Differential Expression of Genes Encoding *Arabidopsis* Phospholipases After Challenge with Virulent or Avirulent *Pseudomonas* Isolates

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Submitted 17 December 2001. Accepted 1 April 2002.

Phospholipase D (PLD; EC 3.1.4.4) has been linked to a number of cellular processes, including Tran membrane signaling and membrane degradation. Four *PLD* genes (α , β , $\gamma 1$, and $\gamma 2$) have been cloned from *Arabidopsis thaliana*. They encode isoforms with distinct regulatory and catalytic properties but little is known about their physiological roles. Using cDNA amplified fragment length polymorphism display and RNA blot analysis, we identified *Arabidopsis* *PLD* $\gamma 1$ and a gene encoding a lysophospholipase (EC 3.1.1.5), *lysoPLI*, to be differentially expressed during host response to virulent and avirulent pathogen challenge. Examination of the expression pattern of phospholipase genes induced in response to pathogen challenge was undertaken using the *lysoPLI* and gene-specific probes corresponding to the *PLD* isoforms α , β , and $\gamma 1$. Each mRNA class exhibited different temporal patterns of expression after infiltration of leaves with *Pseudomonas syringae* pv. *tomato* with or without *avrRpm1*. *PLD* α was rapidly induced and remained constitutively elevated regardless of treatment. *PLD* β was transiently induced upon pathogen challenge. However, mRNA for the *lysoPLI* and *PLD* $\gamma 1$ genes showed enhanced and sustained elevation during an incompatible interaction, in both *ndr1* and overexpressing NahG genetic backgrounds. Further evidence for differential engagement of these *PLD* mRNA during defense responses, other than gene-for-gene interactions, was demonstrated by their response to salicylic acid treatment or wounding. Our results indicate that genes encoding *lysoPLI*, *PLD* $\gamma 1$, and *PLD* β are induced during early responses to pathogen challenge and, additionally, *PLD* $\gamma 1$ and *lysoPLI* are specifically upregulated during gene-for-gene interactions, leading to the hypersensitive response. We discuss the possible role of these genes in plant–pathogen interactions.

Additional keyword: *RPM1*.

The aggregation of lipid molecules into thermodynamically stable bilayers provides the fundamental basis for cellular compartmentation. The membranes of plant vegetative cells constitute between 5 to 10% lipids by dry weight (Ohlrogge and Browse 1995) and they are organized as a semipermeable, fluid mosaic structure, with the bulk matrix being composed

largely of phospholipids (Singer and Nicolson 1972). Emerging evidence indicates that membrane lipids not only serve as a passive, hydrophobic barrier for compartmentalization, but also play important and active roles in cellular processes such as signal transduction cascades and enzyme activation (Munnik et al. 1998; Ryu and Wang 1998). Many of these functions involve the specific catabolism of phospholipids by hydrolases, which are grouped in families, designated phospholipase A, C, and D (PLA₁/A₂, PLC, and PLD), depending upon their site of cleavage (Chapman 1998).

Phospholipase was originally discovered in plants (Hanahan and Chaikoff 1947) but has been most extensively studied in animals, primarily because it is rapidly activated in response to external stimuli (Cockcroft 1984; Liscovitch and Cantley 1995; Liscovitch et al. 1987). Until recently, plant PLD primarily was regarded as a catabolic enzyme whose major purpose was to degrade membrane phospholipids. Recently, however, an important role for PLD isoforms in transmembrane signal transduction has emerged (Lee et al. 1997; Ryu and Wang, 1996, 1998; Wang et al. 2000). PLD hydrolyzes phospholipids at the terminal phosphoesteric bond to generate phosphatidic acid (PA) and free head groups such as choline (Wang 1999), both of which represent potentially important signaling molecules in plants and animals (Munnik et al. 1998; Munnik 2001). In plants, elevated PA levels caused by increased PLD activity have been demonstrated during wounding (Lee et al. 1997), osmotic stress (Munnik et al. 2000), and elicitation (Laxalt et al. 2001). During the wound response, it is proposed that PLD translocates to the membranes as a result of elevated cytosolic calcium (Ryu and Wang 1998). Activated membrane-associated PLD then catalyzes the formation of phosphatidic acid, eventually leading to an increase in the plant hormone jasmonic acid (JA), which plays a key role in many cellular processes, including wound and defense responses (Creelman and Mullet 1997; McConn et al. 1997). The wounding response model also may be equally applicable to other types of biotic stress, including plants response to pathogens.

