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June 22, 2007

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# AtPLAI Is an Acyl Hydrolase Involved in Basal Jasmonic Acid Production and *Arabidopsis* Resistance to *Botrytis cinerea*\*

Received for publication, January 16, 2007, and in revised form, May 2, 2007. Published, JBC Papers in Press, May 2, 2007, DOI 10.1074/jbc.M700405200

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Intracellular phospholipase A<sub>2</sub> (PLA<sub>2</sub>) plays an important role in regulating oxylipin biosynthesis in mammals, but the molecular and biochemical nature of intracellular PLA<sub>2</sub> is not well understood in plants. *Arabidopsis thaliana* gene *Atlg61850* (*AtPLAI*) encodes a 140-kDa protein that is most similar to mammalian calcium-independent PLA<sub>2</sub>, and additionally contains leucine-rich repeats and Armadillo repeats. *AtPLAI* hydrolyzes phospholipids at both the *sn*-1 and *sn*-2 positions, but prefers galactolipids to phospholipids as substrates. Profiling of lipid species altered in response to the necrotrophic fungus *Botrytis cinerea* revealed decreases in the levels of phosphatidylglycerol and digalactosyldiacylglycerol, suggesting that hydrolysis of plastidic polar lipids might provide precursors for pathogen-induced jasmonic acid (JA) production. Disruption of *AtPLAI* by T-DNA insertion reduced the basal level of JA, but did not impede pathogen-induced production of JA, free linolenic acid, or hydrolysis of plastidic lipids. Still, *AtPLAI*-deficient plants exhibited more damage than wild type plants after *B. cinerea* infection, and pretreatment of plants with methyl jasmonate alleviated pathogen damage to the mutant plants. The study shows that *AtPLAI* is an acyl hydrolase, rather than a specific phospholipase A. *AtPLAI* is involved in basal JA production and *Arabidopsis* resistance to the necrotrophic fungus *B. cinerea*.

As a nonspecific necrotrophic pathogen, *Botrytis cinerea* infects more than 200 plant species (1). It obtains nutrients from dead host cells and attacks plants by producing cell wall-degrading enzymes, toxic reactive oxygen species, and host nonselective toxins, resulting in repression of host defense gene transcription, maceration of plant tissue, or host cell death (2). Plants use both pre-existing and induced defense strategies to

cope with pathogen infection (3). One important defense response is the production of oxylipins, which include a variety of oxygenated fatty acid-derived compounds (4–6). The best known oxylipins are jasmonic acid (JA)<sup>2</sup> and its derivatives, collectively called jasmonates. Jasmonates induce changes in host defense responses, including increased expression of proteinase inhibitors, phenylalanine ammonia lyase, chalcone synthase, and proline-rich cell wall proteins (7–11). JA plays a positive role in plant response to necrotrophic pathogen infection (12).

Great progress has been made in the understanding of oxylipin biosynthesis and its biological functions (4–6, 13). Linolenic acid, the most abundant fatty acid in green plant tissues, is the precursor for JA formation. However, the initial step that makes linolenic acid available for JA biosynthesis is not well understood. Because it is a component of membrane lipids, attached via an ester linkage, linolenic acid is presumed to be released from complex lipids by a lipolytic activity, such as by phospholipase A (PLA), analogous to that in animal systems (4–5, 8). In *Arabidopsis*, a link between PLA<sub>1</sub> and JA biosynthesis was reported, but the identified PLA<sub>1</sub> is involved in anther dehiscence and pollen maturation, rather than in defense-induced JA formation in vegetative tissues (14). The discovery of arabidopsides and other complex plastidic lipids containing oxylipins has led to speculation that a lipoxygenase may act on intact plastidic membrane lipids and that intact oxylipin-containing polar complex lipids may be the substrate for a hydrolytic enzyme (15–17). In any case, studies using different plant systems have implicated a role of PLA-like activity in mediating defense responses. Early studies showed that free fatty acids and lysophosphatidylcholine could modulate 1,3- $\beta$ -glucanase activity and callose deposition in suspension-cultured soybean cells (18). The production of free fatty acids and lysophospholipids is induced in various elicitations, such as cell wall extracts

\* This work was supported by the National Science Foundation Grants MCB-0455318 and IOB-0454866. The Kansas Lipidomics Research Center Analytical Laboratory was supported by National Science Foundation EPSCoR program Grant EPS-0236913 with matching support from the State of Kansas through the Kansas Technology Enterprise Corporation and Kansas State University. The Kansas Lipidomics Research Center also received Core Facility support from K-IDeA Networks of Biomedical Research Excellence (INBRE) through National Institutes of Health Grant P20RR16475 from the INBRE program of the National Center for Research Resources. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>2</sup> The abbreviations used are: JA, jasmonic acid; PLA, phospholipase A; LRR, leucine-rich repeats; GST, glutathione S-transferase; NBD, [12-(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]-dodecanoyl; OPDA, oxophytodienoic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; lyso-PG, lysophosphatidylglycerol; LOX, lipoxygenase; H<sub>2</sub>J, dihydrojasmonic acid; MJT, carboxyl methyltransferase; OPR, 12-oxo-phytyldienoic acid reductase; VSP2, vegetative storage protein; HPL, hydroperoxide lyase; AOS, allene oxide synthase; AOC, allene oxide cyclase; SA, salicylic acid; iPLA<sub>2</sub>, Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub>; cPLA<sub>2</sub>, Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub>; WT, Wassilewskija ecotype; Arm, Armadillo.

from fungus *Phytophthora parasitica* var. *nicotianae* on tobacco cells, hairpin, and fungal *Verticillium dahliae* extracts on suspension-cultured soybean cells, oligosaccharide elicitors on tomato leaves, fungal elicitors on cultured cells of California poppy, by wounding, and by systemin (19–24). However, the gene and biochemical identities of the lipolytic enzymes involved in JA production in defense response are not defined.

In mammalian cells, intracellular PLA<sub>2</sub> plays a critical role in the release of arachidonic acid for the synthesis of eicosanoid oxylipins (25, 26). Intracellular PLA<sub>2</sub> consists of two major types: Ca<sup>2+</sup>-dependent PLA<sub>2</sub> (cPLA<sub>2</sub>) and Ca<sup>2+</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>). In comparison, *Arabidopsis* has no homolog to cPLA<sub>2</sub>, but plant patatins share sequence similarities with the catalytic region of iPLA<sub>2</sub> (27). The term patatins refers to a group of closely related vacuolar storage proteins in potato tubers; these proteins possess nonspecific acyl hydrolase activity, releasing fatty acid groups from several types of lipids, including phospholipids and galactolipids (28). Patatin-like proteins are present in other plant species and have been implicated in defense responses. For example, the expression of patatin-like genes was induced in tobacco cells infected with tobacco mosaic virus, bacterium *Erwinia carotovora*, fungus *B. cinerea*, or treated with a cell death-inducing protein elicitor,  $\beta$ -megaspermin (29, 30). In addition, acyl hydrolyzing activity increased before cell death and before the accumulation of 12-oxophytodienoic acid (OPDA) and JA, suggesting that patatin-like proteins mediate the production of JA and defense responses in tobacco (29, 30). In *Arabidopsis*, the expression of the patatin-like gene *At2g26560* (*AtPLAIIA*) was shown to be induced in response to various abiotic and biotic stresses (31). However, suppression of the gene increased plant susceptibility to pathogen, implying that the patatin-like activity may facilitate, rather than suppress, pathogen infection (32). *Arabidopsis* has nine genes with sequences closely related to patatin (32, 33). But there has been no direct evidence for a positive role for patatin-like enzymes in the production of JA and in plant response to pathogens.

Another gene in *Arabidopsis*, designated *AtPLAI* (*At1g61850*) (33), encodes a protein with a patatin catalytic domain, but an overall sequence more similar to mammalian iPLA<sub>2</sub> than to other *Arabidopsis* patatin-like proteins. Whereas the other nine patatin-like genes encode proteins with 382 to 526 amino acid residues, *AtPLAI* encodes a protein about 800 amino acid residues larger than those patatins. In addition, it contains multiple leucine-rich repeats (LRR) at its N terminus. These distinctive features raise intriguing questions about the enzymatic identity and cellular functions of *AtPLAI*. Here, we show that *AtPLAI* is an acyl hydrolase that uses both phospholipids and galactolipids as substrates, that it is involved in basal jasmonic acid production, and that it promotes *Arabidopsis* resistance to the necrotrophic fungus *B. cinerea*.

## EXPERIMENTAL PROCEDURES

**Plant Materials**—*Arabidopsis thaliana* plants (ecotype Wasilewskija) were grown in growth chambers under a day/night regimen of 23/18 °C and 10/14-h photoperiod under white light of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The T-DNA insertion mutants at gene *AtPLAI* (patatin-like acyl hydrolase 1) at the locus *At1g61850*

were isolated by screening T-DNA insertion lines according to the protocol of the *Arabidopsis* Gene Knock-out Research Facility at the University of Wisconsin (34). The two mutants (*Atplai-1* and *Atplai-2*) have T-DNA inserted at the 2nd exon and the 16th exon of the gene, respectively. The sites of T-DNA insertion were confirmed by sequencing, and the homozygous plants were used in the study.

**Genetic Complementation of Mutant Plants**—A 7.8-kb genomic fragment of DNA containing the coding region of *AtPLAI* (*At1g61850*) and 1.42 kb 5'- and 0.55 kb 3'-untranslated regions was amplified by PCR. The PCR product was cloned into the *AscI* site of the pEC291 binary vector. The resulting vector was introduced into the C58C1 strain of *Agrobacterium tumefaciens*, and the *Atplai-1* mutant plants were transformed as described (35). The measurement of JA in the complemented plants and the pathogen test with *B. cinerea* were performed as described below.

**Treatment of Plants with Pathogen, JA, and Salicylic Acid (SA)**—Fungus strain *B. cinerea* was used for inoculation of 5-week-old soil-grown plants. Fungal spores were prepared and quantified as described (36). For inoculation, each leaf was pricked 3 times using a 23-gauge needle, and each wound was covered immediately with a 10- $\mu\text{l}$  drop of a suspension of 10<sup>5</sup> conidial spores/ml in potato dextrose broth medium. Inoculated plants were incubated at 18 °C under dim light at 100% relative humidity in propagator flats covered with a clear polystyrene lid for the time periods indicated. For chemical treatments, 5-week-old soil-grown plants were sprayed with a solution of 0.01% ethanol and 0.1% Triton X-100 in water containing either 0.5 mM salicylic acid (pH 6.5) or 50  $\mu\text{M}$  methyl jasmonate (pH 6.8) 48 h prior to inoculation with *B. cinerea*. Control plants were sprayed with a solution containing only 0.01% ethanol and 0.1% Triton X-100.

