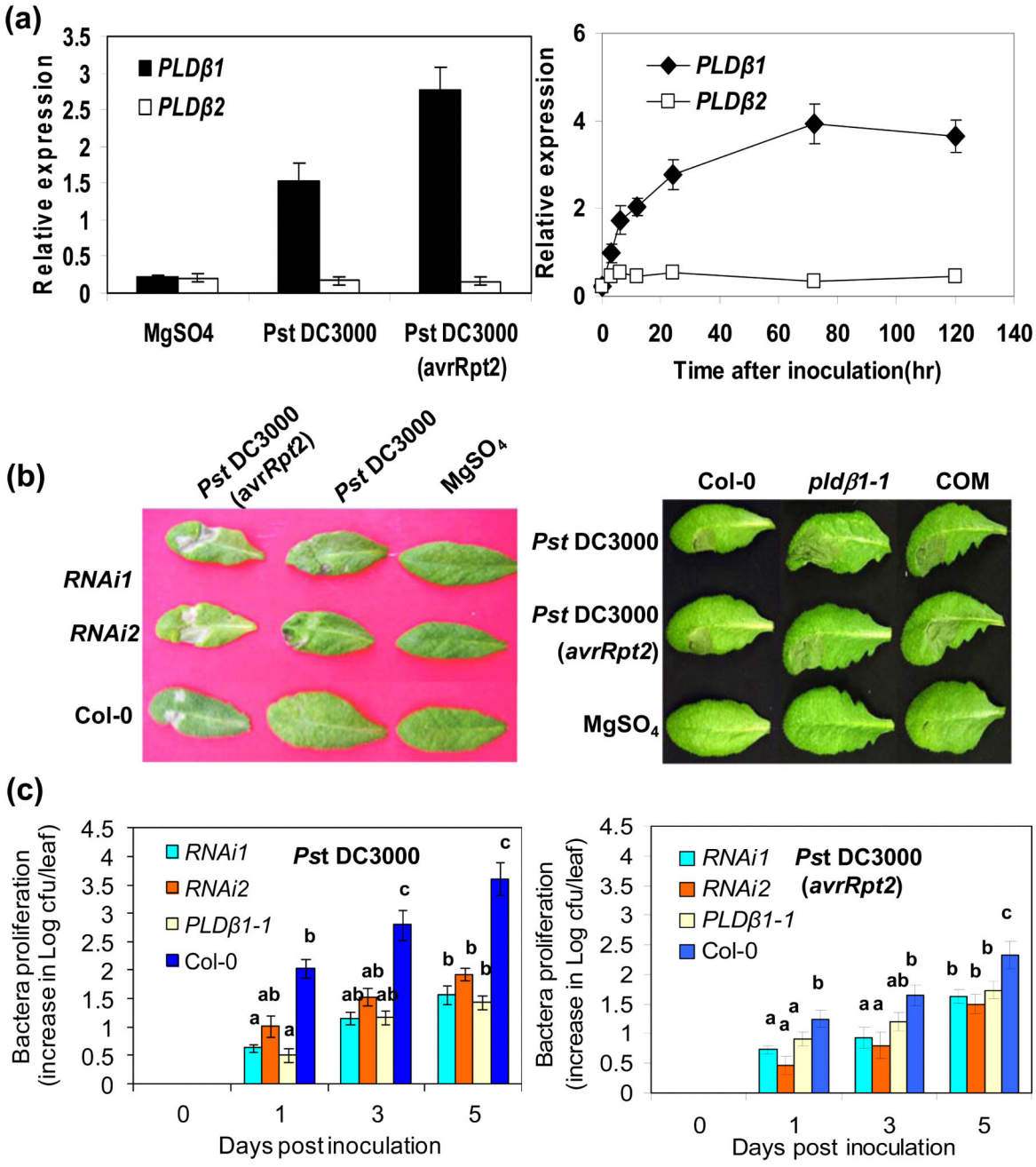

Figure 2. Responses of *PLDβ1* Arabidopsis Mutants to Bacterial Pathogens

Fully expanded leaves of Col-0, *pldβ1-1*, RNAi mutants, and *pldβ1-1* complementation (COM) plants were infiltrated with a suspension containing *Pst* DC3000 with or without *avrRpt2*.

