Phospholipase D and Phosphatidic Acid-Mediated Generation of Superoxide in Arabidopsis

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Phospholipase D (PLD), which hydrolyzes phospholipids into free head groups and phosphatidic acid (PA), may regulate cellular processes through the production of lipid and lipid-derived messengers. We have genetically abrogated PLDs, the most prevalent isoform of PLD in plants, and the depletion of PLDs in Arabidopsis decreased the levels of PA and superoxide production in Arabidopsis leaf extracts. Addition of PA promoted the synthesis of superoxide in the PLD-depleted plants, as measured by chemiluminescence and superoxide dismutase-inhibitable, NADPH-dependent reduction of cytochrome c and nitroblue tetrazolium. The PA-enhanced generation of superoxide was associated mainly with microsomal membranes. Among various lipids tested, PA was the most effective stimulator with the optimal concentrations between 100 and 200 μM. The PA-promoted production of superoxide was observed also in leaves directly infiltrated with PA. The added PA was more effective in stimulating superoxide generation in the PLD-depleted leaves than in the PLD-containing, wild-type leaves, suggesting that PA produced in the cell was more effective than added PA in promoting superoxide production. These data indicate that PLD plays a role in mediating superoxide production in plants through the generation of PA as a lipid messenger.

Phospholipase D (PLD; EC 3.1.4.4) has been identified recently as an important signaling enzyme in various organisms (for review, see Liscovitch et al., 2000; Wang, 2000). Recent results indicate that the cellular activity of plant PLD is regulated by Ca²⁺ (Zheng et al., 2000), polyphosphoinositides (Qin et al., 1997), G proteins (Munnik et al., 1995; Ritchie and Gilroy, 2000), pH changes (Pappan and Wang, 1999), and membrane perturbation (Pappan et al., 1998). The activation of PLD generates phosphatidic acid (PA) and a free head group, both of which are thought to serve directly as cellular messengers. In addition, the formation of PA can lead to the production of other lipid messengers such as diacylglycerol (DAG), free polyunsaturated fatty acids, phosphatidylinositol-4,5-bisphosphate, and jasmonic acid (Wang et al., 2000). PLD has been proposed to participate in cellular events that lead to abscisic acid responses (Fan et al., 1997; Jacob et al., 1999) and the production of ethylene (Lee et al., 1998) and jasmonic acid (Wang et al., 2000). Activation of PLD occurs in response to various biotic and abiotic stress cues, which include water stress (Frank et al., 2000), wounding (Ryu and Wang, 1996; Wang et al., 2000), and pathogen elicitation (Young et al., 1996; van der Luit et al., 2000). In mammalian systems, PLD is activated by a wide variety of signaling events, including protein kinases, polyphosphoinositides, receptor-linked G proteins, and small GTP-binding proteins, such as ADP-ribosylation factor and Rho, and activation of PLD is involved in mediating cellular processes, such as oxidative burst, vesicle trafficking, cytoskeletal organization, and cell proliferation (for review, see Liscovitch et al., 2000). The PA-activated protein kinases include Ca²⁺-dependent and -independent kinases, such as protein kinase C, mitogen-activated protein kinases, and phospholipases (for review, see Liscovitch et al., 2000). The PA-activated protein kinases include Ca²⁺-dependent and -independent kinases, such as protein kinase C, mitogen-activated protein kinases, and Raf kinases. PA has been shown to bind to Raf kinase, and this binding may activate the enzyme by altering its conformation (Ghosh et al., 1996). Moreover, the presence of a PA-specific protein kinase has been documented to mediate the activation of NADPH oxidase (Waite et al., 1997; McPhail et al., 1999). NADPH oxidase catalyzes the NADPH-dependent production of superoxide anion, and it is a multicomponent enzyme composed of several membrane-bound and cytosolic subunits. It becomes active when its four cytosolic proteins, p47-phox, p67-phox, p40-phox, and p21rac, translocate to the membrane. The translocation of p47-phox and p67-phox is prompted by phosphorylation, and p47-phox is a substrate for a newly identified PA-activated protein kinase in animals (Waite et al., 1997). In addition, the membrane-bound p22-phox subunit of flavocytochrome b₅₅₈ is phosphorylated by a PA-activated protein kinase (Regier et al., 1999, 2000). These findings have provided mechanistic insights into the role of PLD...
in the defense response, because the activation of PLD is a documented, key event in signal transduction leading to the reactive oxygen release in neutrophils.