**cDNA Cloning and Expression of Recombinant Protein in *Escherichia coli***—The cDNA library CD4-16 was obtained from the *Arabidopsis* Biological Resource Center (ABRC) in Ohio State University. The full-length cDNA of the *AtPLAI* gene was obtained with PCR using the cDNA library as a template and primers of forward 5'-CGCGTCGACTGATGTCTTCTCATGTTC-3' and reverse 5'-CTTGCGGCCGCGTCTTTC-TATACATTAGG-3'. The cDNA was cloned into a pGEMT-vector (Promega) and both strands were sequenced. The coding sequence of *AtPLAI* (3798 bp) was amplified with PCR and cloned into the *SmaI* and *Sall* sites of the pGEM-4T-1 vector (Amersham Biosciences) after the glutathione *S*-transferase (GST) coding sequence. The sequence of the GST fusion construct was verified before it was introduced into *E. coli* strain BL-21(DE3). The cells were grown to an A<sub>600</sub> of 0.7 and induced with 0.4 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside for 12 h at 22 °C. The purification of the GST fusion protein was performed as described previously (37) and the protein concentration was determined with the Bradford method (38). The purity of the protein was analyzed by 8% SDS-PAGE, followed by Coomassie Blue staining.

**Enzyme Activity Assay Using Phospholipid Substrates**—Lipids fluorescently labeled at the *sn*-2 position, 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl-amino)dodecanoyl]-phospholipids (NBD-lipids), including NBD-phosphatidylcholine



(PC), NBD-phosphatidylethanolamine (PE), NBD-phosphatidylglycerol (PG), and NBD-phosphatidic acid (PA) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). To prepare the lipid substrate, NBD-lipids in chloroform were dried under a nitrogen stream and emulsified in distilled water containing 0.05% Triton X-100 by sonication. Acyl hydrolyzing activities were assayed in a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, and 0.05% Triton X-100. Sixty  $\mu$ M NBD-lipids were used as substrate and 10  $\mu$ g of purified protein was added to the mixture in a final volume of 200  $\mu$ l. The reaction was incubated at 30 °C for 60 min and was then stopped by adding 700  $\mu$ l of chloroform/methanol (2:1, v/v) and 200  $\mu$ l of 2 M KCl. After vortexing and separation by centrifugation, chloroform was evaporated from the organic phase under a nitrogen stream; the lipid was dissolved in chloroform and spotted on a TLC plate (Silica Gel 60, Merck, Darmstadt, Germany). The TLC plate was developed in chloroform/methanol/ammonium hydroxide/water (65:39:4:4, v/v/v/v). Lipids on the plate were visualized under ultraviolet light. The spots corresponding to free fatty acids and lysophospholipids were scraped into vials. The lipid in the vials was extracted with methanol and their fluorescence was measured at 460 nm (excitation) and 534 nm (emission). For activity assays using radioactive phosphatidylinositol (PI) as a substrate, 0.6  $\mu$ Ci of 1-stearoyl-2-arachidonoyl-1-[arachidonoyl-<sup>14</sup>C]PI (PerkinElmer Life Sciences) and 3  $\mu$ mol of unlabeled soy PI (Sigma) were mixed in chloroform, the solvent was evaporated under a nitrogen stream, and the lipid was emulsified in 0.5 ml of H<sub>2</sub>O containing 0.05% Triton X-100 by sonication. The enzyme assay was performed as described above, using 20  $\mu$ l of the radioactive substrate. After separation on a TLC plate, the lipids on the plate were visualized by exposure to iodine vapor. The spots corresponding to the lipid standards were scraped into vials, and their radioactivity was determined by liquid scintillation counting.

**Galactolipid Preparation and Enzyme Assays**—Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were purified from *Arabidopsis* leaves. Lipid was extracted from young *Arabidopsis* leaves and separated as described (39). For enzyme assays, the solvent of the lipid extract was evaporated under a nitrogen stream, and MGDG or DGDG was emulsified in water containing 0.05% Triton X-100 by sonication. The enzyme assay mixture contained 50 mM Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, 0.05% Triton X-100, 100  $\mu$ M galactolipid substrates, and 10  $\mu$ g of enzyme protein in a final volume of 200  $\mu$ l. The reaction was incubated for 1 h at 30 °C and then stopped by adding 700  $\mu$ l of chloroform/methanol (2:1, v/v) and 200  $\mu$ l of 2 M KCl. Lipids from the chloroform phase were separated by TLC in chloroform/methanol/ammonium hydroxide/water (65:39:4:4, v/v/v/v). Lipid spots were visualized by exposure to iodine vapor. Free fatty acid spots were scraped and methylated by adding 1.5 ml of 1.5% sulfuric acid in methanol containing 0.01% butylated hydroxytoluene and triheptadecanoin (C17:0) as an internal standard and heating at 90 °C for 60 min. Methylated fatty acids were extracted by successively adding 1.0 ml of H<sub>2</sub>O and 1.0 ml of hexane. Fatty acid methyl esters were quantified using an Agilent 6890 gas chromatograph with flame ionization detection and an HP-

INNOWax column (30 m length  $\times$  0.25-mm inner diameter, 0.25- $\mu$ m film thickness; Agilent). Also, the non-enzymatic product formation was subtracted from the enzyme-produced products.

**Preparation of Oxylin-rich Total Lipid from Wounded Plants and Enzyme Assay**—Leaves of 4-week-old plants were wounded mechanically with a forceps and total lipids were extracted 45 min after wounding as described (39). The lipids were emulsified in distilled water containing 0.05% Triton X-100 by sonication and used as a substrate. 30  $\mu$ g of the lipids (1  $\mu$ g/ $\mu$ l) and 5  $\mu$ g of purified AtPLAI were added to each reaction in a final volume of 200  $\mu$ l. The other assay conditions for acyl hydrolyzing activities were as described above. Control reactions contained the same amount of lipids and empty vector bacterial proteins and were subjected to the same purification process as those reactions containing AtPLAI. After the reaction the lipids were extracted and the organic phase was analyzed by the mass spectrometry.

**Real Time Quantitative Reverse Transcriptase-PCR**—RNA was isolated from liquid nitrogen-frozen plant leaves using a cetyltrimethylammonium bromide extraction method as described previously (40). Ten micrograms of RNA was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad) containing a blend of oligo(dT) and random hexamer primers. Quantitative reverse transcriptase-PCR was performed on 50 ng of cDNA with the iQ™ SYBR Green Supermix (Bio-Rad). Amplification of the *Arabidopsis* polyubiquitin gene (*UBQ10*) transcripts derived from gene *At4g05320* were used as reference. The gene specific primer pairs used for the PCR are (from 5' to 3'): *AtPLAI* (*At1g61850*): forward, ACACCGACAAGTGAC TGGGAAGATAG, reverse, GTACCTGAGCTTTCGATGAGAGGCAA; lipoxygenase2 (*LOX2*, *At3g45140*): forward, AGAGCTGGAGCTGGTGTGTGTTAAG, reverse, GAACACCCATTCCGTAACACCAT; lipoxygenase 3 (*LOX3*, *At1g17420*): forward, TATGGATTTGCGGCAGAGATCGGA, reverse, AGGCTCAGAACTCGGAACCAACAA; allene oxide synthase (*AOS*, *At5g42650*): forward, TGGTGGCGAGGTTGTTTGTGATTG, reverse ATTAACGGAGCTTCCTAACGGCGA; allene oxide cyclase 1 (*AOC1*, *At3g25760*): forward, CTTAAGCCCAGTGGAGTGTGAAGC, reverse, TATACAGGACACGAGAAAGATAAGACT; allene oxide cyclase 2 (*AOC2*, *At3g25780*): forward, GCACTGGAG7CCTAGCGGAGTTATAAGT, reverse, TATATCATTACACAGCGATACGAGAAAC; hydroperoxide lyase (*HPL*, *At4g15440*): forward, CGTCGAACAAA-CAGTGTGCAGCTA, reverse, TAAATCGGCAACGAGCAAGGAAGC; 12-oxo-phytodienoic acid (OPDA) reductase (*OPR3*, *At2g06050*): forward, CGGATTTGGTTTTCGCGGTTCAAGA, reverse, TCCGTGTAGCCAACAACCTGGA-TCT; jasmonic acid carboxyl methyltransferase (*MJT*, *At1g19640*): forward, TATGTAAGCTCGCCACGATAC-GCT, reverse, AACACGATCAACCGGCTCTAACGA; *PR1* (*At2g14610*): forward, CGTGGGAATTATGTGAACGAG-AAGCC, reverse, CACGTCCTTTATGTACGTGTGTA-TGC; polyubiquitin (*UBQ10*, *At4g05320*): forward, CACA-CTCCACTTGGTCTTGCGT, reverse, TGGTCTTTCCGG-TGAGAGTCTTCA. PCR amplification was performed with

a MyiQ<sup>TM</sup> Single Color Real Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions.

**Analysis of JA and Free Linolenic Acid**—Approximately 50–100 mg of fresh *Arabidopsis* tissues were sealed in 1.5 ml of snap-cap vials. After being frozen in liquid nitrogen, the leaves were ground to powder, and 500  $\mu$ l of 1-propanol/H<sub>2</sub>O/concentrated HCl (2:1:0.002, v/v/v) with internal standards was added, followed by agitation for 30 min at 4 °C. Dichloromethane (1 ml) was added, followed by agitation for another 30 min and then centrifugation at 13,000  $\times$  *g* for 1 min. The bottom layer was used for JA and linolenic acid analysis. Dihydrojasmonic acid (H<sub>2</sub>JA) and heptadecanoic acid (C17:0) were used as internal standards for JA and linolenic acid quantification, respectively.