(a) Expression of *PLDβ1 and PLDβ2* in Col-0 plants infected with *P*st DC3000 pathogens or *P*st DC3000(avr Rpt2) pathogens (2 × 107 cfu/mL) for 12 h. Leaves treated with 10 mM MgSO4 were used as a control (mock). The relative expression level of *PLDβ1* and *PLDβ2*

was analyzed by quantitative PCR. Expression levels were normalized with respect to the housekeeping genes *UBQ* and *Tubulin*. Data bars represent the mean (±SD) of three repeats.
(b) Defensive response of Arabidopsis plants with altered *PLDβ1* expression to virulent and avirulent *P*st DC3000 (2×10^7 cfu/mL). The photographs show representative leaves inoculated with bacterial pathogen for 12 h.
(c) Proliferation of the virulent *P*st DC3000 (*left panel*) and avirulent *P*st DC3000 (*avrRpt2*) (2×10^5 cfu/mL) (*right panel*) pathogens in Col-0, *pldβ1-1, RNAi* plants. Bacterial growth in five leaf discs was measured. The bacterial numbers expressed as the increase of colony-forming units (cfu) per leaf disc after bacterial inoculation (*i.e.* Log cfu post inoculation-Log cfu at time zero). Data represent the average of three samples ± SD. Different letters indicate sample groups with significant differences ($p < 0.05$) from each other.

