Involvement of Phospholipase D in Wound-Induced Accumulation of Jasmonic Acid in Arabidopsis

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Multiple forms of phospholipase D (PLD) were activated in response to wounding, and the expressions of PLDα, PLDβ, and PLDγ differed in wounded Arabidopsis leaves. Antisense abrogation of the common plant PLD, PLDα, decreased the wound induction of phosphatidic acid, jasmonic acid (JA), and a JA-regulated gene for vegetative storage protein. Examination of the genes involved in the initial steps of oxylipin synthesis revealed that abrogation of the PLDα attenuated the wound-induced expression of lipoxygenase 2 (LOX2) but had no effect on allene oxide synthase (AOS) or hydroperoxide lyase in wounded leaves. The systemic induction of LOX2, AOS, and vegetative storage protein was lower in the PLDα-suppressed plants than in wild-type plants, with AOS exhibiting a distinct pattern. These results indicate that activation of PLD mediates wound induction of JA and that LOX2 is probably a downstream target through which PLD promotes the production of JA.

INTRODUCTION

Jasmonic acid (JA) and related compounds are a new class of plant hormones that play an important role in regulating many cellular processes, such as wound and defense responses (Farmer and Ryan, 1992; Bell et al., 1995; Creelman and Mullet, 1997; McConn et al., 1997). The production of JA is a tightly regulated process, and the concentrations of JA in unperturbed plant tissues are often very low. However, JA accumulates in wounded plants or in plants and cultured cells treated with pathogen elicitors; it acts as a signal activating the expression of various genes, such as proteinase inhibitors, thionin, and enzymes in phytoalexin metabolism (Creelman and Mullet, 1997). The pathway for de novo JA biosynthesis, beginning with free α-linolenic acid, has been well elucidated (Vick, 1993; Creelman and Mullet, 1997; also see Figure 1). But when and how linolenic acid is made available for JA synthesis is not well understood. Linolenic acid, the most abundant fatty acid in leaves, is mostly present in an esterified glycerolipid form (Browse and Somerville, 1991). Free fatty acids are not generally found in large amounts in healthy, intact plant cells. The release of linolenic acid from membranes has been thought to be an important step in controlling JA synthesis. An increase in free linolenic acid was observed in cultured cells of several plant species after treatment with fungal wall elicitors (Gundlach et al., 1992) and in wounded plants (Conconi et al., 1996; Ryu and Wang, 1998). A phospholipase A (PLA)–like activity has been proposed to mediate the release of linolenic acid from membranes (Farmer and Ryan, 1992), and the presence of such a wound-inducible PLA activity has been noted in tomato and other plant species (Lee et al., 1997; Narváez-Vásquez et al., 1999).

Recent studies have suggested that activation of phospholipase D (PLD) also may play an important role in mediating wound-induced lipid hydrolysis (Ryu and Wang, 1996, 1998; Lee et al., 1997). PLD hydrolyzes phospholipids at the terminal phosphoesteric bond, generating phosphatidic acid (PA) and free head groups, such as choline (Wang, 1999). This enzyme is involved in various regulatory processes, such as those leading to hormone action (Fan et al., 1997; Jacob et al., 1999), cell proliferation (Daniel et al., 1999), membrane trafficking, secretion (Colley et al., 1997; Jones et al., 1999), and defense response (Waite et al., 1997; Wang, 1999). Wounding of castor bean leaves rapidly activates PLD-mediated hydrolysis, as indicated by a rapid accumulation of PA and choline (Ryu and Wang, 1999). Wound-induced production of PA has been found at both the wound site and sites distal to wounding in several plant species examined, including castor bean, tomato, soybean, sunflower, broad bean, and pepper (Lee et al., 1997). The activation in castor bean appears to result from intracellular translocation of PLD from cytosol to membranes, mediated by an increase in cytoplasmic Ca2+ concentrations (Ryu and Wang, 1996). The regulated increase of PLD-mediated hydrolysis points to its importance in the wound response.