The PLD superfamily members share a catalytic core comprising four key domains, including the “HDK” or phosphatidyltransferase motif (Morris et al. 1996; Waksman et al. 1996). Plant PLDs also share a C2 or Ca²⁺-lipid binding domain found in a range of different proteins. There are three main plant PLD isoforms. PLD α is polyphosphoinositide (PPI) independent when assayed at millimolar concentrations of Ca²⁺. In contrast, PLD β and PLD γ require a PPI cofactor and are most active at

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micromolar concentrations of Ca^{2+} . These specific properties lead to the prediction that plant PLDs are engaged in distinct physiological processes. To address this issue, four PLDs encoded by distinct genes recently have been cloned from *Arabidopsis thaliana*: *PLD α* , *PLD β* , *PLD γ 1*, and *PLD γ 2* (Dyer et al. 1995; Pappan et al. 1997a; Qin et al. 1997, 1999), and have been further classified based upon differences in sequence identity and their in vitro biochemical properties (Pappan et al. 1997a, 1997b, 1998; Pappan and Wang 1999b; Qin et al. 1997; Wang, 1999).

Over the past decade, our understanding of the molecular basis of plant-pathogen interactions has rapidly accelerated. Many resistant interactions are consistent with the "gene-for-gene" model proposed by Flor (Flor 1971). According to this model, resistance requires a dominant or semidominant resistance (*R*) gene in the plant and a corresponding avirulence (*avr*) gene in the pathogen. Simple model pathosystems comprising a bacterial pathogen carrying an *avr* gene and a genetically tractable host carrying the corresponding *R* gene have proved useful for studying this complex molecular and biochemical signal transduction process, which ultimately results in restriction of pathogen growth and elaboration of cell death recognized as the hypersensitive reaction (HR) (Yang et al. 1997).

Early responses identified during plant-pathogen interactions leading to an HR constitute an array of biochemical events, including changes in ion fluxes, increases in reactive oxygen species, and induction of nitric oxide, JA, and salicylic acid (SA) (Alvarez 2000; Bolwell 1999; Bush 1995; Delledonne et al. 2001; Lamb and Dixon 1997; McDowell and Dangel 2000; Nurnberger and Scheel 2001). Accumulating evidence suggests that Ca^{2+} appears to have an early and indispensable role in the plant cell's response to microbial and elicitor challenge (Blume et al. 2000; Grant et al. 2000). Possible targets of elevated calcium include Ca^{2+} -dependent phosphatases and kinases (McAinsh and Hetherington 1998; Trewavas and Malho 1998), calmodulin and Ca^{2+} /calmodulin-dependent kinases (Harding et al. 1997; Harding and Roberts 1998; Heo et al. 1999), and phospholipases D (Wang 2000), the latter probably through binding to their N terminal C2 domain. The distinct structural and biochemical properties of PLD isoenzymes (Wang 2000) suggest that they are subject to unique control and activation mechanisms. Possible mechanisms include regulating the activity of preexisting PLDs, regulating PLD synthesis and degradation (Munnik et al. 1998), or differential expression of PLD isoforms.

Fig. 1. Identification of cDNA amplified fragment length polymorphism (AFLP) fingerprints corresponding to the differential regulation of *PLD γ 1* and *lysoPL1* after challenge with MgCl_2 or the particular *Pseudomonas syringae* derivative at the time (min) indicated. ^{32}P -labeled fragments were visualized by autoradiography after separation on denaturing polyacrylamide gels. cDNA-AFLP display was as described by (Durrant et al. 2000). Primer combinations used were 5' GACTGAGTAC-CAATTCG 3':5' GATGAGTCCTGAGTAAAG 3' and 5' GACTGAGTACCAATTCG 3':5' GATGAGTCCTGAGTAAACG 3'.

The advent of high-density microarrays and open-ended transcription profiling techniques, such as SAGE (Velculescu et al. 1995) and cDNA amplified fragment length polymorphism (AFLP) display (Bachem et al. 1996), have now made it possible to investigate the degree of transcriptional reprogramming undertaken by the host in response to pathogen challenge, and how these transcripts underpin basal resistance responses.

Using cDNA-AFLP to examine differential expression patterns in *Arabidopsis thaliana* challenged with virulent and avirulent *Pseudomonas syringae* strains, we identified fragments corresponding to different phospholipases. In this study, we examine the expression patterns of *PLD α* , *PLD β* , *PLD γ 1*, and a previously undescribed lysophospholipase gene (*lysoPL1*) in *Arabidopsis* leaves inoculated with virulent or avirulent pathogens. We provide evidence that transcripts encoding *PLD β* , *PLD γ 1*, and *lysoPL1* are selectively engaged during the early stages of pathogen challenge and that *PLD γ 1* and *lysoPL1* transcripts are, in addition, specifically enhanced during an incompatible interaction. We discuss a model for how expression of these genes may change the physiology of the challenged cell.

RESULTS

cDNA-AFLP revealed differential phospholipase gene expression upon pathogen challenge.