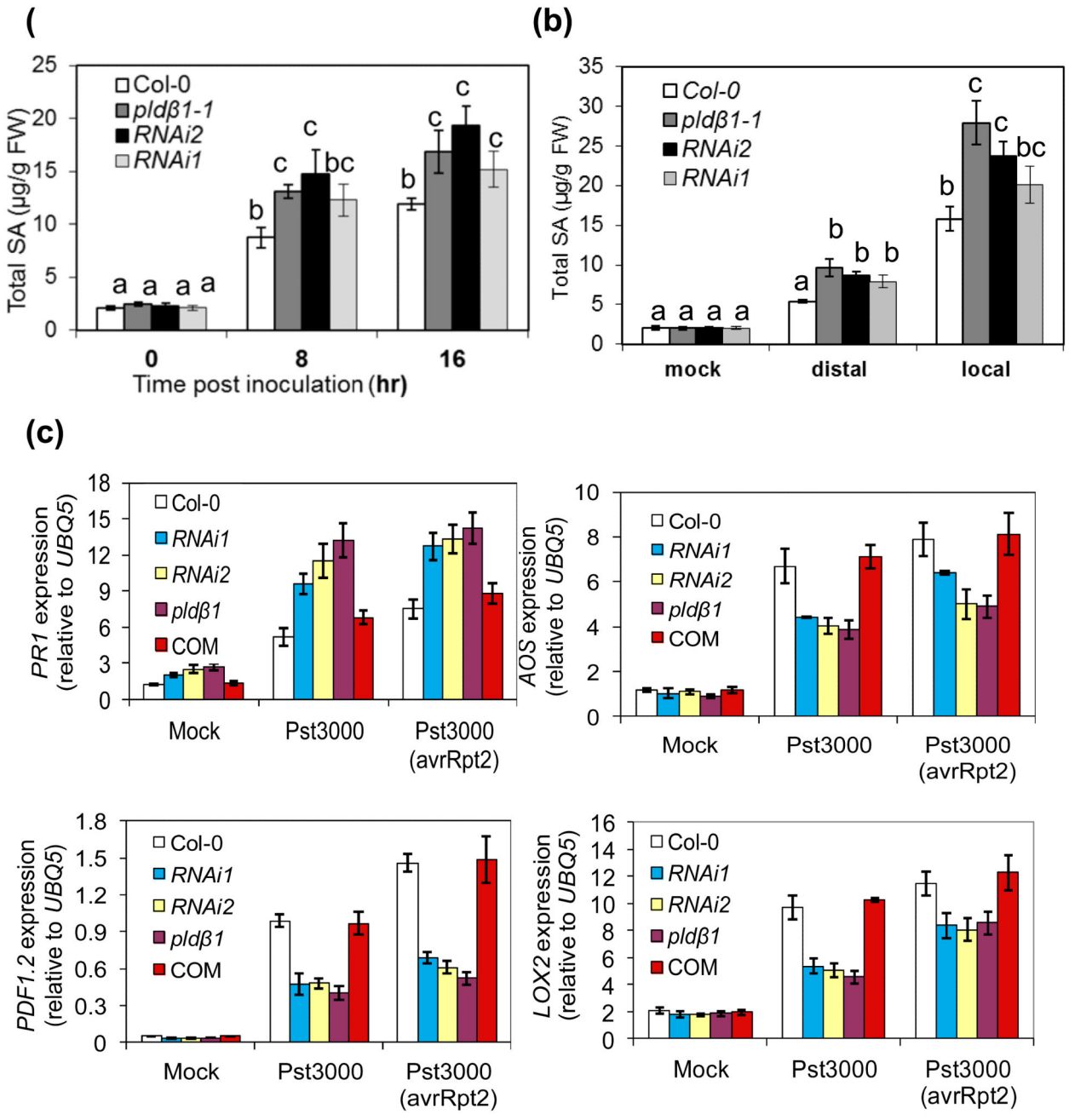

Figure 3. Salicylic Acid in Pathogen-Inoculated *PLDβ1* Arabidopsis Mutants

Total SA (free SA + SA Glycosides) in the wild-type, *pldβ1-1*, *RNAi1*, and *RNAi2* were determined by HPLC. Data are presented as means ± SD (n = 3). Different letters indicate sample groups with significant differences ($p < 0.05$) from each other.

(**a**) SA levels in inoculated leaves as a function of time post *Pst* DC3000 inoculation (2×10^7 cfu/ml).

(**b**) SA levels in leaves from local and distal leaves from *Pst* DC3000-inoculated plants for 12 h.

(**c**) Expression of defense-related genes in Col-0, *pldβ1-1*, *RNAi1*, *RNAi2*, and *pldβ1-1*-complementation (COM) plants infected with *Ps*t DC3000 pathogens (**pst**) or *Ps*t DC3000(Avr Rpt2) (**Rpt**) pathogens (2 × 107 cfu/mL) for 12 h. Leaves treated with 10 mM MgSO4 (m) were used as a control. The relative expression level of *PR-1* was analyzed by quantitative PCR. Expression levels were normalized with respect to the housekeeping genes. Data bars represent the mean (±SD) of three repeats.