Plant extracts were first separated by high performance liquid chromatography, equipped with a reversed-phase column (C18 Gemini 5 $\mu$ , 150  $\times$  2.00 mm, Phenomenex, CA), using a binary solvent system composed of water with 0.1% formic acid and methanol with 0.1% formic acid as a mobile phase at a flow rate of 0.3 ml/min and a gradient of linearly increasing methanol content from 30 to 100% at 30 min. A hybrid triple quadrupole/linear ion trap mass spectrometer (ABI 4000 Q-Trap, Applied Biosystems, Foster City, CA) outfitted with an electrospray ion source, was used in multiple reaction monitoring mode. JA, H<sub>2</sub>JA, linolenic acid, and C17:0 eluted at 12.44, 13.74, 20.7, and 22.01 min, respectively.

JA, linolenic acid, H<sub>2</sub>JA, and C17:0 can easily lose one proton from the carboxylic group and become negatively charged precursors, so they were analyzed in negative scan mode. An appropriate precursor-to-product ion transition that represents a major fragmentation path and is unique for each phytohormone was identified, and MS/MS conditions, including collision energy, collision cell exit potential, declustering potential, and entrance potential were optimized to produce maximal signal. The unique precursor ions and product ions were identified using authentic compounds. Precursor ions ([M-H]<sup>−</sup>) of JA, linolenic acid, H<sub>2</sub>JA, and C17:0 are *m/z* 209, 277, 211, and 269, respectively, which were monitored by Q1. After collision-induced dissociation in Q2 under the optimized conditions, the precursor ions of JA, linolenic acid, H<sub>2</sub>JA, and C17:0 (*m/z* ratio of 209, 277, 211, and 269) give rise to a major fragmentation ion of *m/z* 59, 259, 59, and 251 for JA, linolenic acid, H<sub>2</sub>JA, and C17:0 in the MS/MS spectra of authentic compounds. Therefore, the diagnostic product ions *m/z* ratios of 59, 259, 59, and 251 were selected to be monitored by Q3, and the precursor-to-product ion transitions of 209  $\rightarrow$  59, 277  $\rightarrow$  259, 211  $\rightarrow$  59, and 269  $\rightarrow$  251 are used to quantify JA and linolenic acid. The peak area of the main diagnostic product ion was used for quantification.

**Lipid Profiling**—The processes of lipid extraction, lipid analysis, and lipid quantification were performed as described (41, 42). Briefly, after inoculation of *B. cinerea*, leaf samples were harvested for lipid analysis at the indicated time intervals. Lipid samples were analyzed on an electrospray ionization triple quadrupole mass spectrometer (API 4000, Applied Biosystems, Foster City, CA). The molecular species of phospholipids and galactolipids were quantified in comparison to the two internal standards using a correction curve determined between stand-

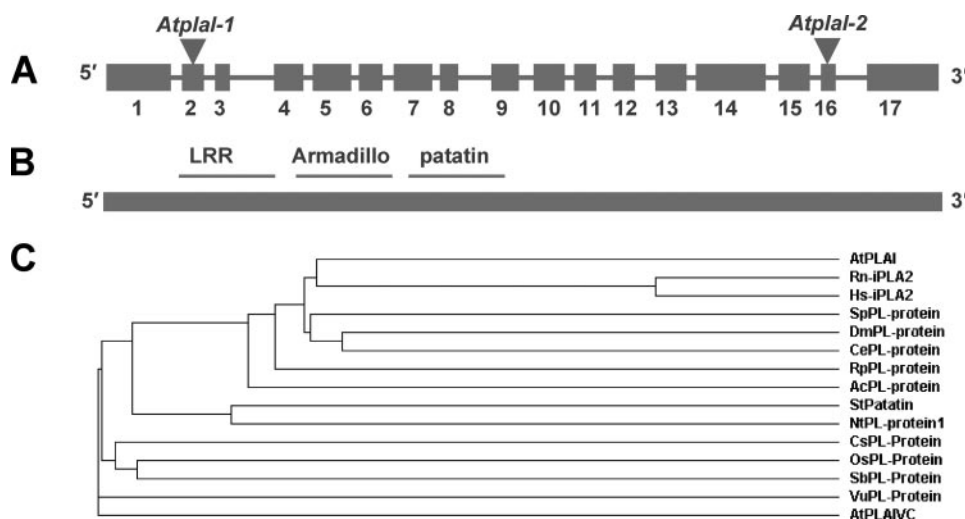
ards (41–43). Five replicates of each treatment for each phenotype were processed and analyzed. Paired values were subjected to Student's *t* test to determine the statistical significance.

Multiple acyl precursor and head group neutral loss ion scanning was used to quantify the levels of major oxylipin containing galactolipid molecular species (17). Briefly, scans of the head group and oxylipin acyl anions were performed in the oxylipin-containing galactolipid mass range (*m/z* 750–1050). Head group neutral loss scans of 179 and 341 (to identify the [M + NH<sub>4</sub>]<sup>+</sup> ions of MGDG and DGDG, respectively) were used to quantify the total amount of the oxylipin-containing species of each nominal mass. By integrating the specific precursor oxylipin acyl chain scans with the neutral loss head group scan, the individual molecular species were quantified. For quantification purposes, internal standards of 16:0/18:0 MGDG (2.01 nmol), 18:0/18:0 MGDG (0.39 nmol), 16:0/18:0 DGDG (0.49 nmol), and 18:0/18:0 DGDG (0.71 nmol) were used.

For analysis of AtPLAI hydrolysis *in vitro* using the mixture of leaf lipids, total lipids were prepared from *Arabidopsis* leaves as described (39). Solvent was evaporated from the lipids in chloroform under a nitrogen stream, and the lipids were emulsified in distilled water containing 0.05% Triton X-100 by sonication. Acyl hydrolyzing activities were assayed as described above. Thirty  $\mu$ l of lipid mixture ( $\sim$ 1  $\mu$ g/ $\mu$ l) were used as substrate and 10  $\mu$ g of purified AtPLAI was added to the mixture in a final volume of 200  $\mu$ l. Control reactions contained the same amount of lipids and empty vector bacterial proteins that were subjected to the same purification process as AtPLAI. After the reaction, the resulting lipids were extracted and the organic phase was analyzed by the triple quadrupole mass spectrometry as described above. The differences in lipid content between the control and AtPLAI reactions were divided by the amount of enzyme to calculate the specific activity.

## RESULTS

**AtPLAI Hydrolyzes Phospholipids and Galactolipids**—A full-length cDNA of AtPLAI has a complete open reading frame for a protein of 1257 amino acid residues, with a predicted pI of 5.55 and molecular weight of 139,279. The coding sequence agrees with that of the annotated AtPLAI gene with 17 exons and 16 introns, a total exon length of 3798 bp, and an intron length of 2,111 bp (Fig. 1A). The sequence of AtPLAI is most similar to intracellular calcium-independent PLA<sub>2</sub> $\gamma$  (iPLA<sub>2</sub> $\gamma$ ) in humans and rats, but it also contains unique domain structures (Fig. 1, B and C). AtPLAI contains one patatin domain between residues 456 and 702 and two other identifiable domains: four LRR from residues 134–156, 157–179, 180–202, and 203–225; and three repeats of Armadillo (Arm) between residues 269–310, 355–393, and 394–435 (Fig. 1B). The last Arm repeat is about 20 amino acid residues upstream of the patatin domain. The Arm repeat is a  $\sim$ 40 amino acid tandemly repeated motif first identified in the *Drosophila melanogaster* segment polarity gene *armadillo* (44). The amino acid sequence of AtPLAI is 33% identical and 51% similar to human iPLA<sub>2</sub> $\gamma$ . AtPLAI is more closely related to a family of proteins including iPLA<sub>2</sub> from mammals, nematode, fruit fly, yeast, and bacteria, than to the group of nine patatin-like proteins in *Arabidopsis* (Fig. 1C).



**FIGURE 1. *Arabidopsis* AtPLAI gene, T-DNA insertion, protein domains, and sequence similarities with proteins in other organisms.** A, gene structure of AtPLAI. The exons are labeled numerically. The sites of two T-DNA insertion mutants are indicated by the solid triangle. B, domain structure of the deduced AtPLAI protein. The bars represent the relative length of each domain or domains. C, cladogram of patatin-like proteins from various organisms based on protein sequences obtained with CLUSTALW program from the ExPASy Proteomics Server. AtPLAI, *Arabidopsis* patatin-like acyl hydrolase I (At1g61850); AtPLAIVC, *Arabidopsis* patatin-like gene (At4g37050); StPatatin, a potato class I patatin precursor (P11768, *Solanum tuberosum*); NtPL-Protein, a patatin-like protein from tobacco (T03841, *Nicotian tabacum*); CsPL-Protein, a patatin-like protein from cucumber (T10260, *Cucumis sativa*); OsPL-Protein, a rice patatin-like protein (AAP04195, *Oryza sativa*); SbPL-Protein, a sorghum patatin-like protein (AAD22170, *Sorghum bicolor*); VuPL-Protein, a cowpea patatin-like protein (AAK18751, *Vigna unguiculata*); AcPL-Protein, a putative patatin-like protein from bacteria *Anabaena circinalis* (CAC01602); CePL-protein, a putative patatin-like protein from *Caenorhabditis elegans* (AAK68250); DmPL-protein, a putative patatin-like protein from *D. melanogaster* (AAF50194); Hs-iPLA2 $\gamma$ , human intracellular membrane-associated calcium-independent phospholipase A $_2$  $\gamma$  (EAL24384, *Homo sapiens*); Rn-iPLA2 $\gamma$ , a putative rat intracellular membrane-associated calcium-independent phospholipase A $_2$  $\gamma$  (XP\_234092, *Rattus norvegicus*); RpPL-protein, a patatin B1 precursor from bacteria *Rickettsia prowazekii* (CAA15046); SpPL-protein, a putative patatin-like protein from fission yeast (CAB16355, *Schizosaccharomyces pombe*).

AtPLAI contains the conserved serine hydrolase motif GX SXG at residues 486–490, and a conserved Asp residue at 681. These serine and aspartic acid residues constitute a catalytic dyad essential for the acyl hydrolase activity of this family of lipases (27, 45). To determine whether AtPLAI encodes an active enzyme, AtPLAI was fused to a GST and expressed in *E. coli*. A protein of 165 kDa corresponding to the predicted size of the GST-AtPLAI protein was purified to apparent homogeneity (Fig. 2A) and was assayed for its ability to hydrolyze various phospholipids. Fluorescent phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylserine (PS), were used as substrates, and the formation of free fatty acids and lysophospholipids was quantified. Both fluorescent free fatty acids and lysophospholipids were generated by the enzyme (Fig. 2B). Because the fluorescent label NBD was at the *sn*-2 position, the detection of both fluorescent lysophospholipids and fluorescent free fatty acid indicates that the enzyme can release fatty acids at both *sn*-1 and *sn*-2 positions.