We used cDNA-AFLP display (Durrant et al. 2000; Sanchez et al. 2000, M. de Torres Zabala, I. Fernandez-Delmond, P. Sanchez, and M. Grant *unpublished data*) to catalogue differential gene expression patterns of mRNA samples isolated at various times after challenge with *P. syringae* pv. *tomato* strain DC3000. Treatments were designed to discriminate innate (DC3000:*hrpA*), compatible (DC3000), and incompatible (DC3000:*avrRpm1*) interactions from mock challenge (10 mM MgCl_2). Infiltration of leaves at high bacterial inoculum (2×10^8 CFU/ml) results in the simultaneous exposure of virtually all cells within the leaf to the challenging bacterium. We initially identified phospholipase molecular signatures corresponding to *PLD γ 1* and a lysophospholipase we named *lysoPL1* (GI:14532651) as being differentially expressed either after pathogen challenge or during the establishment of an incompatible interaction (Fig. 1).

PLD γ 1 is differentially expressed in an avirulent interaction.

Using a cDNA clone for *PLD γ 1* (GI:2653884) (Wang et al. 2000), we demonstrated that pathogen challenge induces *PLD γ 1* mRNA over mock inoculation beginning approximately 60 min postinoculation, regardless of whether or not the pathogen carries the *avrRpm1* gene (Fig. 2). In the pres-

Fig. 2. *PLD γ 1* mRNA is induced in a gene-for-gene interaction. *PLD γ 1* expression in *Arabidopsis* leaves after mock inoculation or challenge with *Pseudomonas syringae* pv. *tomato* DC3000 or *P. syringae* pv. *tomato* DC3000:*avrRpm1*. Lanes correspond to samples harvested at the designated time, in minutes, after pathogen challenge. **A**, RNA blot of 14 μg of total mRNA derived from each treatment probed with ^{32}P -labeled *PLD γ 1*. **B**, The same RNA blot probed with ^{32}P -labeled *actin 2* (GI:17381173) as a loading control.

ence of *avrRpm1*, the *PLDγ1* transcript increase was sustained 4 h postinfiltration, indicating specific engagement of *PLDγ1* in a gene-for-gene interaction. The timing of induction correlates strongly with the increase in $[Ca^{2+}]_{cyt}$ previously demonstrated for this interaction (Grant et al. 2000). To test whether increased $[Ca^{2+}]_{cyt}$ or reactive oxygen species played a role in *PLDγ1* induction, plants were coinfiltrated with either 2.5 mM lanthanide ion solution or 7 μM diphenyleneiodonium chloride (DPI) and the bacteria. La^{3+} ions are capable of blocking plasma membrane calcium channels, and may promote Ca^{2+} release from the cytosol. However when inhibitors of calcium channels (La^{3+}) or the oxidative burst (DPI) were coinfiltrated, we could not detect any differences in the *PLDγ1* induction profile for either DC3000 or DC3000:*avrRpm1* challenge (data not shown).

mRNA for *PLDα* and *β* are differentially regulated upon pathogen challenge.

A role for PLD isoforms previously has been identified in wounding and elicitation; therefore, we investigated the expression patterns of other *Arabidopsis* PLD isoforms in their response to pathogen challenge. Gene-specific probes for *PLDα* (GI:18400879) (Dyer et al. 1995), *β* (GI:15284210) (Pappan et al. 1997), and *γ2* (GI:5052356) (Qin et al. 1999) were generated by identifying and amplifying unique regions of the corresponding genes (discussed below). The expression levels of *PLDα* and *β* in *A. thaliana* accession Col-5 is shown in Figure 3 (*γ2* could not be detected).

Pathogen challenge with DC3000, DC3000:*avrRpm1*, or $MgCl_2$ resulted in a rapid increase in *PLDα* levels that remained constitutively expressed for the remainder of the experiment (Fig. 3A). Given that no detectable differences between pathogen and mock challenge could be detected, our data suggest that the initial rapid increase in *PLDα* expression probably was due to an underlying stress response rather than specific to pathogen challenge. In contrast, infiltration with either DC3000 or DC3000:*avrRpm1* elicited a rapid and transient induction of *PLDβ* message between 30 and 60 min postinfiltration (Fig. 3B). There was no discrimination between either pathogen, nor after challenge with DC3000:*hrpA* or *Escherichia coli* (data not shown), suggesting that the mRNA accumulates in response to recognition of some endogenous microbial molecular pattern. In all cases, *PLDβ* mRNA levels were significantly lower than *PLDα* or *γ1*, and therefore required substantially longer exposure to detect the transcripts.

Sustained expression of a lysophospholipase gene during an incompatible interaction.