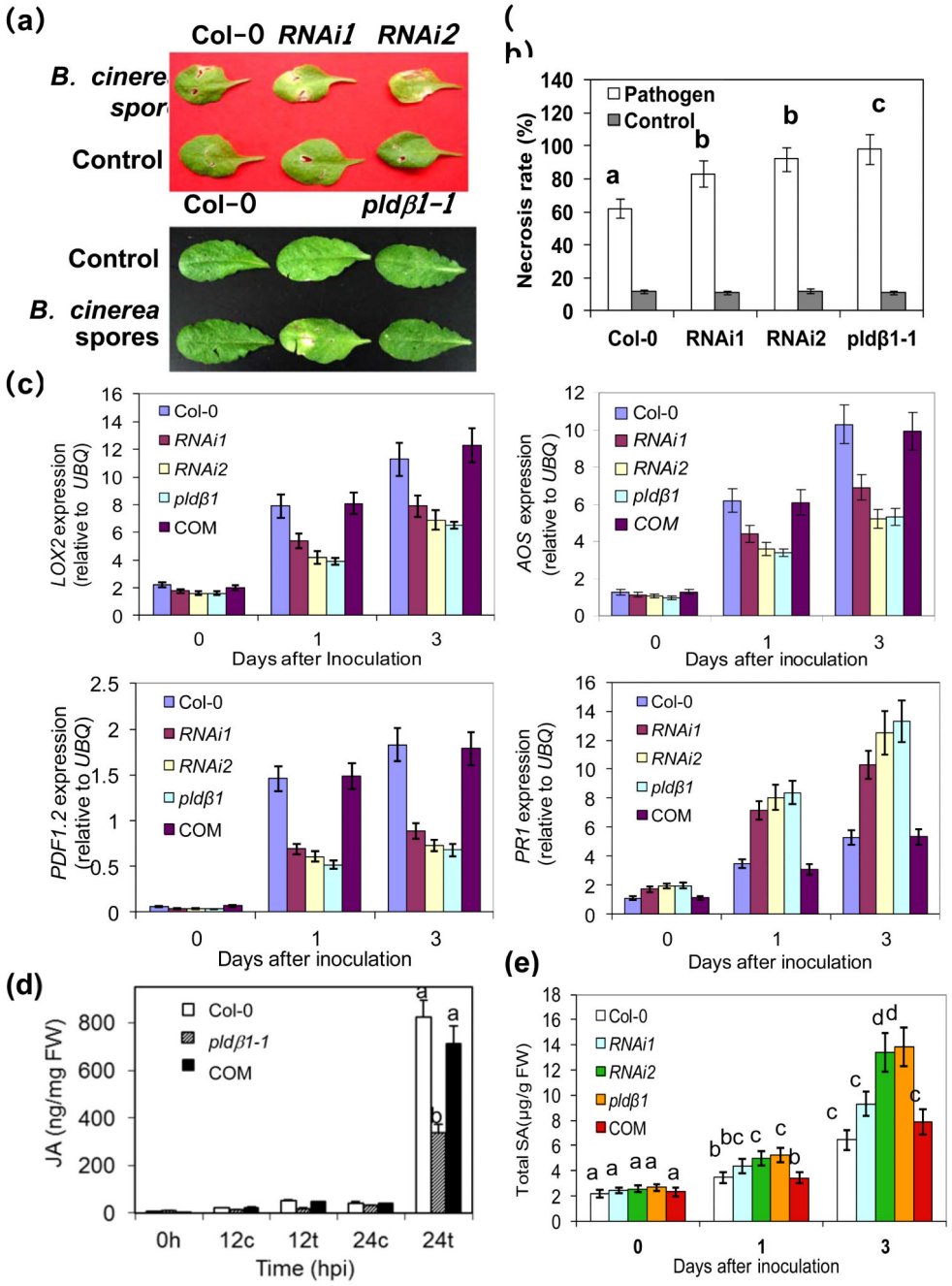

Figure 4. Responses of *PLDβ1* Arabidopsis Mutants to *B. cinerea*

(**a**) Leaves of three week-old Col-0, *pldβ1-1*, *RNAi*, and *pldβ1-1*-complementation (COM) plants were inoculated with *B. cinerea* spores (5×10^7 spores/ml) or water (control). The photographs show representative leaves 5 days post-inoculation.

(**b**) Lesions scored at 5 days post inoculation. The lesion developing rate was the number of leaves with a necrotic area among the inoculated leaves in each line. Data are presented as mean ± SD (n = 25). Different letters indicate sample groups with significant differences ($p < 0.05$) from each other.

(c) Expression of defense-related genes in Col-0 and *PLDβ1* mutants infected with fungal pathogens by quantitative PCR. Expression levels were normalized with respect to *ubiquitin 5* (*UBQ5*). Data bars represent the mean (±SD) of three repeats. Leaves treated with water were used as control.

(d) JA levels in wild-type (Col-0), *pldβ1-1*, and *pldβ1*-complementation (COM) plants at various hours post inoculation (hpi) with *B. cinerea*. C indicates control (*i.e.* mock-inoculated) and T indicated treated (*i.e.* pathogen-inoculated). Three week-old soil-grown seedlings were treated with *B. cinerea* spores. Values are means ± SE of three independent replicate experiments.

(**e**) Salicylic acid in *Botrytis cinerea* spore-inoculated *PLDβ1* mutants. Samples were harvested after inoculation *Botrytis cinerea* spores (5×10^7 cfu/m1) for different time. Total SA (free SA + SA Glycosides) in the wild-type, *pldβ1-1, RNAi1, RNAi2,* and *pldβ1-1* complementation plants were determined by HPLC. Data are presented as means ± SD (n = 3).