On the basis of the analysis of wound activation of PLD and induction of various lipid metabolites, Ryu and Wang...
Wound Induction of Multiple PLDs and Their Role in Wound-Induced PA Production

The expression of PLoα gene in Arabidopsis was suppressed by introducing a PLoα antisense gene. When measured by a PLD assay specific for PLoα activity (Pappan et al., 1997a), the leaves of PLoα antisense plants displayed <3% of the PLoα activity in wild-type plants (Figure 2A). The lack of activity resulted from the loss of PLoα protein, as attested by the absence of an immunoreactive PLoα band in the soluble and membrane-associated fraction in

**RESULTS**

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the antisense plants (Figure 2B). The observation of the loss of PLDα protein was supported by the lack of PLDα mRNA (denoted PLDα in Figure 3), indicating that PLDα gene expression was abrogated in the antisense plants. The transcript of the introduced PLDα antisense gene was readily detectable (Anti-PLDα fragment in Figure 3). The antisense transcript contained no open reading frame; thus, no translation was expected. A previous study showed that the PLDα-depleted leaves had the normal phosphatidylinositol 4,5-bisphosphate (PIP2)-dependent activities that are characteristic of PLDβ and PLDγ (Pappan et al., 1997a; Qin et al., 1997). The present study indicated that the expressions of PLDβ, PLDγ1, and PLDγ2 (see below) were the same for the antisense and the wild-type plants (Figure 3). These results show that the antisense suppression is specific to the PLDα isoform.

After wounding, membrane-associated PLDα activity increased, accompanied by a decrease in soluble PLDα activity in wild-type leaves (Figure 2A). The PLD specific activity increased ~30% in the microsomal fraction and decreased 15% in the soluble fraction 1 hr after wounding. Immunoblotting with a PLDα-specific antibody showed a clear increase in membrane-associated PLDα protein 30 min after wounding, whereas the soluble PLDα protein decreased slightly after wounding (Figure 2B). This decrease in soluble PLDα protein was consistent with the small decrease of PLDα activity overall in this fraction. This inverse change in membrane-associated and soluble PLD was also observed in wounded castor bean leaves (Ryu and Wang, 1996) and suggests that PLDα translocates from the cytosol to membranes in response to wounding (Figure 1). The increased association of PLDα with membranes could bring it into contact with lipid substrates, thereby rapidly activating PLD-mediated hydrolysis in response to wounding.

The wound activation of PLD was documented by a rapid increase in its lipid product, PA (Figure 4). PLDα-abrogated and wild-type leaves contain similar amounts of PA before wounding, but the PA amount in wild-type leaves was substantially more than in PLDα-deficient leaves after wounding. The wound-induced PA in wild-type plants was ~2.5 times that of PLDα-deficient leaves 15 min after wounding. That is, >60% of the wound-induced PA is derived from PLDα activation at this time point and in this wounded condition. PA in the PLDα-deficient leaves increased markedly,
albeit less than in wild-type leaves, after wounding. In particular, the difference in the wound-increased PA between PLDα-deficient and wild-type plants became smaller in the later phases than in the early phases after wounding, the wound-induced PA in wild-type leaves being only 1.4-fold that of PLDα-deficient leaves 1 hr after wounding. These results indicate that PLDα was not the only PLD responsible for the wound-increased PA; other PLDs also contributed to the increase, particularly in later responses to wounding.

The potential involvement of other PLDs in the wound response was examined by monitoring temporal changes in their transcripts after wounding (Figure 3). Four PLDs—PLDα, β, γ1, and γ2—have been identified in Arabidopsis (Qin et al., 1997, 1999). In unwounded Arabidopsis leaves, all four PLD transcripts were detectable. The patterns of wound-induced accumulation of PLDβ, γ1, and γ2 mRNA were almost identical between the antisense and wild-type plants, indicating that the loss of PLDα gene expression did not affect the wound-induced expression of the other PLD genes. Expression of these PLD genes responded differently to wounding. The greatest increase for PLDβ mRNA occurred 30 min after wounding, whereas for PLDγ1 and γ2 transcripts was at 60 min after wounding. In contrast, PLDα mRNA showed a slight increase at 3 and 6 hr after wounding but showed no obvious change in the early phases of wounding (Figure 3). These results suggest that increased gene expression was involved in wound induction of PLDβ and PLDγs, but not PLDα, in the early phases of wound responses.