A novel phospholipase signature corresponding to a gene encoding a predicted lysophospholipase like protein (*lysoPL1*;

GI:14532651) was initially identified by cDNA-AFLP as being upregulated upon pathogen challenge (Fig. 1). Lysophospholipases specifically cleave lysophospholipid and lysophosphatidic acid at the *sn*-1 position. RNA blot analysis revealed that the *lysoPL1* was rapidly induced beginning 60 min postchallenge but barely increased with the $MgCl_2$ control inoculation (Fig. 4). In the compatible interaction, *lysoPL1* levels remained high for 2 h before decreasing to background levels. In contrast, *lysoPL1* transcript levels remained elevated at 4 h during an incompatible interaction. The induction pattern is remarkably similar to that exhibited by *PLDγ1*, suggesting that both genes are co-regulated during an incompatible host response. The timing of induction correlates with the initiation of increased $[Ca^{2+}]_{cyt}$ during the *avrRpm1/RPM1* interaction; however, pharmacological challenges as described above for *PLDγ1* failed to compromise expression patterns (data not shown). As with *PLDγ1*, the *lysoPL1* expression pattern was identical whether the challenge was DC3000, DC3000:*hrpA*, or *E. coli* (data not shown), indicating that initial engagement of the *lysoPL1* is not due to delivery of type III effector proteins and is probably a component of the host's innate response to microbial occupation of the apoplast. Subsequently however, in the presence of *avrRpm1*, the *lysoPL1* transcript remained strongly induced. Interestingly, the hybridization pattern in Figure 4 reveals that at least two *lysoPL*-like genes are simultaneously recruited in a co-regulated manner during the resistance response.

To further investigate the specificity of phospholipase gene induction in the *avrRpm1-RPM1* interaction, we compared gene expression in a transgenic Niederzens (Nd-0) accession carrying the *RPM1* gene under control of its native promoter with the wild-type Nd-0 which is *rpm1^{null}* (Grant et al. 1995). The RNA blot (Fig. 5) confirms the requirement for both a functional *avrRpm1* and *RPM1* gene product to sustain the elevated levels of both *lysoPL1* and *PLDγ1*.

lysoPL1 is a member of an extended gene family in *Arabidopsis*.

Given that this is the first report describing the specific induction of a *lysoPL* during a gene-for-gene interaction, we investigated the nature of the *lysoPL1* gene family in *Arabidopsis*. A BLAST P search of the *Arabidopsis* database indicated a number of gene products predicted to be similar to *lysoPL1*, although only one (GI:15233200) revealed sufficient overall nucleotide level homology to be detectable under the hybridization conditions used in these experiments. A multiple alignment of the six most related *Arabidopsis* *lysoPL1* proteins based upon a PSI Blast cut off of greater than 50% amino acid identity is shown in Figure 6. The InterPro motif corresponding to the esterase/lipase/thioesterase family active site is un-

Fig. 3. Expression patterns of *PLDα* and *PLDβ* after pathogen challenge. Total mRNA was isolated from leaves of plants at the times (min) indicated after mock inoculation or challenge with virulent (DC3000) or avirulent (DC3000:*avrRpm1*) pathogens. **A**, RNA blot probed with ^{32}P -labeled *PLDα*. **B**, RNA blot probed with ^{32}P -labeled *PLDβ*. **C**, Loading controls using ^{32}P -labeled *actin 2* probe, which displayed constitutive expression regardless of pathogen treatment.

Fig. 4. A novel lysophospholipase gene is induced in a gene-for-gene manner. Total mRNA was isolated from pathogen challenged leaves at the time points (minutes after inoculation) indicated. Control leaves were challenged with 10 mM $MgCl_2$. **A**, The *lysoPL1* mRNA is rapidly upregulated in response challenge with DC3000 or DC3000:*avrRpm1*, but remains elevated in the incompatible interaction. The additional coregulated species at the higher molecular weight probably corresponds to GI:15233200. **B**, The same RNA blot probed with a loading control, the *Arabidopsis actin 2* gene (GI:17381173).

derlined. Notably, the majority of the protein constitutes an α/β hydrolase fold structure (Ollis et al. 1992; Remington et al. 1992).

***PLD γ* and *lysoPLI* mRNAs are strongly and differentially induced by application of SA.**

To examine the regulation of *PLD γ* and *lysoPLI* in other defense or abiotic responses, we compared gene expression profiles after wounding or after challenge with SA, a key inducer of systemic-acquired resistance (Alvarez et al. 2000). Selective engagement of the *PLD* family and *lysoPLI* within and between treatments is shown in Figure 7. *PLD α* , β , and γ wound induction profiles were similar to those determined by mock challenge. Our results were comparable with those obtained by Wang and associates (2000), who demonstrated that *PLD β* and γ mRNA levels increased within 30 min of wounding; however, the level of induction we observed was significantly lower and we did not detect a second phase of *PLD β* induction. In contrast, no significant recruitment of *PLD γ* or *lysoPLI* mRNA occurred on wounding, even after 28 h. *PLD γ* message levels were, however, dramatically elevated within 2 h of treatment with 5 mM SA. Surprisingly, and analogous to pathogen challenge, *lysoPLI* also was induced by SA application, although induction was delayed by an hour with respect to *PLD γ* . The magnitude and timing of *lysoPLI* and *PLD γ* genes in defense responses suggests an important role for their protein products in defense responses.