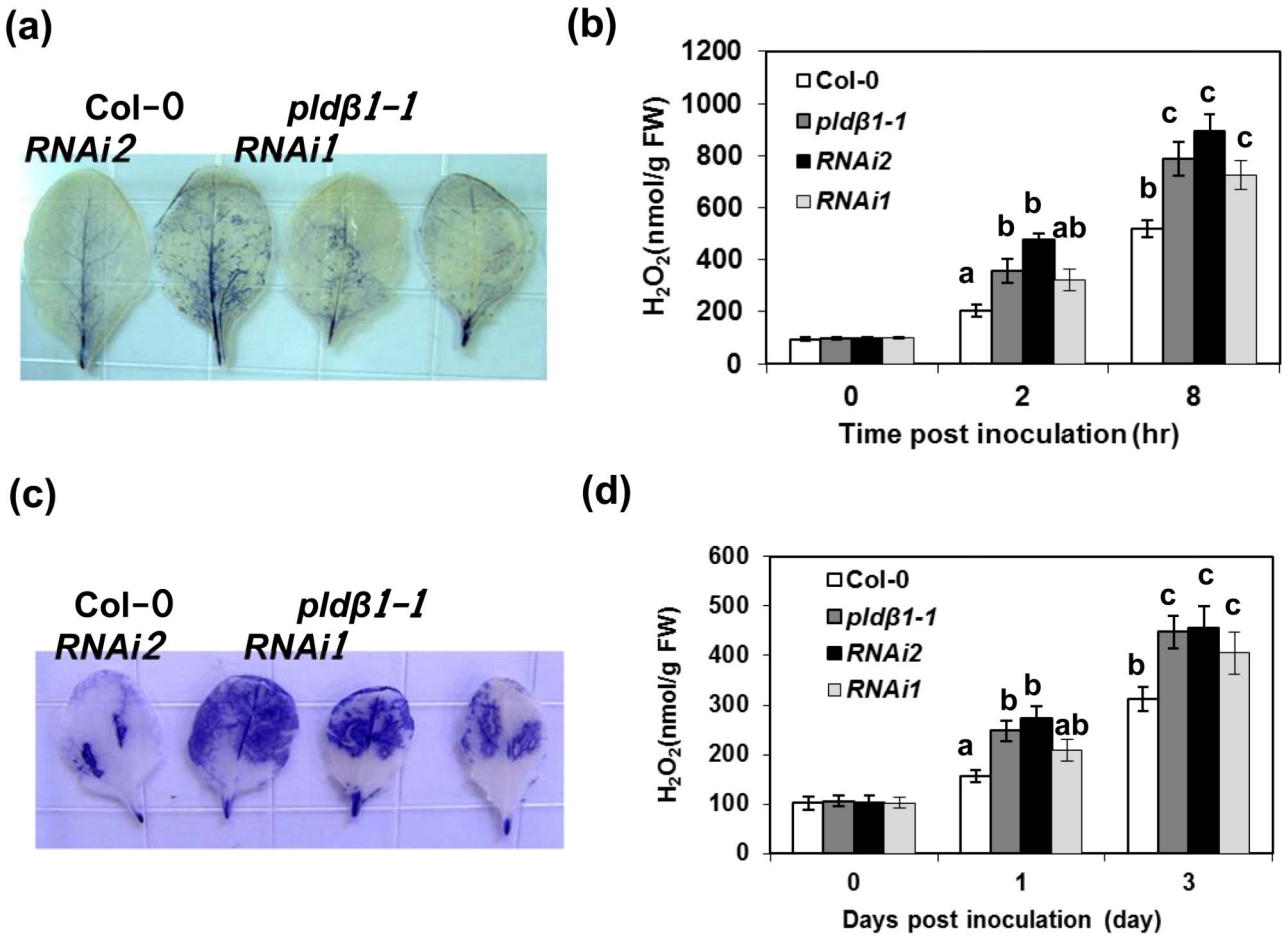

Figure 5. ROS Generation in Arabidopsis Response to Pathogen Infection

The wild-type (Col-0), *pldβ1-1*, *RNAi1*, and *RNAi2* plants were inoculated with *P*st DC3000 (2×10^7 cfu/ml) or *Botrytis cinerea* spores. Data are presented as means ± SD (n = 5).

(**a**) Nitroblue tetrazolium staining of 5-week old Arabidopsis leaves 12 h post inoculation with *P*st DC3000 bacterial pathogen (2×10^7 cfu/ml).

(**b**) H_2O_2 quantification in *P*st DC3000-infected leaves 12 h post inoculation.

(**c**) Nitroblue tetrazolium staining of 5-week old Arabidopsis leaves 12 h post inoculation with *B. cinerea*.

(**d**) H_2O_2 quantification in *B. cinerea* spore-inoculated leaves.

Different letters indicate sample groups with significant differences ($p < 0.05$) from each other.