To verify the hydrolytic activity at *sn*-1 and *sn*-2 positions, radioactive 1-stearoyl-2-arachidonyl-1-[*arachidonyl*- $^{14}$ C]]PI was used. Consistent with results from the fluorescent lipid assays, both radioactive free fatty acid and lyso-PI were generated (Fig. 2C). The amount of [ $^{14}$ C]lyso-PI formed was 5-fold higher than that of [ $^{14}$ C]fatty acid, suggesting that the enzyme prefers hydrolysis of PI at the *sn*-1 to *sn*-2 position. The relative amount of NBD-fatty acids versus NBD-lysophospholipids indicates that the enzyme preferred to release the fatty acid at

the *sn*-1 position as compared with the *sn*-2 position when PC, PE, or PG was the substrate but favored the *sn*-2 position when PA or PS were the substrates (Fig. 2B). The enzyme also hydrolyzed galactolipids, MGDG and DGDG (Fig. 2D). The activity of AtPLAI toward galactolipids was actually much higher than that of all the phospholipids tested (Fig. 2, B–D).

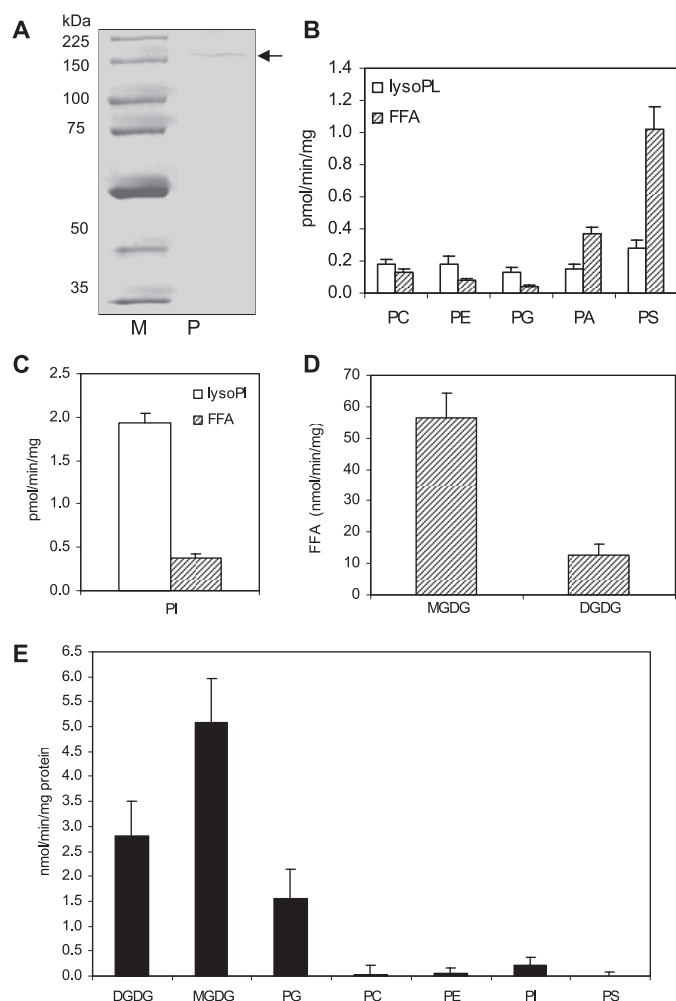
To determine lipid selectivity, total lipids extracted from plant leaves were used as substrates for the enzyme, and the resulting decrease in phospholipids and galactolipids was analyzed with electrospray ionization triple quadrupole mass spectrometry. The lipids that AtPLAI hydrolyzed most were MGDG, DGDG, and PG (Fig. 2F), which are predominantly localized in chloroplasts. By comparison, AtPLAI displayed much lower activity toward PC or PE, the two most common extraplasmidic phospholipids in leaves. Taken together, these results indicate that AtPLAI is an acyl hydrolase preferring galactolipids,

rather than strictly an A-type phospholipase.

To investigate if AtPLAI hydrolyzed oxygenated galactolipids, an oxylipin-rich lipid mixture prepared from wounded *Arabidopsis* leaves was used as substrates. The major OPDA-containing galactolipid species in the substrate were arabidopside species, A (OPDA/dnOPDA MGDG), B (OPDA/OPDA MGDG), C (OPDA/dnOPDA DGDG), and D (OPDA/OPDA DGDG) (17), and their amounts (mean  $\pm$  S.D.) were  $0.092 \pm 0.033$ ,  $0.093 \pm 0.01$ ,  $0.035 \pm 0.014$ , and  $0.493 \pm 0.066$  nmol/reaction, respectively. AtPLAI hydrolyzed both oxylipin- and non-oxylipin-containing galactolipids (Fig. 3). However, the percentage of oxylipin-DGDG and -MGDG hydrolyzed by the enzyme was approximately two to three times higher than that of non-oxylipin-DGDG and -MGDG (Fig. 3B). These results indicate that AtPLAI can use diverse lipid species including arabidopsides as substrates, and it displays a preference for arabidopsides to non-OPDA-containing MGDG or DGDG.

**Abrogation of AtPLAI Increases Susceptibility to *B. cinerea***—Two T-DNA insertion mutants of AtPLAI were isolated by screening the Wassilewskija (WT) ecotype of *Arabidopsis*. Atplai-1 contains a T-DNA insertion at nucleotide 438 from the start codon, corresponding to 14 amino acid residues after the start of the LRR domain (Fig. 1A). Atplai-2 has the insertion at nucleotide 3288, or 394 amino acid residues after the patatin domain (Fig. 1A). The T-DNA insertions resulted in loss of the expression of AtPLAI as indicated by the absence of the detectable transcript quantified by real time PCR (Fig. 4A). Both mutant alleles co-segregated with kanamycin resistance and

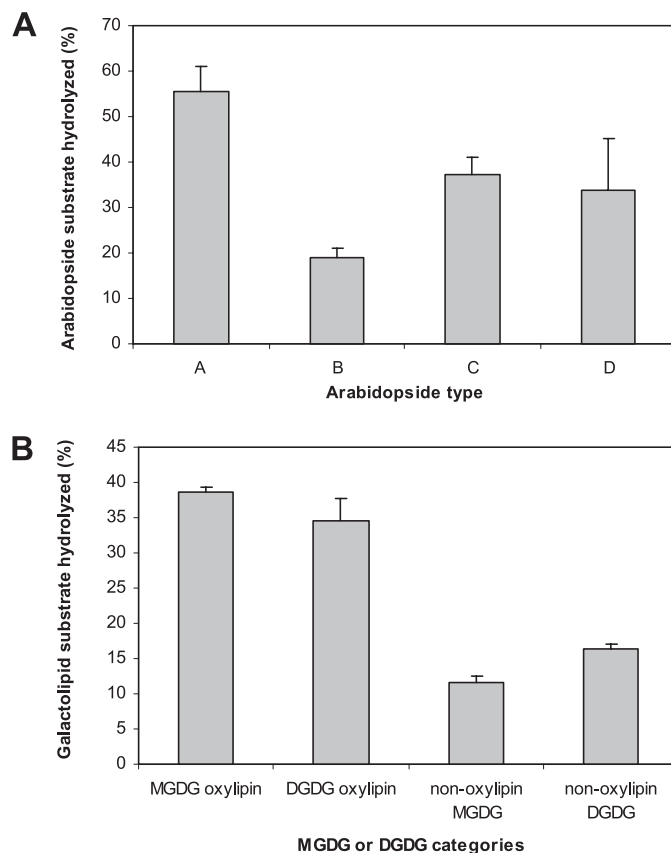




**FIGURE 2. Acyl hydrolyzing activity of AtPLAI on various substrates.** A, Coomassie Blue staining of an 8% SDS-PAGE gel loaded with affinity purified GST-AtPLAI from *E. coli*. M, protein marker; P, purified protein. B, fluorescent fatty acid and lysophospholipid released by AtPLAI when NBD-phospholipids were used as substrates. C, production of radioactive fatty acid and lyso-PI by AtPLAI when 1-stearoyl-2-arachidonyl-1-[arachidonyl- $^{14}$ C]PI was used as substrate. D, AtPLAI-catalyzed formation of free fatty acids for MGDG and DGDG. E, hydrolysis of polar glycerolipids by AtPLAI using total lipids from *Arabidopsis* leaves, followed by mass spectrometry analysis. Total *Arabidopsis* lipids were incubated with purified AtPLAI or the fraction from empty vector bacteria that were subjected to the same purification process as AtPLAI. Resultant lipids were quantified, and the differences between the control and AtPLAI reactions were used to calculate the specific activity. Values are mean  $\pm$  S.D. ( $n = 3$ ).

susceptibility in a 3:1 ratio, suggesting that each mutant contains a single T-DNA insertion in the genome. The mutant *Atplai-1* was complemented by introducing the genomic DNA of *AtPLAI* with its own promoter.