In contrast, *PLD α* is induced by a variety of stress signals and would appear to function in general stress responses.

***PLD γ* and *lysoPLI* transcripts are still up-regulated in mutants affecting defense responses.**

To further investigate the induction of *PLD γ* and *lysoPLI* genes, we examined expression in the *ndr1* genetic background and SA-degrading NahG plants. Many, but not all, R proteins of the RPM1 class require NDR1, a small membrane-associated protein of unknown biochemical function (Century et al. 1995, 1997). The *ndr1* mutant is impaired in resistance to DC3000:*avrRpm1*; however, the ability to generate an HR is retained (Century et al. 1995). Salicylate hydroxylase-expressing NahG plants do not accumulate SA, are incapable of developing systemic acquired resistance (SAR), and do not show pathogenesis-related (*PR*) gene activation upon pathogen infection (Delaney et al. 1994). However, *RPM1*-mediated resistance is an extremely rapid response and is unaffected in NahG plants. We examined the expression of *PLD γ* and *lysoPLI* after DC3000:*avrRpm1* challenge of Col-5, NahG, and *ndr1-2* plants. Expression patterns in *ndr1-2* are indistinguishable from Col-5 (Fig. 8), suggesting that *PLD γ* and *lysoPLI* induction is signaled through pathways effecting the HR rather than restriction of pathogen growth. NahG plants also exhibited an identical pattern of *PLD γ* and *lysoPLI* gene expression, suggesting that signaling pathways regulating these genes during an incompatible response are independent of those responsible for their differential induction after SA application (Fig. 7).

DISCUSSION

Eukaryotic lysophospholipid acyltransferases and transacylases appear to participate not only in fatty acid remodeling systems but also in the synthesis and degradation of some bioactive lipids and their precursors. In mammalian systems, the interaction of extracellular signal molecules with cell surface receptors often leads to the activation of PLD and subsequent hydrolysis of phosphatidylcholine and other phospholipids to generate PA, a secondary messenger. (Liscovitch et al. 2000).

Bacterial phytopathogens deliver type III effector proteins extracellularly or intracellularly by means of the type III secretion system (Grant and Mansfield 1999). These proteins are predicted to mimic or interfere with host proteins to redirect plant metabolism toward nourishment of the invading pathogen. Recently, two putative plant lipases, EDS1 and PAD4, have been shown to interact both independently and together in host defense signaling pathways active against a number of diverse plant pathogens, including bacterial phytopathogens, suggesting a possible role for lipids in signaling host defense to pathogens (Falk et al. 1999; Feys et al. 2001; Jirage et al. 1999). Here we have demonstrated differential regulation of plant *PLD* isoforms and *lysoPLI* after pathogen challenge. Significantly, *lysoPLI*, *PLD β* , and γ mRNA levels are induced upon pathogen challenge but with markedly different temporal responses, indicating that their expression occupies different steps in the pathogen response pathway. Transcripts for *lysoPLI* and *PLD γ* accumulate in a coregulated manner during an incompatible interaction and, additionally, after challenge with SA, suggesting a role for their protein products in defense responses.

A rapid, early transient increase of $[Ca^{2+}]_{cyt}$ in response to pathogen challenge and a subsequent sustained increase in cytosolic calcium during an incompatible interaction have recently been demonstrated in vivo (Blume et al. 2000; Grant et al. 2000). Although we found no evidence of a role for $[Ca^{2+}]_{cyt}$ in accumulation of *PLD γ* or *lysoPLI* transcripts, elevated $[Ca^{2+}]_{cyt}$ may be required to activate their protein product. Conventional PLDs are active at millimolar concentrations of Ca^{2+} (20 to 100 mM), although *PLD β* and γ are active at neutral pH and micromolar Ca^{2+} concentrations. Therefore, it is possible that plant PLDs may respond to high Ca^{2+} concentrations provided locally by influx of apoplastic Ca^{2+} , caused by wounding, an incompatible interaction, or other stress inducers. However, biochemical analyses of PLD are restricted by the difficulty of distinguishing between isoforms and lack of pharmacological inhibitors of PLD in plants and animals.

PLD α was the most abundant transcript detected, exhibiting a rapid, constitutive induction under a variety of treatments. In all our experiments, the expression levels of *PLD α* increased rapidly above background under all challenges and remained elevated throughout the experiment, suggesting no specific role for transcriptional control of *PLD α* during early responses to *Pseudomonas* pathogens of *Arabidopsis* spp.