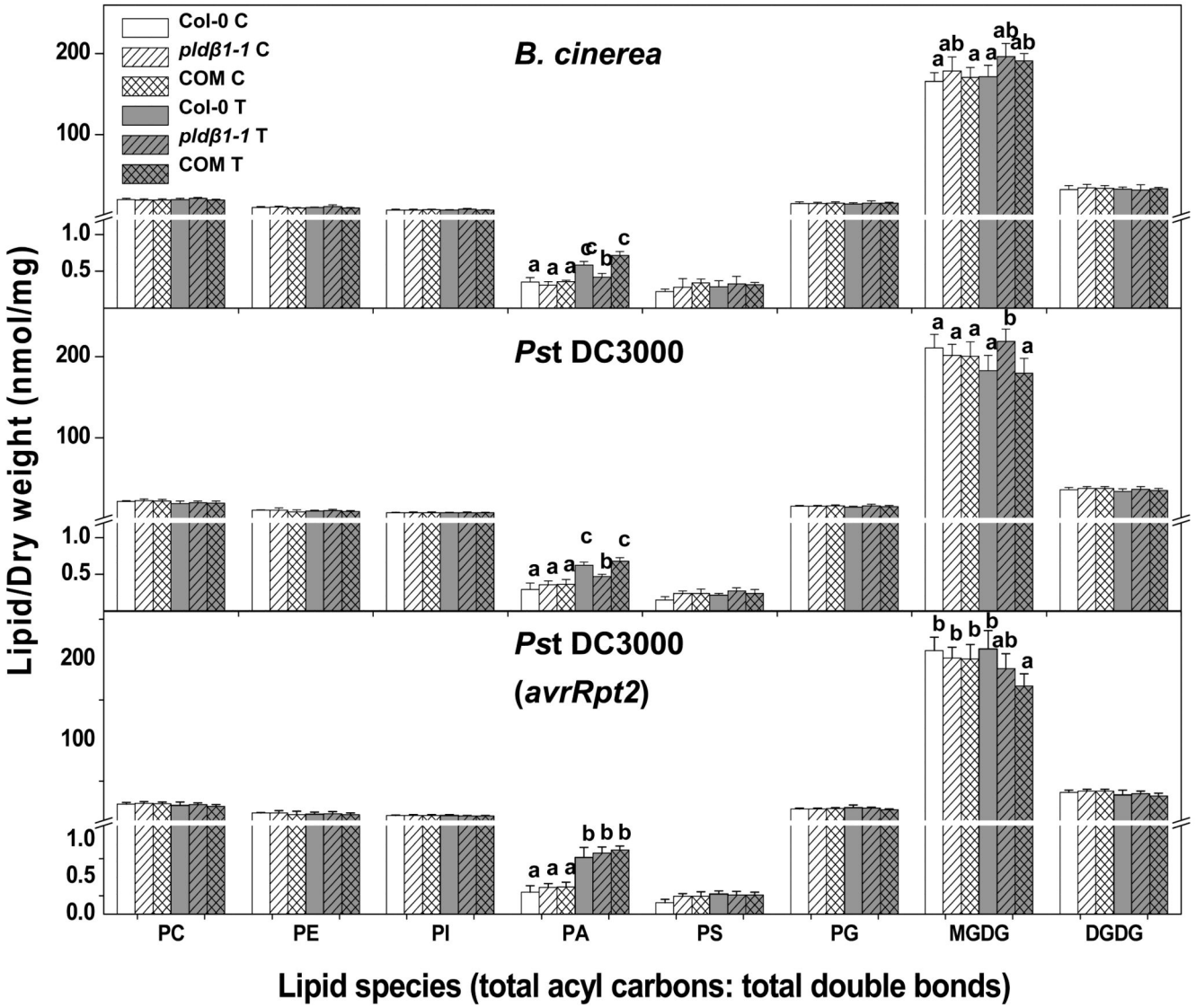

Figure 6. Phospholipid and Galactolipid Content from Leaves of Wild-type and *pldβ1-1* Arabidopsis as affected by *B. cinerea*, *Pst* DC3000, or Avirulent *Pst* DC3000 (*avrRpt2*) Infection

Col-0, *pldβ1-1*, and *pldβ1* complementation (COM) plant were inoculated with control solutions (-C) or with pathogens (-T). Control solutions were water for *Botrytis cinerea* spores (5×10^7 spores/ml) and 10 mM $MgSO_4$ for virulent strain *Pst* DC3000 and avirulent strain *Pst* DC3000 (*avrRpt2*) (2×10^7 cfu/ml). Leaf samples were collected for lipid profiling 24 h post inoculation. Values are means ± SE (n = 5). Different letters indicate sample groups with significant differences ($p < 0.05$) from each other.