The occurrence of the mammalian NADPH oxidase-like activity has been indicated in plants (Lamb and Dixon, 1997). Genes homologous to the neutrophil oxidase catalytic unit gp91-phox have been cloned from Arabidopsis and rice (Oryza sativa; Keller et al., 1998; Torres et al., 1998). Diphenylene iodonium (DPI) and α-naphthol, two known inhibitors of mammalian NADPH oxidase, inhibited the plant oxidative burst (Levine et al., 1994; Dwyer et al., 1996; Orozco-Cárdenas et al., 2001). In addition, studies have also indicated that the oxidative burst in neutrophils and plant defenses share some common mechanisms of activation. Many signaling components activating the neutrophil oxidase are thought to be operational in the plant oxidative burst. These include phospholipases A and C, G proteins, and protein kinases/phosphatases (Dwyer et al., 1996; Lamb and Dixon, 1997).

Recent results have revealed that PLD in plants is a family of heterogenous enzymes. Four types of PLD have been characterized from Arabidopsis: the conventional plant PLD, now known as PLDα, and novel types of plant PLD, PLDβ, PLDγ, and PLDδ (Pappan et al., 1997a, 1997b; Qin et al., 1997; Wang, 2000). These PLDs displayed unique regulatory and catalytic properties (Qin et al., 1997; Pappan and Wang, 1999). PLDα is the most prevalent form and is present in all plant tissues examined (Pappan et al., 1997a). Its activity increases in response to many perturbations, some of which are related to tissue disruption such as wounding, extraction, storage conditions, and homogenization (Ryu and Wang, 1996). This high sensitivity of PLDα to tissue handling can be problematic for the measurement of in vivo activation of PLD and may overwhelm the PA changes resulting from a localized activation of PLD or a specific PLD isoform in vivo. We have generated Arabidopsis depleted of PLDα by introducing a PLDα antisense gene, and showed that virtually all PLDα activity was lost in the antisense-suppressed leaves (Fan et al., 1997; Pappan et al., 1997a). The deficiency of this most common PLD impaired the plant’s ability to generate PA (Wang et al., 2000), thus providing a unique and effective system to determine the participation of PLD and PA in cell function. Results of this study have provided evidence for a role of PLD/PA in mediating superoxide production in Arabidopsis.

RESULTS
Antisense Suppression of PLDα Lowered the Level of Superoxide Production

To determine whether PLD was involved in the generation of superoxide, we compared the superoxide level in the leaf extracts between PLDα-deficient and wild-type Arabidopsis. It was reasoned that if PLD played a role in activating superoxide production, the loss of PLDα might impede superoxide production in the PLDα-suppressed tissues. Lucigenin-dependent chemiluminescence (LDC) has been used in plant and animal systems as an indicator for the presence and synthesis of superoxide (Auh and Murphy, 1995; Murphy et al., 1998). In this study, we first examined several parameters to verify the utility of LDC for measuring superoxide in Arabidopsis extracts. Inclusion of superoxide dismutase (SOD), which converts O2− to H2O2, in the assay mixtures, abolished LDC generated from leaf extracts (Fig. 1A), and addition of the reactive oxygen species H2O2 had little effect on LDC (data not shown), indicating the assay’s specificity for superoxide. The production of LDC was NADPH dependent, and no superoxide was detected in the absence of NADPH. In addition, NADH could not substitute NADPH for LDC; NADH at 80 μM supported about 10% LDC of NADPH at the same concentration. The self-suicide inhibitor of mammalian NADPH oxidase, DPI, abolished LDC (Fig. 1A). These results indicate that the LDC assay measured the presence of superoxide, and that superoxide in this assay system was produced mainly through an NADPH-dependent pathway.