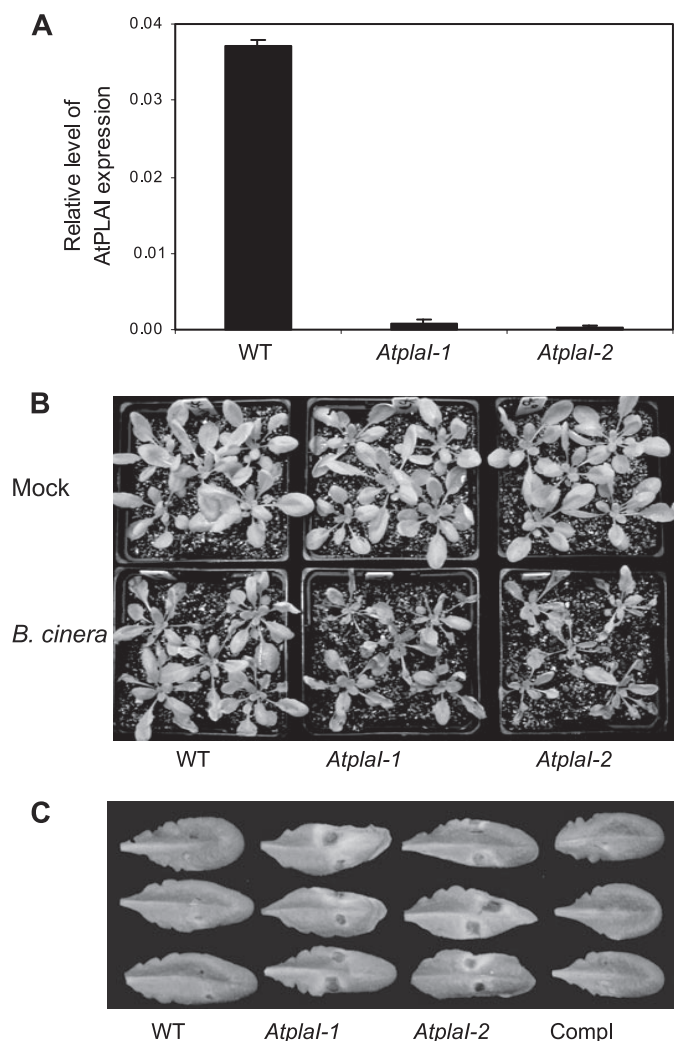
The response of the two T-DNA insertion mutants to *B. cinerea* was compared with WT plants. Both WT and mutant leaves developed necrosis at the site of inoculation. However, the mutant plants showed more susceptibility to *B. cinerea*, as indicated by increased lesion size, necrosis, and maceration of leaves after fungal infection than WT plants (Fig. 4, B and C). WT plants developed only mild symptoms of disease; no clear hypersensitive reaction was observed in the first 2 days after fungal infection, and the lesions on later days were small as compared with those on leaves of the mutant plants (Fig. 5A). The infection with *B. cinerea* resulted in stunted growth for



**FIGURE 3. Hydrolysis of galactolipids by AtPLAI using total lipids from wounded *Arabidopsis* leaves, followed by mass spectrometry analysis.** Total *Arabidopsis* lipids were incubated with 5  $\mu$ g of purified AtPLAI protein at 30  $^{\circ}$ C for 2 h. Resultant lipids were quantified, and the differences between the control and AtPLAI reactions were used to calculate the ratio of hydrolyzed over total in each category of galactolipids. Values are mean  $\pm$  S.D. ( $n = 5$ ). A, percentage of major OPDA-containing arabidopsides as substrates by AtPLAI. Arabidopsis A, OPDA/dnOPDA MGDG; B, OPDA/OPDA-MGDG; C, OPDA/dnOPDA-DGDG; D, OPDA/OPDA-DGDG. dnOPDA, nor-oxophytodienoic acid. B, percentage of non-oxylipin MGDG and DGDG versus oxylipin-containing MGDG and DGDG hydrolyzed by AtPLAI. The total oxygenated MGDG and DGDG per reaction were  $0.271 \pm 0.096$  and  $0.562 \pm 0.193$  nmol (mean  $\pm$  S.D.), and the amount of regular, non-oxygenated MGDG and DGDG were  $64.058 \pm 5.269$  and  $25.615 \pm 2.825$ , respectively.

many of mutant plants; growth retardation occurred in a much smaller fraction of WT plants (Fig. 5C). Compared with WT plants, the mutant plants had fewer new leaves emerging after fungal infection (Fig. 5B) and the new leaves were also smaller (Fig. 4B). When the genomic *AtPLAI* gene fragment was introduced into the mutant *Atplai-1*, the transformed plants behaved like wild type, displaying less susceptibility to the fungal infection than the mutants (Fig. 4C). Results from the genetic complementation show that the defect in the mutant plants is caused by the lack of *AtPLAI*. Collectively, the above results show that disruption of *AtPLAI* renders *Arabidopsis* more susceptible to *B. cinerea*.

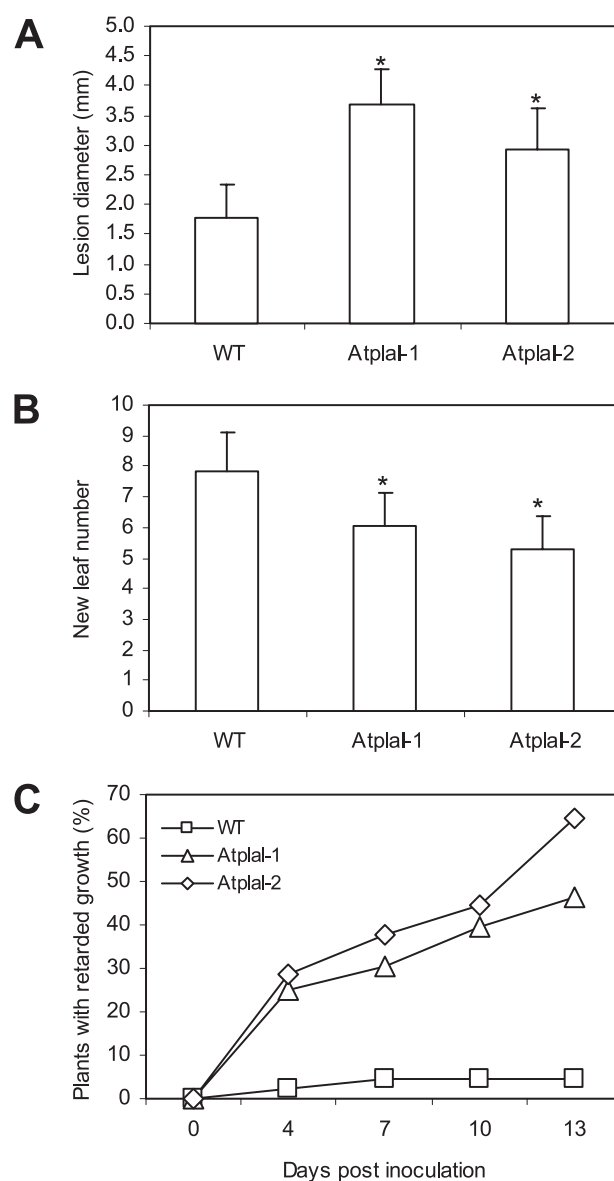
**Knockout of *AtPLAI* Decreases the Basal JA Level**—To gain insight as to how the defect in *AtPLAI* compromises the defense response, we tested the hypothesis that ablation of the acyl hydrolase *AtPLAI* might compromise JA production. Because the two knock-out lines responded similarly to the pathogen, most of the following comparative analyses were performed only on WT and *Atplai-1* to reduce the number of samples to be



**FIGURE 4. Disease symptoms of *Atplal-1* and WT plants infected with *B. cinerea*.** A, expression level of the *AtPLAI* gene in WT and two mutant plants as determined by real-time PCR. B, representative plant phenotypes after inoculation with *B. cinerea* for 3 days. Plants were pricked with a needle and then the wounds were covered with water or *B. cinerea* spore suspension at  $10^5$  spores  $\text{ml}^{-1}$ . Mutant plant leaves showed more lesions, necrosis, and shrinkage when compared with WT plants. C, damage on leaves at 3 days after inoculation with *B. cinerea*. Leaves from plants complemented with *AtPLAI* resembled the wild type in response to *B. cinerea* infection.

analyzed. Without fungal infection, the basal level of JA in both mutant leaves was significantly lower than that of WT leaves with about 50% less in *Atplal-1* (Fig. 6A). The basal level of JA in the complemented *Atplal-1* plants was restored to that in WT (Fig. 6A), confirming that the decreased level of JA was due to the loss of *AtPLAI*.

To further determine the effect of the *AtPLAI* mutations on JA production, the expression of several genes involved in oxylipin synthesis and response in WT and mutant was compared by quantitative real-time PCR. Without infection, the expression levels of lipoxygenases 2 and 3 (*LOX2* and *LOX3*), JA carboxyl methyltransferase (*MJT*), and a 12-oxo-phytodienoic acid reductase (*OPR3*) were ~3-fold higher in WT than in mutant plants (Fig. 6B). The level of a vegetative storage protein (*VSP2*), a JA-responsive gene, was also higher in WT than in the mutant plants. The attenuated expression of genes involved in oxylipin response is consistent with the decreased level of JA in

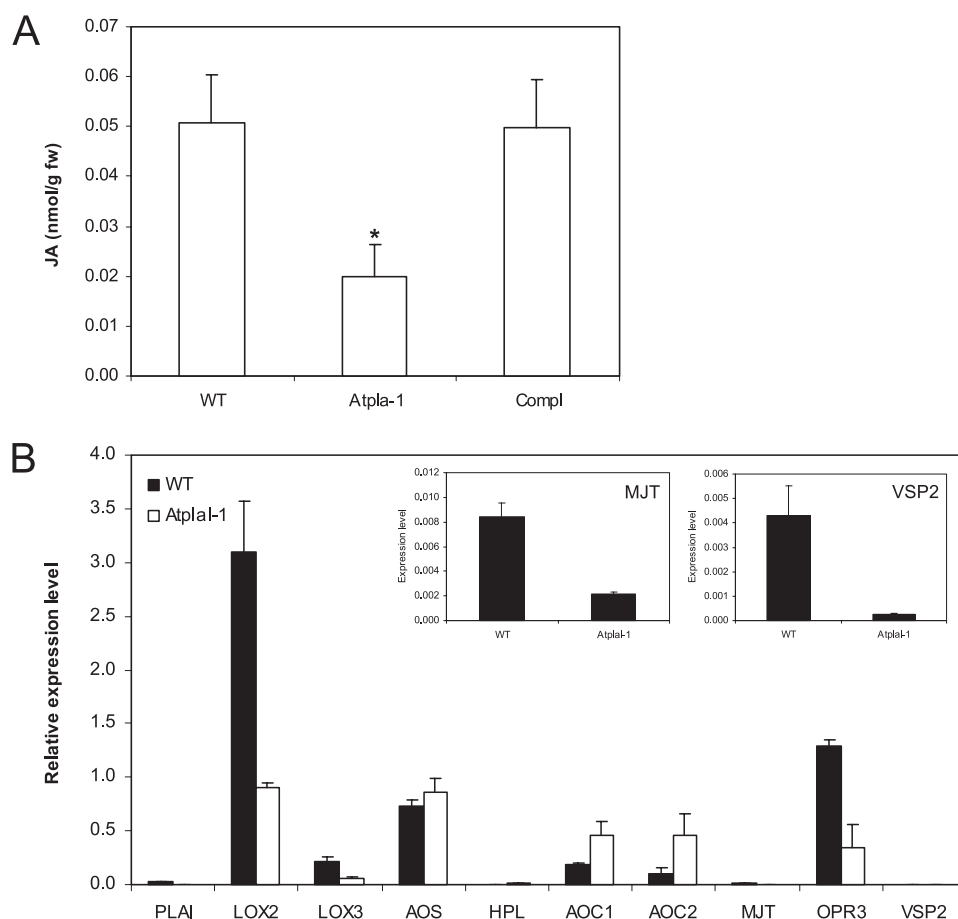


**FIGURE 5. Measurements of damage caused by fungal infection.** A, lesion diameters on leaves of plants at 3 days after inoculation with *B. cinerea*. B, number of new leaves that emerged from the plants by 7 days after inoculation. C, percentage of plants with retarded growth after inoculation with *B. cinerea*. Data are means obtained from representative experiments performed with 50 plants per genotype. Values are the mean  $\pm$  S.D. ( $n = 50$ ). Statistically significant differences between the wild type and either mutant line are marked with asterisks ( $p < 0.05$ , according to Student's *t* test).

the mutant plants. On the other hand, the levels of hydroperoxide lyase (*HPL*) and allene oxide synthase (*AOS*) were similar between the mutant and WT plants, whereas allene oxide cyclases 1 and 2 (*AOC1*, *AOC2*) appeared higher in the mutant than in WT plants.

