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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 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, 1996). 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 Ca^{2+} 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

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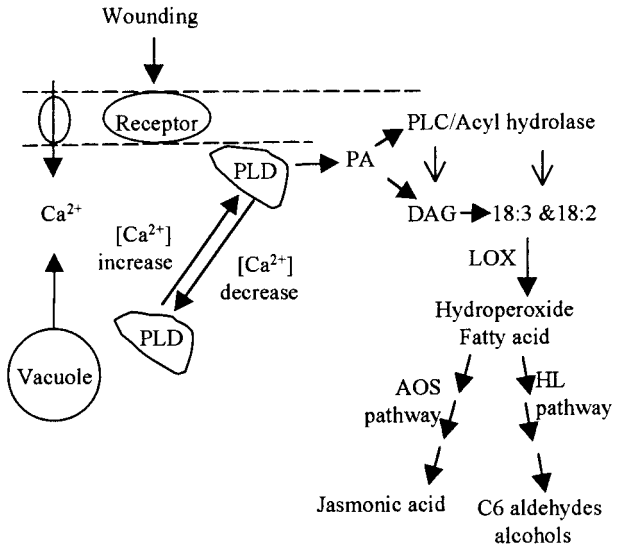

Figure 1. Working Model Depicting Activation and the Role of Phospholipase D in Mediating Lipid Hydrolysis and Production of JA in Plant Response to Wounding.

Wounding induces translocation of phospholipase D (PLD) to membranes via an influx of Ca^{2+}. PLD associated with membranes becomes active and releases free polyunsaturated fatty acids from membrane phospholipids by initiating the lipolytic process and by increasing the activities of acyl-hydrolyzing enzymes. AOS, allene oxide synthase; DAG, diacylglycerol; HL, hydroperoxide lyase; LOX, lipoxygenase; PLC, phospholipase C; 18:3, linolenic acid; 18:2, linoleic acid.

(1998) proposed a working model to account for the role of PLD in the wound response (Figure 1). PLD activation may promote the release of polyunsaturated fatty acids through two interwoven processes. First, the PLD-mediated formation of PA may initiate a lipolytic pathway, consisting of PLD, PA phosphatase, and acyl-hydrolyzing enzymes. In this pathway, phospholipids are converted sequentially into PA, diacylglycerol (DAG), and free fatty acids, including the substrate for JA synthesis. Consistent with this proposed pathway is the observation that wound-induced PA production in castor bean occurs before DAG and linolenic acid are produced (Ryu and Wang, 1998). This PLD-initiated process has also been proposed to occur in deteriorating membranes of senescent and aging plant tissues (Paliyath and Droillard, 1992; Samama and Pearce, 1993). Second, PA produced by PLD may directly stimulate acylhydrolase or PLA activities. In several plant species, the wound induction of PA precedes the induction of lysophosphatidylcholine and lysophosphatidylethanolamine, which could result from PLA activity (Lee et al., 1997). PA has been shown to be an activator of PLA_2 in mammalian systems (Bauldry and Wooten, 1997; Kinkaid et al., 1998). This and the first pathway are not mutually exclusive, and operation of either process releases arachidonic acid in mammalian cells (Ishimoto et al., 1994). Thus, linolenic acid for wound-induced JA synthesis perhaps is derived directly from PA released by PLD or from other lipids whose hydrolysis is activated by PLD-produced PA.