The superoxide level produced in PLDα-suppressed leaves was about 50% lower than that in wild-type leaves (Fig. 1A). The dependence of superoxide synthesis on the amounts of protein was compared between PLDα-deficient and wild-type lines (Fig. 1B). A near-linear increase of superoxide production occurred as the protein concentrations increased from 5 to 30 μg. The differences in LDC between PLDα-deficient and wild-type leaves could be measured reliably, and no significant fluctuation was noted within the first 15 min of the reaction (data not shown).

Suppression of PLDα Compromised the PA-Generating Ability

One possibility for the lower level of superoxide production in the PLDα-suppressed Arabidopsis is reduced production of PA that serves as a stimulator of superoxide synthesis. PLDα protein was undetect-
able by immunoblotting with PLDα-specific antibodies (Fig. 2A). In a similar manner, the transcript of PLDα in the PLDα-suppressed leaves was also underdetectable by RNA blotting (Fan et al., 1997; Wang et al., 2000). PLDα activity in the antisense leaf extracts was less than 5% of that in extracts of wild-type leaves (Fan et al., 1997; Pappan et al., 1997b). The antisense suppression was specific to PLDα, and the expression and activities of PLDβ or γ were similar in the PLDα-suppressed and wild-type plants (Pappan et al., 1997a; Wang et al., 2000).

To determine PA levels in the PLDα-suppressed and wild-type Arabidopsis, we measured the contents of total phospholipids and PA in the leaf homogenates that were used for assaying superoxide synthesis. No substantial difference was observed in total phospholipid content between the PLDα-suppressed and wild-type plants. The PA level in the homogenate of PLDα-suppressed leaves was approximately 30% lower than that in the homogenates of wild-type leaves (Fig. 2B), confirming that the PA level was decreased in the PLDα-deficient extracts.

**Addition of PA Promoted Superoxide Production**

If the lower level of PA in the PLDα-suppressed leaves was a contributor to the decreased level of superoxide, exogenous PA might be able to restore the lost superoxide generation. When water-soluble diC₈PA (200 μM) was added directly to the extraction buffer, superoxide production was stimulated by about 60% in the PLDα-deficient leaves as measured by LDC. This PA promotion was confirmed further by two other methods, SOD-inhibitory, NADPH-dependent reductions of cytochrome c (cyt c) and nitroblue tetrazolium (NBT), both of which gave about 40% stimulation (Fig. 3A). The lower effects of these two methods could have been caused by the presence of other interfering enzyme in the assay mixtures, as observed in other systems (Murphy et al., 1998). For example, the presence of cytochrome c reductases in the plasma membrane of leaf extracts could decrease the differences of superoxide-generating enzymes between PLDα-antisense and wild-type Arabidopsis.
To determine the subcellular association of PA-promoted superoxide-generating activity, the proteins extracted from the PLDα-antisense leaves were separated into soluble and microsomal fractions. Superoxide-synthesizing activity was present in both fractions (Fig. 3B). The PA-promoted activity was associated entirely with the membrane fraction. Superoxide production in the soluble fraction was slightly lower for extracts of PA-treated leaves than for extracts of untreated leaves (Fig. 3B).

Superoxide-synthesizing activity first increased and then decreased with increasing PA concentrations. This effect of PA was similar to the results with PA and DAG as activators in animal systems (Qualliotine-Mann et al., 1993). The optimal concentration of diC<sub>8</sub>-PA to activate superoxide production was between 100 and 200 μM, and no significant stimulation was observed when PA concentrations were 300 or 400 μM (Fig. 4). At all PA concentrations tested, wild-type leaves were much less responsive to exogenous PA than were PLD-suppressed leaves. The optimal stimulation of superoxide production was about 15% in wild-type leaves, whereas the PA treatment promoted an 80% increase in the superoxide synthesis in PLDα-antisense leaves.