**Loss of *AtPLAI* Does Not Impede Pathogen-induced JA Production**—When plants were infected with *B. cinerea*, both WT and the mutants exhibited biphasic increases in JA (Fig. 7A). The first increase occurred at 1 h after inoculation, and the second increase was at 24 h after inoculation. The level of JA at the second peak in WT was ~40 times higher than the earlier one (Fig. 7A). The increase at 1 h after inoculation was caused, in part, by wounding that was caused by 3 pricks with a





**FIGURE 6. The basal level of jasmonic acid in plants and the relative expression level of *AtPLAI* and several other genes in the oxylipin pathway in WT and *Atplai-1* plants.** A, the basal level of jasmonic acid in plants without any treatment. Statistically significant differences between WT and mutant are marked with asterisks ( $p < 0.05$ , according to Student's *t* test). Values are means  $\pm$  S.D. ( $n = 5$ ). B, the relative level of gene expression in plants without treatment. *PLAI*, *AtPLAI*; *LOX2*, lipoxygenase 2; *LOX3*, lipoxygenase 3; *AOS*, allene oxide synthase; *HPL*, hydroperoxide lyase; *AOC1*, allene oxide cyclase 1; *AOC2*, allene oxide cyclase 2; *MJT*, jasmonic acid carboxyl methyltransferase; *OPR3*, OPDA reductase 3; *VSP2*, vegetative storage protein 2. Their accession numbers in the GenBank<sup>TM</sup> data base are listed under "Experimental Procedures." The two insets show the expression of the *MJT* and *VSP2* genes in plants because their expression level did not match the scale of other genes in the figure. Values are means  $\pm$  S.D. ( $n = 3$ ).

23-gauge needle used for inoculation. In control plants that were pricked, but not inoculated with *B. cinerea*, JA also increased at 1 h (Fig. 7B). However, unlike pathogen-infected plants, no second peak was observed in control WT or mutant plants (Fig. 7B). Thus, the second, major peak of increase in JA resulted from *B. cinerea* infection. The *AtPLAI* mutant plants displayed wound- and pathogen-induced increases in JA, at a magnitude and time course similar to that of WT plants (Fig. 7A). After fungal infection, the genes involved in oxylipin synthesis, such as *LOX2*, *AOS*, *OPR3*, and *AOC2* displayed a similar pattern of induction in both WT and mutants (data not shown).

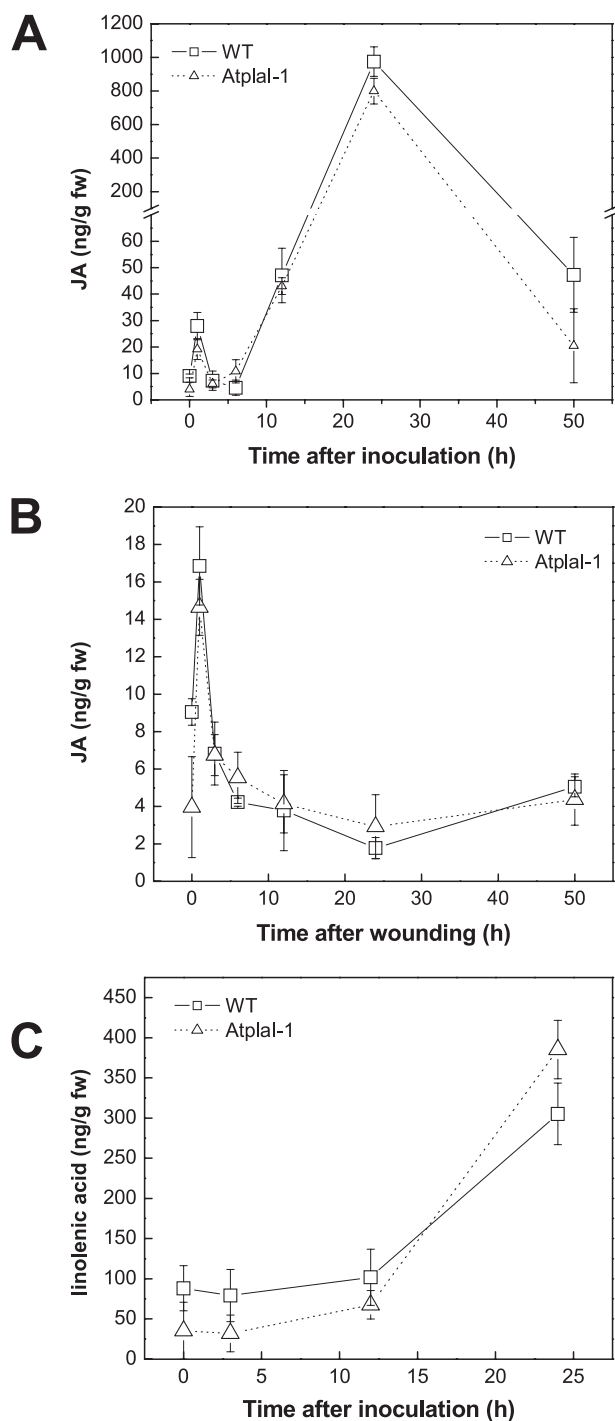
The level of free linolenic acid before and after fungal infection was measured to determine whether ablation of *AtPLAI* affected the formation of free linolenic acid, a potential substrate for JA synthesis. Without inoculation, the basal level of free linolenic acid in WT was more than 50% higher than that in *Atplai-1* (Fig. 7C). Whereas it remained lower in the mutants than in WT in the earlier hours after inoculation, the levels of free linolenic acid became higher in the mutant than WT at 24 h

after infection. The rise in the level of free linolenic acid coincided with the large increase in JA in both WT and mutant plants (Fig. 7, A and C). The higher levels of linolenic acid at later times in the mutants as compared with WT could be due to increased lipid hydrolysis (Fig. 8 and 9) related to the greater tissue damage by fungal infection in the mutant plant (Figs. 3 and 4).

**Plastidic Lipids PG and DGDG Are Decreased Earlier than PC and PE after *B. cinerea* Infection**—To investigate the lipid changes after *B. cinerea* infection, we quantitatively profiled polar glycerolipid species in WT and *AtPLAI*-deficient leaves following *B. cinerea* inoculation. Approximately 140 phospholipid and galactolipid species were analyzed, which included the major membrane lipid classes, PC, PE, PG, PI, MGDG, and DGDG, and minor classes, PS, PA, lyso-PC, lyso-PE, and lyso-PG, as well as minor acyl species within each head group class. This lipid profiling analysis is capable of detecting small changes in lipid species, as demonstrated recently by adding small, known amounts of specific lipid species an *Arabidopsis* lipid extract (46). WT and *Atplai-1* leaves contained similar amounts of polar glycerolipids, measured as either nanomole/mg of dry weight (Fig. 8A) or mol % of total lipids analyzed (data not shown).

After WT and *Atplai-1* leaves were inoculated with *B. cinerea*, no significant changes in phospholipids or galactolipids were detected in 0, 1, 3, 6, or 12 h post-infection (Fig. 8A and data not shown). At 22 h post *B. cinerea* inoculation, there was no significant decrease in other phospholipids analyzed, except that PG decreased significantly in both WT and the mutant (Fig. 8A). In both genotypes, the PG level in *B. cinerea* infected leaves was about 15% lower than that of control leaves that were pricked but inoculated with mock solution. The decrease came primarily from the predominant species 34:4 PG (Fig. 8B) that is the 18:3-16:1 acyl species (41). The major galactolipid MGDG displayed no change, but the level of DGDG decreased in both genotypes after the pathogen infection (Fig. 8A). The decrease in DGDG comes primarily from linolenic acid-containing species, 34:3 DGDG and 36:6 DGDG (Fig. 8B), which are composed primarily of 18:3-16:0, and 18:3-16:3 acyl groups, respectively (41).

When plants were infected with *B. cinerea* for 2 days or more, *Atplai-1* displayed greater decreases in phospholipids PC and PE and more increases in lyso-PC and lyso-PE than did WT



**FIGURE 7. Jasmonic acid level in plants of WT and *Atplal-1* in response to *B. cinerea* and wounding.** A, change in JA level in WT and *Atplal-1* leaves within 50 h after inoculation with *B. cinerea*. B, change in JA level in WT and *Atplal-1* leaves in response to wounding that was caused by 3 pricks with a 23-gauge needle used for inoculation. C, change in linolenic acid in plants in response to infection by *B. cinerea*. Five-week-old plants grown in growth chamber were used for inoculation with fungal pathogen *B. cinerea*. Samples of inoculated leaves were taken at different time points post-inoculation. Levels of JA and linolenic acid in plants were determined using mass spectrometry.