PLD α activity in *Arabidopsis* spp. normally is associated with metabolically active or rapidly growing tissues (Fan et al. 1999), although it also has been implicated in senescence and defense responses. *PLD α* was shown to cluster in membranes

Fig. 5. Sustained accumulation of *lysoPL* and *PLD γ* mRNA requires a functional *RPM1* gene. Total mRNA was isolated at the time points (hours postinoculation) indicated for Niederzens (Nd-0) or Nd-0:*RPM1* leaves challenged with DC3000:*avrRpm1*. RNA blots were probed with **A**, *lysoPLI* or **B**, *PLD γ* .

adjacent to the bacterial cells in resistant interactions 12 h after infiltration (Young et al. 1996). Given *PLD α* 's activity at physiological concentrations of Ca^{2+} or at noncytoplasmic acidic pH (Pappan and Wang 1999), it may play a role in membrane degradation during the late stages of the HR rather than in the generation of key signaling intermediates.

The pattern of *PLD β* and *PLD γ* and *lysoPLI* expression is suggestive of an earlier role in the defense process. *PLD β* is suggested to be under tight transcriptional control by Fan and associates (1999), who were unable to detect *PLD β* protein when investigating its distribution in *Arabidopsis* tissues. *PLD β* was rapidly and transiently induced by both pathogen challenge and wounding (Fig. 5B), indicating a degree of cross talk between these biotic and abiotic stress treatments. *PLD β* mRNA also has been shown to increase maximally 30 to 60 min after wounding before slowly decreasing (Wang et al. 2000). Recently, the role of *PLD β* has been extensively studied in plants' response to stress, wounding, and elicitation (Laxalt et al. 2001). In this study, *PLD β* mRNA rapidly increased ninefold, and *PLD* activity threefold, 2 h after treatment of tomato suspension cells with the fungal elicitor xylanase. Tomato leaves responded with a similar pattern but increases were significantly attenuated compared with cell suspension cultures. Here, we found some evidence for an *Avr* gene dependent on increase in *PLD β* ; however, the increase was less than twofold and not consistently reproducible. Comparison of the strength of the *PLD β* transcript in Figure 3B (DC3000:*avrRpm1*) with the corresponding loading control in Figure 3C indicates a possible biphasic induction of *PLD β* in a gene-for-gene interaction. In view of the results

from xylanase elicitation, further investigation into a possible role for *PLD β* in incompatible interactions is warranted.

A notable property of signaling enzymes, and the foundation for transcriptomics, is that treatments that usually activate them rapidly enhance the expression of their genes. In this study, we have revealed a temporally controlled, specific role for transcripts encoding these two potential signaling molecules, *lysoPLI* and *PLD γ* , in an incompatible interaction. If the observed increase in *PLD γ* can be translated into an increase in *PLD γ* activity, the resultant catabolic products can lead to generation of various lipid messengers, such as PA, lysophospholipids, N-acyl ethanolamine (NAE), diacylglycerol, and nonesterified fatty acids (precursors of JAs). A number of possible roles for *lysoPLI* and *PLD γ* in generating signaling molecules are outlined in Figure 9 and discussed below. An increase in *PLD γ* activity associated with the incompatible interaction could play dual roles through the generation of the secondary signaling molecules PA and NAE. In animal cells, NAE triggers nitric oxide release (Liscovitch et al. 2000). This is significant because nitric oxide has been shown to potentiate reactive oxygen-mediated defense responses during the *avrRpm1*-*RPM1* interaction (Delledonne et al. 1998). Additionally, Sang and associates (2001) recently demonstrated that *PLD* plays a key role in mediating superoxide production through the generation of PA as a lipid messenger. In plants, activation of *PLD* precedes that of *PLA* acyl hydrolases (Lee et al. 1997; Ryu and Wang 1998). *PLD γ* -mediated release of PA could activate *lysoPLI*, resulting in the production of linolenic acid for JA biosynthesis (Fig. 9). Alternatively, the activation of *lysoPLI* in the incompatible in-

Fig. 6. Boxshade of a Clustal W (Thompson et al. 1994) alignment corresponding to the most related lysophospholipases predicted from the Arabidopsis database. Selection criteria were based on a PSI Blast specification of greater than 50% identity at the amino acid level. *lysoPLI* (GI:14532651) is preceded by an asterisk. Identical amino acid residues corresponding to 50% or greater of the related lysophospholipases are shaded black, and similar ones are shaded gray. Numbers correspond to predicted amino acid residues, and names are derived from the corresponding GI accession number. Predicted functional motifs, determined by InterPro Scan, include the ubiquitous esterase/lipase/thioesterase family active site between residues 39 and 134 (InterPro Entry IPR000379), indicated by a dashed line, and the α/β hydrolase fold (Ollis et al. 1992; Remington et al. 1992), which lies between residues 65 and 287.

teraction may function to promote the release of polyunsaturated fatty acids lipolytically by hydrolysis of the derivatives of the sequential activities of PLD and PA phosphohydrolase, as speculated to occur in degradative processes (e.g., senescence) (Ryu et al. 1997). This activity may result in feedback inhibition of PLD α activity because it is inhibited by some lysophospholipids (lysoPE and lysoPI). Significantly, another member of the *lysoPL* family appears to be co-regulated with *lysoPL1* (Fig. 4), suggesting common functions for this family in the incompatible interaction.