It is evident that multiple lipolytic enzymes are activated after wounding (Conconi et al., 1996; Ryu and Wang, 1996, 1998; Lee et al., 1997; Narváez-Vásquez et al., 1999), and discerning the role of different lipolytic reactions is a central, yet extremely challenging facet of understanding the function and regulation of membrane lipid hydrolysis. In addition, PLD is a multiple gene family encoding several distinct isoenzymes, and three types of PLDs—PLDα, PLDβ, and PLDγ1—have been characterized in Arabidopsis (Pappan et al., 1997a, 1997b, 1998; Qin et al., 1997; Wang, 1999). PLDα is the conventional plant PLD and is polyphosphoinositide independent when assayed at millimolar concentrations of Ca^{2+} (Pappan et al., 1997a). In contrast, the newly identified PLDβ and PLDγ1 require a polyphosphoinositide cofactor and are most active at micromolar concentrations of Ca^{2+} (Pappan et al., 1997b; Qin et al., 1997). These findings raise questions about the role of individual PLDs in wound responses. Judging from the results of cloning, purification, activity distribution, and expression studies, PLDα is more prevalent and widespread than PLDβ and PLDγ1 in plant tissues (Pappan et al., 1997a; Fan et al., 1999; Wang, 1999). We have generated PLDα-deficient Arabidopsis by introducing an antisense gene of this *PLD*. Virtually all PLDα in leaves is lost, but the antisense plants possess normal amounts of the polyphosphoinositide-dependent PLD activity (Pappan et al., 1997a). In this study, we examined the role of PLDs in a wound signaling pathway by determining the effect of PLDα abrogation on PA and JA production in wounded Arabidopsis and by monitoring the expression *PLDα*, *PLDβ*, and *PLDγ*s in response to this wounding. The results provide evidence that activation of PLD is involved in mediating wound induction of JA and that multiple PLDs are activated in response to wounding. In addition, this study indicates that lipoxygenase 2 (*LOX2*) is probably one of the targets through which PLD promotes the wound induction of JA.

RESULTS

Wound Activation of Multiple PLDs and Their Role in Wound-Induced PA Production

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

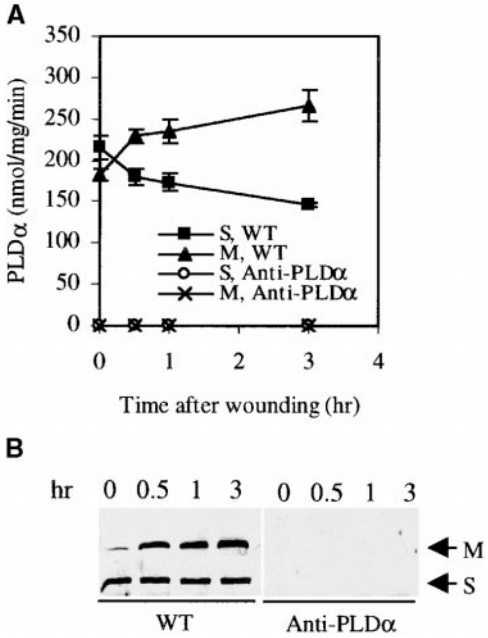

Figure 2. Wound-Induced Membrane Association of PLDα in Arabidopsis Leaves.

(A) PLDα activity in microsomal (M) and soluble (S) fractions after wounding in wild-type (WT) and *PLDα*-suppressed (Anti-PLDα) Arabidopsis leaves. Error bars indicate ±SE.

(B) Immunoblotting analysis of microsomal (M) and soluble (S) PLDα at various intervals after wounding in wild-type (WT) and *PLDα*-suppressed (Anti-PLDα) Arabidopsis leaves.

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.

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 (PIP_2)-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 inversed 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,

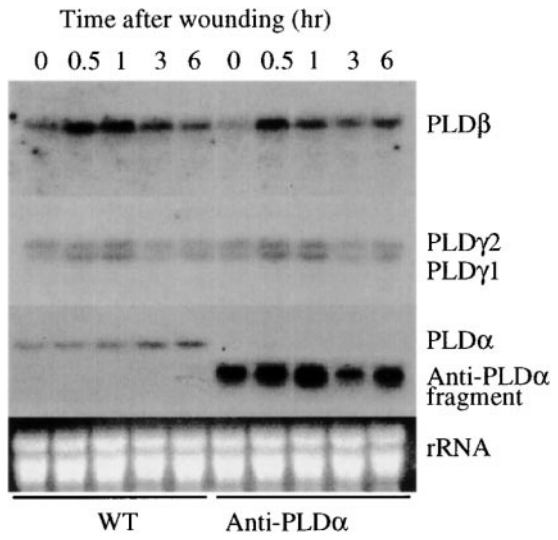

Figure 3. Effect of Wounding on the Expression of Multiple *PLD* Genes.