The increases in PLDβ and γ gene expression were reflected also by the amounts of wound-increased PIP2-dependent PLD activity possessed by PLDβ and γs (Pappan et al., 1997b; Qin et al., 1997). The increase occurred in microsomal fractions but not in soluble ones, and approximately twofold increases were observed at 3 hr after wounding in wild-type (Figure 5A) and PLD-deficient leaves (data not shown). Immunoblotting with a PLDγ antibody showed an increase in membrane-associated PLDγ (Figure 5B). But PLDβ in unwounded and wounded leaves was undetectable with a PLDβ antibody (data not shown), which is consistent with an earlier study (Fan et al., 1999) and indicates that the amount of this isoform present was very low. The wound-increased PIP2-dependent PLD activity, which did not occur in the first 30 min after wounding, lagged behind the increases in PLDβ and γ transcripts (Figure 3) and PLDα activity (Figure 2A). These temporal changes in the transcripts, protein content, and activity of PLDα, β, and γ suggest that the wound-induced expression of PLDβ and γ genes contributed mainly to the increased PIP2-dependent PLD activity, whereas the increased membrane association was primarily responsible for the early phases of PLDα activation.

Figure 4. Wound-Induced Increase in PA in PLDα-Suppressed and Wild-Type Arabidopsis Leaves.

Phospholipids were separated by thin-layer chromatography, and PA was isolated and quantified by gas chromatography. Values are means ± SE (n = 5). WT, wild type; wt, weight.

Figure 5. Wound-Induced Increase in PIP2-Dependent PLD in Wild-Type Arabidopsis Leaves.

(A) PIP2-dependent PLD activity in leaf microsomal (M) and soluble (S) fractions after wounding. Error bars indicate ± SE.

(B) Immunoblotting of microsomal (M) and soluble (S) PLD with affinity-purified PLDγ antibodies at various intervals after wounding. Microsomal PLD was from the pellet of 100,000 g centrifugation of the 6000 g supernatant, and soluble PLD was from the supernatant after 100,000 g centrifugation. Proteins were resolved on an 8% SDS-polyacrylamide gel, and PLD in the blot was made visible by using alkaline phosphatase as the stain.
Evidence for PLD in Modulating Wound Induction of JA and Oxylipin Pathways

The role of PLDα activation in wound responses was determined by comparing wound induction of JA in PLDα-deficient and wild-type plants (Figure 6). The concentration of JA in unwounded leaves was very low (data not shown) and increased greatly after wounding. The wound-induced JA was substantially greater in wild-type than PLDα-deficient leaves. At 1 and 2 hr after wounding, the JA content in wounded wild-type leaves was approximately twice that in PLDα-suppressed plants grown in the greenhouse (Figure 6A). Wounding was also performed on plants grown in a growth chamber to verify the attenuated wound induction of JA. The wound-increased JA was ~50% greater in wild-type than in PLDα-deficient leaves (Figure 6B). Because the antisense and wild-type plants had similar temporal patterns for the JA increase after wounding, the decrease in JA was not the result of a slower response to wounding in the PLDα-suppressed plants; rather, the PLDα-depleted plants had less overall ability to accumulate JA.