Our results also revealed the presence of two regulatory pathways operating on *lysoPL1* and *PLD γ 1* expression. The *avrRpm1*–*RPM1* interaction results in a rapid cell death, even in plants overexpressing the *nahG* gene, suggesting that the HR is independent of SA. In the *ndr1* genetic background, the *avrRpm1*–*RPM1* interaction elicits an HR but renders the host susceptible to DC3000:*avrRpm1*. The expression pattern of both *lysoPL1* and *PLD γ 1* during an incompatible interaction was indistinguishable in wild-type, NahG overexpressing, or the *ndr1* genetic backgrounds (Fig. 8), suggesting transcriptional control associated with the HR. Interestingly, both genes were rapidly up-regulated upon treatment with SA (Fig. 5) in the same time frame as *PR1* mRNA (a marker of SAR; data not shown), revealing an additional role in defense responses. *PLD γ 1* induction preceded *lysoPL1* induction by 60 min, suggesting a possible role for lysoPL1 in generating secondary messengers for the defense signaling network or in the recycling process (Fig. 9). Ishiguro and associates (2001) recently demonstrated that the JA biosynthetic precursor, linolenic acid (LA), could be generated from the *sn*-1 position of phospholipids by PLA1 activity. LysoPL may, therefore, also generate LA for JA biosynthesis.

Clearly, the expression pattern of this class of *lysoPLs* suggests an important role in plant–pathogen interactions. The physiological role of this novel pathway during pathogen challenge warrants further consideration; however, the redundancy of the *lysoPL1* gene family makes such an approach technically demanding.

MATERIALS AND METHODS

Bacterial strains and infiltration of plants.

P. syringae pv. *tomato* strain DC3000 containing the empty vector plasmid pVSP61 (Innes et al. 1993) and DC3000 with

avrRpm1 cloned into pVSP61 (DC3000:*avrRpm1*) was used to infiltrate plants. Rifampicin (50 μ g/ml) and kanamycin (25 μ g/ml) were used to select for the bacterium and plasmid, respectively. Bacterial inoculums were prepared as previously described (Grant et al. 2000). Final cell densities were adjusted to approximately 0.26 at 600 nm (approximately 2×10^8 CFU/ml) in 10 mM MgCl₂. Finally, the leaves were infiltrated on the abaxial surface with a needleless 1-ml syringe.

A minimum of five leaves on three plants was infiltrated for each time point. The samples were harvested at the appropriate time after challenge and frozen in liquid N₂. All experiments were repeated at least three times. Total RNA was isolated from frozen plant tissue using a standard protocol (Sanchez et al. 2000).

SA treatment. Five-week-old plants were sprayed with a solution of 5 mM SA (sodium salt; Sigma-Aldrich, Dorset, U.K.) and harvested after the appropriate time interval.

Wounding. Plants were wounded by squeezing with serrated forceps at a minimum of five sites along each leaf lamina. Sufficient pressure was exerted to cause visible darkening and moisture accumulation at the wound site.

Plant material and growth conditions.

Plants were sown in Levingtons F2 compost and were vernalized for 2 days at 4°C. Plants were grown under short-day conditions in a controlled environment chamber (12 h of light, 22°C day, 20°C night) with white fluorescent light of approximately 150 μ E m⁻² sec⁻¹ for 5 to 6 weeks before use. Initial cDNA-AFLP display utilized Nd-0 and Nd-0 transformed with the *RPM1* disease resistance gene to analyze differential gene expression after challenge with virulent (DC3000) and avirulent (DC3000:*avrRpm1*) bacteria (Grant et al. 1995; Sanchez et al. 2000).

cDNA-AFLP.

cDNA-AFLP display on plants challenged with DC3000, DC3000:*hrpA*, or DC3000:*avrRpm1* was carried out as described (Durrant et al. 2000; Sanchez et al. 2000; M. de Torres Zabala, P. Sanchez, I. Fernandez-Delmond, and M. Grant, unpublished data). The differentials corresponding to the *PLD γ 1*

Fig. 7. Phospholipase gene signatures respond differentially to wounding or salicylic acid (SA). The mRNA accumulation of phospholipases **A**, *PLD α* , **B**, *PLD β* , **C**, *PLD γ 1*, and **D**, *lysoPL1* was examined by RNA blot analysis after wounding or spraying with SA (5 mM) at the times indicated (hours post treatment = hpt). Only **A**, *PLD α* mRNA responded significantly to wounding. mRNA for both **C**, *PLD γ 1* and **D**, *lysoPL1* were rapidly and strongly induced by SA application, with *PLD γ 1* message accumulating significantly earlier than *lysoPL1* transcripts.