RNA gel blot of *PLDα*, *β*, *γ1*, and *γ2* transcripts in Arabidopsis leaves before and after wounding. Total RNA (10 μg per lane) isolated from leaves before and after wounding was probed with *PLDα*, *β*, *γ1*, and *γ2* cDNAs, and rRNA was used to indicate equal loading. Autoradiograms for *PLDα*, *β*, and *γ* blots resulted from exposure for 1, 4, and 2 days, respectively. The anti-*PLDα* fragment marks the transcript from the introduced *PLDα* antisense gene. WT, wild type.

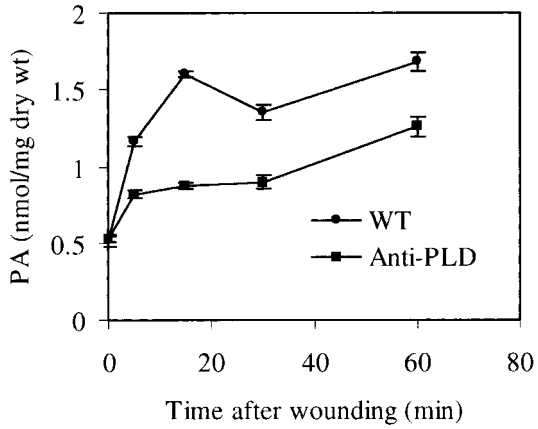

Figure 4. Wound-Induced Increase in PA in *PLDα*-Suppressed and Wild-Type Arabidopsis Leaves.

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.

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 that 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 *PLDγ*s, 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 PIP_2-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 PIP_2-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 PIP_2-dependent PLD activity, whereas the increased membrane association was primarily responsible for the early phases of PLDα activation.

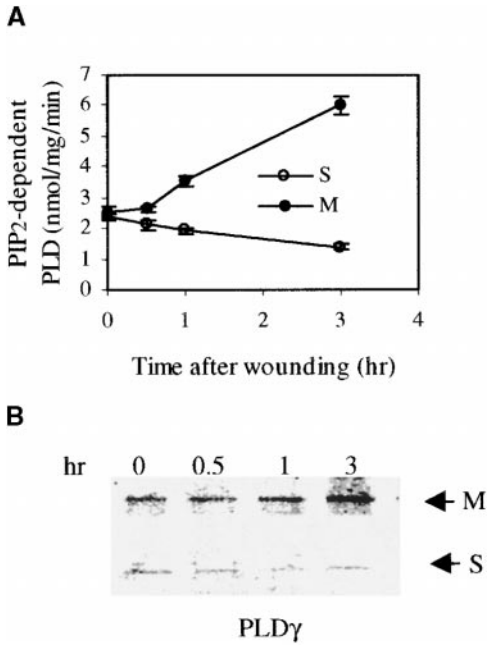

Figure 5. Wound-Induced Increase in PIP_2-Dependent PLD in Wild-Type Arabidopsis Leaves.

(A) PIP_2-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.

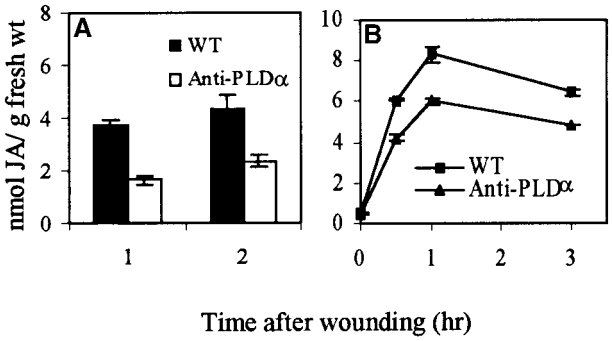

Figure 6. Decreased Wound Induction of JA in *PLDα*-Suppressed Arabidopsis Leaves.