The decreased JA concentrations in PLDα-suppressed plants correlated with decreased amounts of mRNA of JA-inducible genes LOX2 and AtVSP (for Arabidopsis thaliana vegetative storage protein) (Bell and Mullet, 1993; Berger et al., 1996) (Figure 7). The LOX2 transcript increased greatly at 1 hr after wounding in wild-type leaves, and the increase persisted for 4 hr. However, only a slight induction of LOX2 transcript was detected in the PLDα-depleted plants (Figure 7A). Similarly, the amount of AtVSP mRNA in PLDα-depleted plants was substantially less than that in wild-type plants after wounding (Figure 7B). On the other hand, the expression of wound-inducible genes, allene oxide synthase (AOS) and hydroperoxide lyase (HL) (Bate et al., 1998; Laudert and Weiler, 1998), responded similarly to wounding in PLDα-depleted and wild-type plants; no mRNA was detected in the unwounded leaves, and the amount was greatest at 1 hr after wounding (Figure 7B).

To determine the effect of PLDα depletion on systemic response to wounding, JA concentrations in unwounded leaves (hereafter referred to as systemic leaves) of wounded plants were measured in wild-type and antisense plants. No systemic increase of JA occurred in PLDα-abrogated plants, whereas JA increased in wild-type systemic leaves at 1 hr after wounding (Figure 8A). To test whether the systemic JA increase was delayed in PLDα-abrogated leaves, the systemic JA in the antisense plants was measured at 3 hr after wounding but showed no increase. Compared with the JA increase in wounded leaves (Figure 6), the JA increase in wild-type systemic leaves was quite small, consistent with results reported by others (Laudert and Weiler, 1998).

To clarify whether the PLDα abrogation affected the systemic response, we assessed the wound induction of LOX2, AOS, and AtVSP gene expression in systemic leaves. Wounding increased the expression of LOX2, AOS, and AtVSP in systemic leaves, the increase being less in the PLDα-deficient than in the wild-type plants (Figure 8B). In addition, the pattern of systemic induction of AOS was distinct from that of LOX2 and AtVSP. One hour after wounding, the LOX2 and AtVSP mRNAs were much less in PLDα-deficient than in wild-type plants, but the amount of systemic induction of AOS was similar in both genotypes (Figure 8B). This pattern of induction at the earlier phase of wounding was similar to that in wounded leaves: depletion of PLDα rendered LOX2 and AtVSP, but not AOS, less sensitive to wounding induction than in wild-type plants (Figure 7). However, at 3 hr after wounding, further systemic increases in LOX2 and AtVSP were seen in both wild-type and PLDα-deficient plants, whereas a further increase for AOS was observed only in wild-type but not in the antisense plants.

The PLDα-suppressed plants were analyzed also for the ability to respond to methyl JA without wounding to test whether the decreased gene expression was caused by an impaired JA perception or an impaired signaling process in the PLDα-deficient plants. Airborne methyl JA induced the expression of AtVSP and LOX2 in the same manner in wild-type and PLDα-suppressed plants (Figure 9). The basal amount of LOX2 transcript shown in Figures 7 and 8 was invisible here because of the use of a shorter exposure, given the high amounts of methyl JA–induced transcript. This shows that the PLD-depleted plants were fully capable of perceiving the methyl JA signal. Methyl JA is a well-documented inducer for the expression of AtVSP (Berger et al., 1996). These data suggest that the decreased induction of AtVSP resulted from a decrease in JA production rather than from an altered perception of wounding or JA in the PLDα-deficient plants.
DISCUSSION

This study indicates that multiple PLDs are involved in the wound response and that PLDα, β, γ1, and γ2 are expressed differently in response to wounding. The PLDβ gene displayed the strongest wound induction, PLDγ1 and γ2 mRNAs were moderately increased, and the expression of PLDα was least sensitive to wounding. Meanwhile, PLDα is activated in wounding by the increased association of preexisting enzyme with membranes. Such a pattern of changes for the four PLDs in response to wounding could mean that they occupy different steps in the wound and JA signaling pathways. The intracellular translocation of PLDα constitutes an early step in stimulus-induced PLD activation (Ryu and Wang, 1996; Figure 1), because no de novo synthesis of PLD mRNA and protein is required. On the other hand, the increased expression of PLDβ and γ genes may provide PLDs for later phases of wound responses. The changes in wound-induced PA are consistent with this interpretation. Suppression of PLDα only partially blocks wound-induced PA production, and the differences in the concentrations of wound-induced PA between PLDα-deficient and wild-type leaves are greater in the early phases than in the later phases of wounding. Some of the wound-induced PA might also be derived from de novo synthesis or from activation of phospholipase C (PLC) followed by activation of a DAG kinase.