Fig. 8. RNA blots of Col-5, *ndr1*-2, or NahG expressing *Arabidopsis* leaves challenged with DC3000:*avrRpm1* were probed with **A**, *lysoPL1* or **B**, *PLD γ 1* at the times indicated (hpi = hours postinfiltration). *PLD γ 1* and *lysoPL1* mRNA expression patterns are unaffected in *ndr1* genetic backgrounds or in plants overexpressing a bacterial salicylate hydroxylase gene.

Fig. 9. Diagram showing that possible interactions were Phospholipase D (PLD) and lysoPL1 activities and could generate potential secondary signaling molecules during incompatible interactions. The possibility for compartmentalization is ignored in this model. lysoPL1 = lysophospholipase; A₁ = phospholipase A₁; A₂ = phospholipase A₂; C = phospholipase C; JA = jasmonic acid; DAG = diacylglycerol; PA = phosphatidic acid; PAP = phosphatidic acid phosphohydrolase; = fatty acyl acid.

and *lysoPL1* fingerprints were derived from primer combinations 5' GACTGAGTACCAATTCG 3':5' GATGAGTCCTG-AGTAAAG 3' and 5' GACTGAGTACCAATTCG 3':5' GATGAGTCCTGAGTAACG 3', respectively.

RNA blots.

Approximately 14 µg of total RNA was separated by denaturing 1.5% formaldehyde-agarose gel electrophoresis and transferred to nylon membrane (Hybond-NX, Amersham, Buckinghamshire, U.K.). Radioactive DNA probes corresponding to the *lysoPL1*, *PLDγ1* cDNA, or polymerase chain reaction (PCR) products of *PLDα*, *PLDβ*, and *PLDγ2* genes (discussed below) were prepared by random primed labeling, incorporating ³²P-dCTP (>3,000Ci/mmol; Amersham) by using the Stratagene Prime-It II kit. Membranes were prehybridized and hybridized in Church buffer (0.5 M NaPO₄, 7% sodium dodecyl sulfate [SDS], 10 mM EDTA, pH 7.2) prior to washing in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS for 20 min, followed by two washes in 0.1× SSC, 0.1% SDS for 15 min each.

Probes for Northern blots.

The *PLDγ1* cDNA (Wang et al. 2000) was excised from pBSK (Stratagene) and gel purified. A *lysoPL1* cDNA probe was generated by PCR using the cDNA used as a template for the cDNA-AFLP studies based upon the predicted gene sequence. The mRNA and amino acid sequences of *PLDα*, *PLDβ*, and *PLDγ2* from *A. thaliana* were identified by using BLAST (Basic Local Alignment Search Tool) from a Gen-

Bank database. In order to design primers for amplification of the unique regions of the three PLDs, the amino acid sequences of all four cloned PLDs were aligned by using the ClustalW alignment program and the following unique regions were identified: for *PLDα*, 1–399 bp; for *PLDβ*, 1–727 bp; and for *PLDγ2*, 1–775 bp. Primers were designed in these unique intronless regions using the Primer3 program and candidate primers checked to verify that the sequences were unique in the *Arabidopsis* genome. The following primers were used: *PLDα* forward, 5'GGACCAGATCCCGAATTCCTTC3'; *PLDα* reverse, 5'AGCTCCAATCGGGATATCATC3'; *PLDβ* forward, 5'CATCAGCACACTTCGGGTTC3'; *PLDβ* reverse, 5'TTCAATTTTCCTGGCAATC3'; *PLDγ2* forward, 5'ATTGGAGTTTAATCGGAAC3'; *PLDγ2* reverse, 5'AGAAGGTAGGAGGAAGCGAAG3'; Actin 2 (GI:17381173) forward, 5'GTGCCAATCTACGAGGGTTTCT3'; and Actin 2 reverse 5'CTTAATCTTCATGCTGCTTG-GTG3'.

All PCR used *A. thaliana* Col-5 genomic DNA as a template. The MgSO₄ concentrations used for the amplification were as follows: 1.5 mM MgSO₄ for *PLDα*, *PLDγ2*, and actin probes; 5 mM MgSO₄ for *PLDβ*. PCR products were gel purified and sequenced to verify the probes.

ACKNOWLEDGMENTS

We thank P. Dodds for useful discussions and W. Byrne for excellent technical assistance. Support from the BBSRC (32/P13864) and the Central Research Fund, Imperial College is gratefully acknowledged.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Arabidopsis database (<http://www.arabidopsis.org/Blast/>)
 GenBank database (www.ncbi.nlm.nih.gov/blast/blast.cgi)
 ClustalW alignment program (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/Options/clustalw.html>)
 Primer3 program (<http://www.basic.nwu.edu/biotools/Primer3.html>)