(A) Plants grown in a greenhouse. wt, weight.

(B) Plants grown in a growth chamber.

Leaves on plants were wounded with a hemostat. JA was extracted from leaves with methanol, partially purified with tC_{18}-SepPak cartridges, identified by gas chromatography–mass spectrometry, and quantified by gas chromatography. Values are means ±SE ($n = 3$). WT, wild type.

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 wound 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 *PLD*-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.

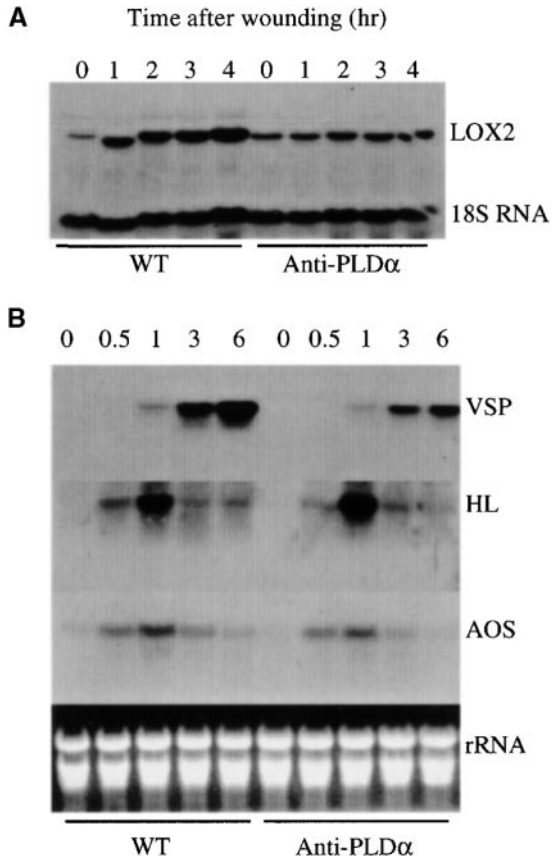

Figure 7. Effect of Wounding on the Expression of JA/Wound-Inducible Genes in PLDα-Abrogated and Wild-Type Arabidopsis Leaves.

(A) Autoradiography of *LOX2* transcripts on an RNA gel blot hybridized with a *LOX2* cDNA probe. The filter was stripped and hybridized with an 18S rRNA probe to indicate the equal loading of total RNA.
(B) Autoradiography of Arabidopsis *VSP*, *AOS*, and *HL* transcripts on an RNA gel blot.
Lanes 0 were total RNA from leaves before wounding, and lanes marked 0.5, 1, 2, 3, 4, and 6 indicate the time (hours) after wounding. Total RNA (10 μg per lane) was loaded, and rRNA was used to indicate the equal loading. WT, wild type.

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 PLA_2, 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, PLA_1, PLA_2, 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

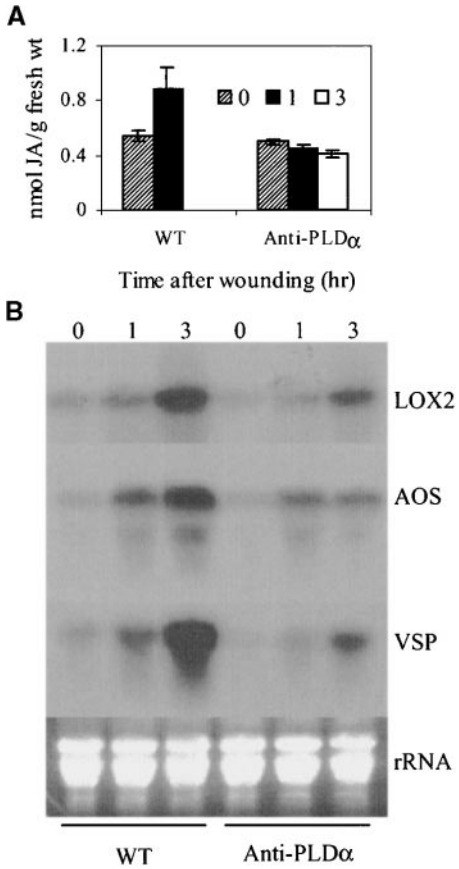

Figure 8. Attenuated Systemic Induction of JA and Expression of *LOX2*, *AOS*, and *VSP* Genes in PLDα-Abrogated Arabidopsis Leaves.