(Fig. 9). *Atplal-1* exhibited greater decreases in PI and PS than did WT 3 days after inoculation (Fig. 9), indicating that different classes of phospholipids have varied susceptibility to degradation during pathogenesis. There was no difference between

WT and *Atplal-1* in changes in MGDG or DGDG (data not shown). This greater increase in phospholipid hydrolysis in *Atplal-1* than in WT plants is correlated to the more severe damage in the mutant, but it is unlikely that this late stage increase in phospholipid hydrolysis is involved in JA biosynthesis because JA accumulation peaked at 24 h after infection (Fig. 7A) and phospholipid hydrolysis peaked between 48 and 72 h after infection (Fig. 9). The similar results in mutant and wild type suggest that other acyl hydrolyzing activities, rather than AtPLAI, contribute to the pathogen-induced membrane lipid loss in disease-damaged tissues.

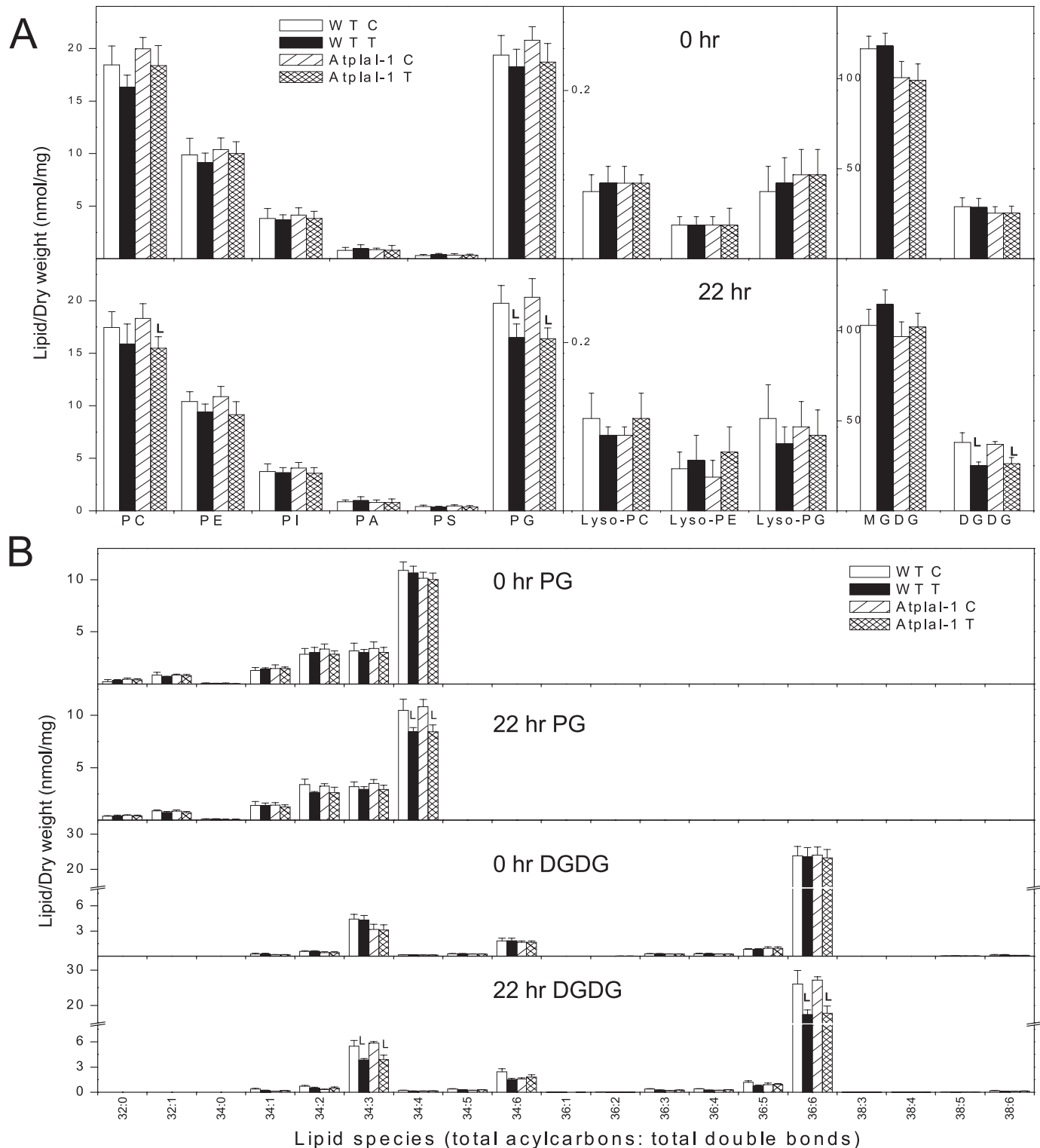
**Pretreatment of Methyl-JA Restores the AtPLAI Mutant Resistance to WT**—The above results indicate that ablation of AtPLAI results in decreases in basal JA, but not in pathogen-induced lipid hydrolysis or JA production. To test whether the decreased resistance to *B. cinerea* in *Atplal-1* results from a compromised ability to respond to JA, WT and mutant plants were treated with methyl-JA prior to *B. cinerea* inoculation. Without methyl-JA pretreatment, larger lesions were found on mutant leaves than WT leaves, but after methyl-JA pretreatment, the lesion size in the mutant plants decreased significantly and became indistinguishable from WT plants (Fig. 10).

The effect of pretreatment with SA on the mutant response to *B. cinerea* was also examined. When WT and mutant plants were pretreated with SA, the lesions on leaves of both WT and mutant plants were larger than control plants of each genotype (Fig. 10). The increase in disease susceptibility was relatively more in WT than in the mutant. The SA effect is consistent with the hypothesis that SA suppresses JA-mediated defense, thus resulting in increased plant susceptibility to *B. cinerea*. The ability of the *AtPLAI* mutants to respond to JA and SA pretreatment suggests that the increased disease susceptibility in *AtPLAI* mutants does not result from the compromised ability to respond to JA or SA.

## DISCUSSION

In *A. thaliana*, AtPLAI is most similar to mammalian iPLA<sub>2</sub>, and additionally it contains Armadillo and LRR domains at its N terminus. Arm-repeat proteins function in various animal processes, including intracellular signaling and cytoskeletal regulation (47). In higher plants, some Arm-repeat proteins function in intracellular signaling like their animal counterparts, but functions for most of the Arm-containing proteins are unknown in plants (48). Proteins with LRRs include tyrosine kinase receptors, cell adhesion molecules, virulence factors, and extracellular matrix-binding glycoproteins (49–50). They are involved in a variety of biological processes, such as signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, disease resistance, apoptosis, and immune response. In particular, the LRR domain has been implicated as a primary pathogen recognition domain for plant resistance genes (51–53). These sequence features raise intriguing questions about the enzymatic identity and cellular function of AtPLAI. Specifically, this study investigated the enzymatic activity of AtPLAI and its role in JA production and defense response.

The results show that AtPLAI is an acyl hydrolase with broad lipid substrate specificity. AtPLAI hydrolyzes phospholipids at

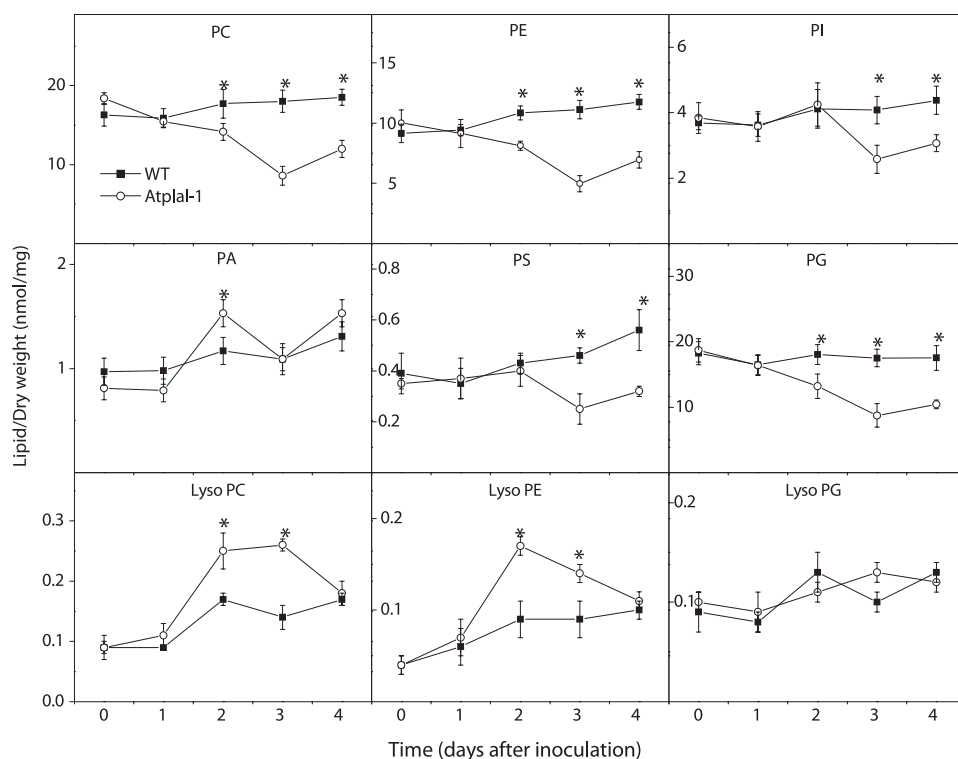


**FIGURE 8. Changes in polar glycerolipids as affected by *B. cinerea* infection.** A, polar lipid classes in WT and *Atpla1-1* leaves without and with *B. cinerea* infection for 0 and 22 h. B, amounts of PG and DGDG species in WT and *Atpla1-1* leaves without and with *B. cinerea* infection for 0 and 22 h. WT and mutant plants were grown at the same time and in the same growth chamber. Five-week-old plants were inoculated with *B. cinerea* with three pricks to each leaf. Infection (T) was initiated by placing a 10- $\mu$ l drop of freshly harvested spore suspension ( $5 \times 10^5$  spores/ml) on each prick. Control (C) leaves were pricked three times and covered with water. Values are mean  $\pm$  S.D. ( $n = 5$ ). The value for the PLD $\alpha$ 1-deficient plant is lower than the value for WT plants at  $p < 0.05$  according to Student's  $t$  test.