The significance of PLD-mediated hydrolysis in wound responses has been demonstrated in this study by the observations that suppression of PLDα substantially reduced the wound induction of JA and the wound/JA-inducible AtVSP and LOX2. Activation of PLD has the potential to affect oxylipin production in various ways. The increase in PA has been suggested to promote the release of polyunsaturated fatty acids by supplying PA as a lipolytic substrate or by activating acyl-hydrolyzing enzymes such as PLA and nonspecific acyl hydrolases (Figure 1). The PLD product PA has been recently identified as a pivotal lipid messenger that activates various lipid-metabolizing enzymes, including PLA2, PLC, and phosphoinositide 5-kinase (Bauldry and Wooten, 1997; Kinkaid et al., 1998; Jones et al., 1999). PA may modulate these enzyme activities by direct binding (Waite et al., 1997; Daniel et al., 1999; Jones et al., 1999), by altering membrane environments (Cornell and Arnold, 1996), or both. PA is a nonlamellar lipid with a tendency to form inverted hexagonal phases, and the bilayer-perturbing property is known to stimulate many lipid-modifying enzymes, including PLDs, PLA1, PLA2, PLC, PA phosphatase, and DAG kinase, as well as G proteins and protein kinases and phosphatases in mammalian systems (Cornell and Arnold, 1996; Escriba et al., 1997). Studies in plants have suggested that the activation of PLD precedes that of PLA/acyl hydrolases (Samama and Pearce, 1993; Ishimoto et al., 1994; Lee et al., 1997; Ryu and Wang, 1998). Ryu et al. (1997) also reported that some lysophospholipids, such as lysophosphatidylethanolamine, are inhibitors of PLDα and animal PLDs. This raises an intriguing possibility that increased PLA/acyl hydrolase activity may lead to a feedback inhibition of PLD activation (Wang, 1999).

In addition, the present study points to another process by which PLD may regulate JA synthesis, that is, through modulating the expression of LOX2. Support for this mechanism is the finding that depletion of PLDα decreased the induction of LOX2 gene expression but had no effect on the wound induction of AOS and HPL in wounded leaves. The
decreased wound induction of LOX2 could have a direct effect on wound induction of JA because LOX2 in Arabidopsis catalyzes the first step in JA synthesis to form 13-hydroperoxylinolenic acid (Vick, 1993; Bell et al., 1995; Creelman and Mullet, 1997). This decrease in LOX expression would lead to a slower rate of supplying the oxygenated fatty acid to AOS, which commits the LOX product for JA synthesis (Figure 1). Additionally, LOX has been reported to utilize fatty acids on glycerolipids (Brash et al., 1987; Perez-Gilabert et al., 1998), and oxidized fatty acids on membrane lipids are more susceptible to cleavage by phospholipases (Banas et al., 1992). Thus, a decrease in LOX could also reduce the release of oxygenated fatty acids from membranes for JA synthesis.