(A) JA concentrations in systemic leaves of wild-type and PLDα-abrogated Arabidopsis. JA was extracted from unwounded leaves of wounded plants with methanol, identified by gas chromatography–mass spectrometry, and quantified by gas chromatography. wt, weight. Error bars indicate ±SE.

(B) RNA gel blotting of *LOX2*, *AOS*, and *VSP* transcripts in unwounded leaves of wounded wild-type and *PLDα* antisense plants. Lower leaves from ~5-week-old Arabidopsis plants grown in a growth chamber were wounded, and RNA was extracted from the upper unwounded leaves. Lanes 0, 1, and 3 mark total RNA from leaves before wounding and at 1 and 3 hr after wounding, respectively. Total RNA (10 μg per lane) was loaded, and equal loading was verified by rRNA.

WT, wild type.

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

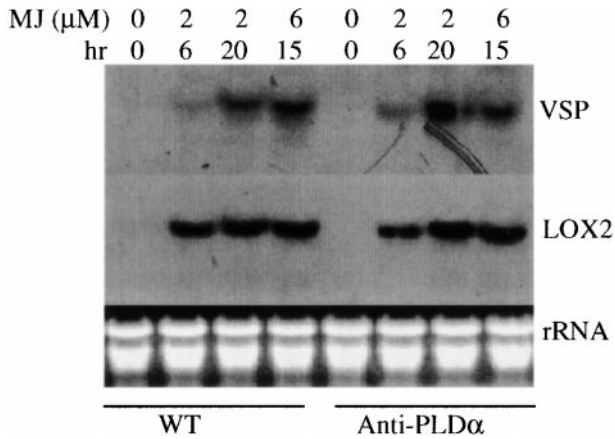

Figure 9. JA-Induced Expression of *LOX2* and *VSP* without Wounding in Arabidopsis Leaves.

Six-week-old plants were pretreated in sealed chambers for a day before airborne methyl JA (dissolved in ethanol) was applied to chambers at final concentrations of 0 (ethanol only), 2, and 6 μM for the times indicated. Total RNA (15 μg per lane) was hybridized with *LOX2* and *AtVSP* (VSP) cDNA probes, and rRNA was used to indicate the equal loading. WT, wild type.

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 plastids in defense responses. Perhaps wounding decompartmentalizes 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 peptide 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 cochromatography 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 DTT). The homogenate was centrifuged at 6000*g* for 10 min at 4°C to remove tissue debris, and the supernatant was centrifuged at 100,000*g* 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 suspended in buffer A and centrifuged again at 100,000*g* 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 $CaCl_2$, 1 mM egg yolk phosphatidylcholine mixed with dipalmitoylglycerol-3-phospho[methyl-^{3}H-choline, and 2 to 10 μg of protein in a total volume of 200 μL. The PIP_2-independent PLD activity was assayed in the presence of 100 μM $CaCl_2$ and mixed lipid vesicles composed of phosphatidylcholine, phosphatidylethanolamine, and PIP_2, with phosphatidylcholine being radioactively labeled (Pappan et al., 1997a). In both assays, release of ^{3}H-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., 1997b). 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 α-^{32}P-dCTP by random priming. Hybridization, washing, and visualization were performed as previously described (Wang et al., 1999). The relative amounts of mRNA for specific genes were estimated by analyzing the intensity of bands on autoradiograms with Kodak (Rochester, NY) 1D image analysis software.

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

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