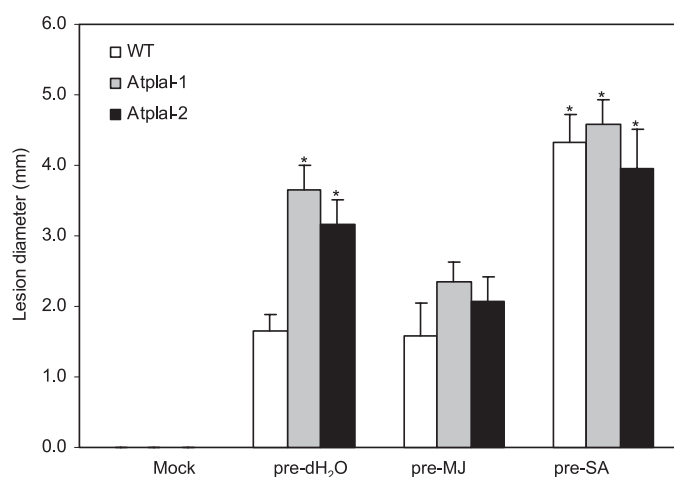
the *sn*-1 and *sn*-2 positions and also is more active toward galactolipids than phospholipids. Galactolipids, MGDG and DGDG, are present primarily in photosynthetic membranes inside

chloroplasts. Using green fluorescent protein-tagging and transient expression systems in tobacco, AtPLAI has been suggested to be localized in chloroplasts (32). Most cellular linole-





**FIGURE 9. Differences in phospholipid levels between WT and *Atplal-1* leaves during *B. cinerea* infection for 4 days.** WT and mutant plants were grown at the same time and in the same growth chamber. Five-week-old plants were inoculated with *B. cinerea* with three pricks to each leaf. Infection was initiated by placing a 10- $\mu$ l drop of freshly harvested spore suspension ( $5 \times 10^5$  spores/ml) on each prick. Values are mean  $\pm$  S.D. ( $n = 5$ ). Significant differences between WT and mutant are marked with asterisks ( $p < 0.05$ , according to Student's *t* test).



**FIGURE 10. Effects of JA and SA treatments prior to inoculation on plant response to *B. cinerea* infection.** Lesion diameter in leaves of plants pretreated with 50  $\mu$ M methyl jasmonate (*pre-MJ*), 0.5 mM salicylic acid (*pre-SA*), or distilled water (*pre-dH<sub>2</sub>O*) 48 h before inoculation with *B. cinerea* as described under "Experimental Procedures." The lesion diameter was measured 3 days after inoculation with the fungus on leaves from 50 inoculated plants per genotype. Statistically significant differences between the WT and mutants pretreated with distilled water (*pre-dH<sub>2</sub>O*) and between the WT (*pre-dH<sub>2</sub>O*) and SA treatment to WT, *Atplal-1*, and *Atplal-2* (*pre-SA*) are marked with asterisks ( $p < 0.05$ , according to Student's *t* test). Values are means  $\pm$  S.D. ( $n = 30$ ).

nic acid is esterified to galactolipids, and some enzymes involved in JA biosynthesis, such as AOS and AOC, are also localized in chloroplasts (4, 5). Thus, the substrate preference and localization of AtPLAI in chloroplasts would suggest that

AtPLAI affects plant functions via its hydrolysis of plastidic lipids. However, ablation of AtPLAI impedes the accumulation of only basal JA, not accumulation of pathogen-induced JA, nor the pathogen-induced hydrolysis of galactolipids or phospholipids.

The effect of *AtPLAI* on basal JA formation is confirmed by genetic complementation that restored the basal JA accumulation to the WT level. In addition, the positive role of AtPLAI in the basal JA formation is supported by a decrease in the basal level of free linolenic acid and by reduced expression of the JA-responsive gene *VSP2*, whose level of expression is indicative of endogenous JA levels in plants (54, 55). The basal levels of expression in WT and *Atplal-1* of other genes potentially involved in JA biosynthesis are varied. The lower level of gene expression for *LOX2*, *LOX3*, *OPR*, and *MJT* is consistent with the decreased basal level of JA in the mutants and the notion that JA biosynthesis is mediated by a positive feedback regulatory system (14,

56–58). On the other hand, the expression of AOS and AOCs did not display the same decrease. The lack of a correlation of AOS expression and JA levels has been observed in other *Arabidopsis* mutants (59, 60). In the case of AOC genes, different forms have been shown to undergo opposite changes during leaf senescence (59). It is possible that *Arabidopsis* leaves have sufficient basal AOS and AOC protein, which may catalyze basal JA formation without altered gene expression.

There is still an open question regarding whether JA biosynthesis starts with complex lipids or free fatty acids. The present results suggest that complex lipids, such as galactolipids, serve as starting precursors for JA synthesis. First, quantitative analysis indicates that the free linolenic acid pool (0.4 nmol/g fresh weight) is not large enough to provide the burst of JA production ( $\sim 4.5$  nmol/g fresh weight) after the infection. Second, AtPLAI is able to hydrolyze oxygenated DGDG and MGDG and is actually selective more for arabinogalactolipids over regular DGDG and MGDG. Third, oxygenated DGDG and MGDG are produced in leaves in response to stimulation. In addition, the result shows DGDG is more oxygenated than MGDG, which is consistent with the changes in lipid species following infection. The early decrease in lipids comes primarily from linolenic acid-containing species, 34:4PG, 34:3DGDG, and 36:6DGDG. These species are made primarily of 18:3-16:1, 18:3-16:0, and 18:3-16:3 acyl groups, respectively (42, 46). By comparison, the level of the major galactolipid MGDG does not decrease, and significant decreases in phospholipids PC and PE occurred 2 days after infection in WT plants. The increased hydrolysis of

PC and PE is consistent with increases in lyso-PE and lyso-PC, which peaked between 48 and 72 h after infection. The greater increase in phospholipid hydrolysis in *AtPLAI*-deficient plants is correlated with more severe disease damage, rather than the burst of JA production. Thus, such increases in phospholipid hydrolysis are likely to be more related to catabolism than to the initial phase of JA production. The results also suggest that another acyl hydrolyzing activity or activities, rather than *AtPLAI*, contributed to the membrane lipid loss in disease-damaged tissues.

Considering the plastidic lipid hydrolysis observed and the substrate preference of *ATPLAI*, the finding that the *AtPLAI*-deficient plants still produce as much pathogen- or wound-induced JA as WT does seems somewhat counterintuitive. One explanation evokes the functional redundancy of acyl-hydrolyzing enzymes. The *Arabidopsis* genome contains nine patatin-like genes, and two of them have been shown to encode acyl hydrolyzing activities. The nine genes are divided into two groups: Group I with five genes (*At4g37050*, *At4g37060*, *At4g37070*, *At5g43590*, and *At2g26560*) and Group II with four genes (*At2g39220*, *At3g54950*, *At4g29800*, and *At3g63200*) (32, 33). *AtPLAI* and Group I proteins have the typical serine hydrolase motif GXSXG and a conserved aspartic acid as a catalytic dyad essential for lipid acyl hydrolase activity (24, 42). Two Group I proteins (*At4g37070* and *At2g26560*) have been shown to possess acyl hydrolyzing activity (32, 33, 61), but their role in enhancing JA production is unknown. On the contrary, *At2g26560* is reported to promote pathogen colonization (33), and *At2g26560* does not promote JA formation.<sup>3</sup> In addition, the Group II proteins do not contain the conserved Ser-Asp catalytic dyad; the serine in the GXSXG motif is substituted with a glycine, and the aspartic acid in the dyad is also replaced with a glycine. No protein in Group II has been reported to have acyl hydrolase activity, so it is unclear if the Group II proteins have acyl hydrolase activity. Thus, whether the nine patatin-like proteins contribute to the basal or pathogen-induced JA in the *AtPLAI* mutant remains to be investigated. Another possibility is that other types of phospholipases may regulate wound- or pathogen-induced JA accumulation. There are other A-types of phospholipases in *Arabidopsis* (62). In addition, previous studies showed that phospholipase D  $\alpha 1$  promotes wound-induced accumulation of free linolenic acid and JA in *A. thaliana* (39, 59). The data suggest that production of basal JA and stimulus-induced JA is regulated by two separable processes.

JA is well documented to play a positive role in plant response to necrotrophic pathogen infection (12, 56). As shown by this study and others, JA increases rapidly after elicitation and wounding (10, 63). However, the roles of basal *versus* pathogen-induced JA on plant defense responses are often not distinguished, and little is known about the role of basal level of JA in defense response. The present results show that the *AtPLAI*-ablated plants are more susceptible to *B. cinerea* although the mutants produce a similar amount of pathogen-induced JA. One possible explanation for the uncoupling between the increase in pathogen-induced JA and the decrease in *B. cinerea*

resistance in *Atplai-1* is that ablation of *AtPLAI* might compromise the plant ability to respond to JA. This is unlikely, however, because pretreatment of *AtPLAI*-deficient mutants with JA restores the mutants ability to resist to pathogen. In addition, when WT and mutant plants were pretreated with SA, the lesions on leaves of both WT and mutant plants were larger than control plants of each genotype. SA and JA activate distinct sets of genes and defense pathways in *Arabidopsis* and were reported to have antagonistic interactions in *Arabidopsis* in response to distinct microbial pathogens (12, 64, 65). The SA effect is consistent with the hypothesis that SA suppresses JA-mediated defense, thus resulting in increased plant susceptibility to *B. cinerea*. These results indicate that *AtPLAI*-disrupted plants have normal ability to respond to JA and SA.

Taken together, the results demonstrate that *AtPLAI* is an acyl hydrolase, rather than a specific PLA. We propose to redefine *AtPLAI* as patatin-like acyl hydrolase. In addition, the study suggests that induced JA alone is not sufficient for defense against *B. cinerea*, and that the basal level of JA is important in defense response. We propose that the acyl hydrolyzing activity of *AtPLAI* is involved in maintaining the homeostatic pool of basal free fatty acids and basal JA that plays a positive role in defense responses.

**Acknowledgment**—We thank Mary Roth, Kansas Lipidomics Research Center, Division of Biology, Kansas State University, for expert technical assistance in lipid profiling.

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**AtPLAI Is an Acyl Hydrolase Involved in Basal Jasmonic Acid Production and  
*Arabidopsis* Resistance to *Botrytis cinerea***

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*J. Biol. Chem.* 2007, 282:18116-18128.

doi: 10.1074/jbc.M700405200 originally published online May 2, 2007

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