Furthermore, the data for the PLD-abrogated plants indicate that different regulatory processes are involved in the wound activation of LOX2 and AOS, particularly in the early phases of wounding. Both LOX2 and AOS are essential enzymes in JA synthesis, and their levels of expression have been correlated with JA production (Bell et al., 1995; Creelman and Mullet, 1997; Laudert and Weiler, 1998). The expression of LOX2 and AOS is wound inducible, as observed with the wounded wild-type leaves (Figure 6B). Unlike LOX2, however, wound induction of AOS is not altered by PLD abrogation in wounded leaves, suggesting that two distinctly different signaling pathways regulate the expression of LOX2 and AOS in the tissue. This explanation is supported also by the systemic results, which show a distinct temporal pattern of induction for AOS that differs from those for LOX2 and VSP. PLDα abrogation decreases the amount of systemic induction of LOX2, but not of AOS, at 1 hr after wounding. Thus, the wound activation of PLD may provide PA, which serves as a messenger increasing LOX expression, but it does not mediate the wound induction of the AOS gene. A recent report suggests that ethylene is a signal in the wound induction of AOS (Sivasankar et al., 2000). On
the other hand, the suppression of PLDα decreased the systemic induction of AOS in a later phase of wounding (Figure 8). The later attenuation of AOS expression could be a consequence of a decrease in or even a lack of systemic JA in the PLDα-abrogated plants.

The PLD-mediated activation of LOX2 may also provide an important connection between the locations of wound perception and JA synthesis. PLDα is present in the plasma and microsomal membranes but not in chloroplasts (Xu et al., 1996; Young et al., 1996). In contrast, LOX2 and AOS, which are encoded by nuclear genes, are localized in the chloroplasts of Arabidopsis (Vick, 1993; Bell et al., 1995; Creelman and Mullet, 1997). This compartmentalization raises a question of how the information on the cell surface is transduced into plasstds in defense responses. Perhaps wounding compartmentalizes the enzymes and lipids involved in JA synthesis, which are initially localized in different compartments, and this may activate JA production. However, studies using the peptidase systemin and plant cell wall-derived oligosaccharides have shown an induction of de novo JA synthesis without direct tissue damage (Farmer and Ryan, 1992; Narváez-Vásquez et al., 1999). Thus, the plasma membrane is probably the initial point that transduces wound or elicitation signals to the induction of de novo JA synthesis. Two pathways of JA synthesis have also been proposed, one localized in the chloroplast and another in the cytoplasm (Creelman and Mullet, 1997; Wang et al., 1999). The regulation of LOX2 expression through PLD activation outside plastids links wounding perturbation to induction of JA synthesis. The connection of PLDα activation with the formation of the defense hormone JA indicates that PLD activation constitutes an important signaling step in plant defense responses.

**METHODS**

**Plant Materials and Growth Conditions**

Seeds of wild-type and PLDα-suppressed Arabidopsis thaliana ecotype Columbia were sown in soil and cold-treated at 4°C overnight. Plants were grown in growth chambers under 14-hr-light/10-hr-dark cycles with cool-white fluorescent light of 100 μmol m⁻² sec⁻¹ at 23 ± 3°C. The greenhouse conditions were a 12-hr photoperiod at 25 ± 5°C. Generation of the phospholipase D (PLD) α-deficient line was described previously (Fan et al., 1997; Pappan et al., 1997a). Before each treatment, the PLDα deficiency of the transgenic plants was confirmed by assaying extracts for PLDα activity and sometimes by using immunoblot analysis with PLDα-specific antibodies (Pappan et al., 1997a).

**Wounding and Analysis of Jasmonic Acid and Phosphatidic Acid**

Leaves on plants were wounded with a hemostat (taped to close only to the first notch) twice on each leaf. Wounded leaves were detached at indicated intervals and stored in liquid nitrogen before extraction of jasmonic acid (JA). For systemic effect, unwounded leaves from the wounded plants were collected. JA was extracted and measured as described previously (Wang et al., 1999), and the wound-induced JA was detected and verified by gas chromatography–mass spectrometry. For determination of phosphatidic acid (PA), wounded leaves were immersed in hot isopropanol (75°C) for 15 min immediately after sampling to terminate any lipolytic activities (Ryu and Wang, 1996). Lipids were extracted and separated as described previously (Ryu and Wang, 1996, 1998). Phospholipids were separated by thin-layer chromatography on Silica Gel 60 (Merck) developed in chloroform/methanol/acetic acid/water (85:15:12.5:3.5 [v/v/v/v]). Individual lipids were visualized with iodine vapor and identified by co-chromatography with authentic standards (Ryu and Wang, 1996, 1998). PA bands were scraped into test tubes and quantified by determining fatty acid content with an HP5890A (Hewlett-Packard, Wilmington, DE) gas chromatograph and using 15:0 fatty acid as an internal standard. Fatty acid methyl esters were separated by gas chromatography with a Supelco 30-m Omegawax 250 capillary column (Restek, Bellefonte, PA) run isothermally at 200°C and were detected with a flame ionization detector.