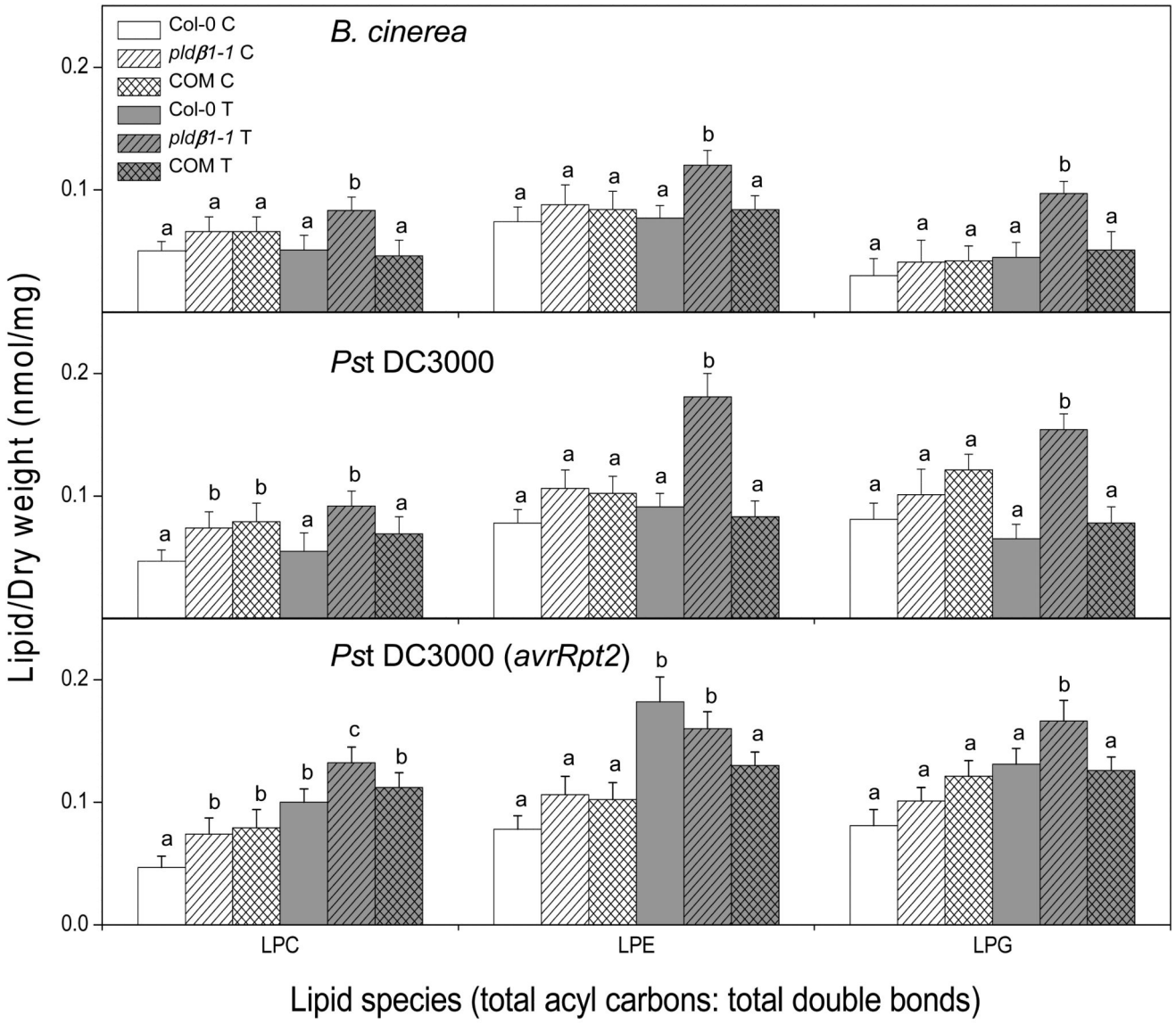

Figure 7. Levels of total LPC, LPE, and LPG from Leaves of Wild-type and *pldβ1-1* Arabidopsis with and without Inoculation of *B. cinerea*, *Pst* DC3000, or avirulent *Pst* DC3000 (*avrRpt2*)

Col-0, *pldβ1-1*, and *pldβ1*-COM plant were inoculated with control solutions (-C) or with pathogens (-T). Control solutions were water for *Botrytis cinerea* and 10 mM $MgSO_4$ for virulent strain *Pst* DC3000 and avirulent strain *Pst* DC3000 (*avrRpt2*). Leaf samples were collected for lipid profiling 24 h post inoculation. Values are means of ±SE (n = 5). Different letters indicate values with significant differences ($P < 0.05$) from each other.