**Protein Fractionation, Assay of PLD Activity, and Immunoblotting**

Total protein from Arabidopsis tissues was extracted by grinding in an ice-chilled mortar and pestle with buffer A (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, and 2 mM DTI). The homogenate was centrifuged at 6000g for 10 min at 4°C to remove tissue debris, and the supernatant was centrifuged at 100,000g for 60 min at 4°C. The resulting supernatant was referred to as the soluble fraction and the pellet as the microsomal fraction. The pellet was resuspended in buffer A and centrifuged again at 100,000g to reduce cytosolic contamination. Protein concentration was determined by a dye binding assay according to the manufacturer’s (BioRad, Hercules, CA) instructions.

PLD activity was determined by procedures described previously (Pappan et al., 1997a). Briefly, the PLDα, phosphatidylinositol 4,5-bisphosphate (PIP2)-independent activity was assayed in the presence of 100 mM Mes, pH 6.5, 0.5 mM SDS, 1% (v/v) ethanol, 25 mM CaCl₂, 1 mM egg yolk phosphatidylcholine mixed with dipalmitoyl-glycerol-3-phospho[methyl-3H-choline, and 2 to 10 μg of protein in a total volume of 200 μL. The PIP₂-independent PLD activity was assayed in the presence of 100 μM CaCl₂ and mixed lipid vesicles composed of phosphatidylcholine, phosphatidylethanolamine, and PIP₂, with phosphatidylycholine being radioactively labeled (Pappan et al., 1997a). In both assays, release of ³H-choline into the aqueous phase was quantitated by scintillation counting.

The protein extracts were separated by 8% SDS-PAGE and transferred onto polyvinylidene difluoride filters. The membranes were blotted with PLDα, PLDβ, or PLDγ antibodies, followed by incubation with a second antibody conjugated to alkaline phosphatase, according to a published procedure (Fan et al., 1999). PLDα and PLDβ antibodies were produced in rabbits against the C-terminal 13-amino acid peptide of the respective Arabidopsis PLDα and PLDβ (Pappan et al., 1997a). PLDγ antibodies were raised against a 13-amino acid peptide near its C terminus and were affinity-purified against Arabidopsis PLDγ expressed in Escherichia coli (Fan et al., 1999). The proteins recognized by antibodies were made visible by staining the phosphatase activity with a Bio-Rad immunoblotting kit.
Treatment of Plants with Methyl JA; RNA Isolation and Blotting

Six-week-old Arabidopsis plants in pots were placed in a sealable 8-liter glass jar for 1 day for acclimation before methyl JA (Sigma) was placed in the jar. Tissues were collected for RNA extraction after treatment. Total RNA was isolated from Arabidopsis leaves by a cetyltrimethylammonium bromide extraction method (Fan et al., 1997; Wang et al., 1999). Equal amounts of total RNA were separated by 1% formaldehyde–agarose denaturing gel electrophoresis and transferred to nylon membranes. PLD gene-specific probes were as reported previously (Qin et al., 1997; Fan et al., 1999). Probes for LOX2, AOS, HL, and AtVSP were based on cDNA inserts from the respective expressed sequence tag clones obtained from the Arabidopsis Resource Center (Ohio State University, Columbus). The inserts were verified and isolated for labeling with the Arabidopsis Resource Center (Ohio State University, Columbus).

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