Specificity of PA and Other Lipids in Reconstituting Superoxide Production

To investigate the specificity of PA in the activation of superoxide formation, several cellular phospholipids were tested for their abilities to promote the activity of NADPH-dependent superoxide synthesis. The major membrane phospholipids tested, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylylycerol, and phosphatidylinositol (PI), had no effect, whereas the minor plant membrane phospholipids, phosphatidyl-Ser (PS) and lysoPA, stimulated the superoxide-synthesizing activity by approximately 20% (Fig. 5A). DAG was more effective than PS and lysoPA, and the superoxide level was 50% higher than that in untreated transgenic leaves. Similar to the PA treatment, the optimal concentration of DAG for promoting superoxide production was between 100 and 200 μM in PLDα-deficient leaves. The protein extracted from wild-type leaves was less responsive to the added DAG than that from PLDα-antisense and wild-type plants.
deficient leaves. Concentrations of DAG above 100 μM inhibited the superoxide-synthesizing activity in wild-type leaves (Fig. 5B). DAG and PA showed no significant synergistic effect on promoting superoxide generation in Arabidopsis (data not shown).

PAs with different fatty acids were tested to determine the effect of acyl composition on the superoxide synthesis activity. Except for egg yolk PA, which contained a mixture of varied fatty acids, the other PAs used in the study were synthetic. Stimulation was observed with diC₈PA (PA8:0), dipalmitoyl-PA (PA16:0), distearoyl-PA (PA18:0), dioleoyl-PA (PA18:1), and egg yolk PA, whereas dilinoleoyl-PA (PA18:2) showed little effect on both the wild-type and antisense plants (Fig. 6). This result indicates that the acyl composition of PA has some effect on the stimulation of superoxide synthesis, but it is not clear why the polyunsaturated dilinoleoyl-PA was not as effective as the other PAs.

**PA Enhanced Superoxide Burst in Leaves**

The above results showed the PA promotion of superoxide production in leaf extracts. To determine if PA had the same effect in vivo, dioleoyl-PA was infiltrated into leaves on plants. The treated leaves were detached and stained for superoxide formation. The cytochemical staining (brown precipitates) was due to reduction of NBT by superoxide and has been used as an indicator for the tissues actively generating reactive oxygen (Jabs et al., 1995). Infiltration of the control buffer and dioleoyl-PA into leaves with the pressure of a needleless syringe resulted in an increase of reactive oxygen species in the wound area (Fig. 7). A clear difference in the NBT staining between the buffer- and PA-infiltrated leaves occurred between 30 and 60 min after the treatment. PA enhanced the oxidative burst in both PLDₐ-deficient and wild-type leaves. Judged from the staining intensity, this PA enhancement was greater in the PLDₐ-antisense leaves than in wild-type leaves. The PA effect was transient; 2 h after treatment, no obvious difference was noted between the buffer- and PA-infiltrated leaves. This could mean that the infiltrated PA was metabolized rapidly.

**Figure 5.** Lipid specificity and DAG concentration effect on superoxide production. A, Effect of phospholipids and DAG on superoxide-producing activity from PLDₐ-suppressed and wild-type leaf extracts. Lipids (200 μM) were added to the extraction buffer prior to homogenization. PC, PE, and PI were from soybean, and PS was from bovine brain, whereas dipalmitoyl-phosphatidylglycerol, 1-oleoyl-lysophosphatidic acid (LPA), and diactanoyl-DAG were synthetic. B, Superoxide production as affected by dioctanoyl-DAG concentration in PLDₐ-suppressed and wild-type leaves. Superoxide production was measured using 10 μg of proteins from 10,000g supernatant and was expressed as percentage of that without lipid treatment. The activity of superoxide production was expressed as a percentage of that measured without phospholipid addition. Values are means ± se of three separate extractions.

**Figure 6.** Effect of acyl composition of PA on superoxide production. PAs (200 μM) with varied fatty acids were added to extraction buffer prior to homogenization. The activity without lipids (control) was set as 100%. PA8:0, dioctanoyl-PA; PA16:0, dipalmitoyl-PA; PA18:0, distearoyl-PA; PA18:1, dioleoyl-PA; PA18:2, dilinoleoyl-PA; and PA e.y., PA from egg yolk. The percentages are means ± se of three experiments.
Role of PLD in Mediating Reactive Oxygen Production

The data of this study collectively indicate that PLD plays a role in the production of reactive oxygen. First, suppression of the predominant form of PLD, PLDα, led to reduced superoxide synthesis. Second, addition of exogenous PA, the lipid product of PLD activation, to leaves promoted the activity of reactive oxygen production. In particular, the added PA reconstituted the lost superoxide-generating activity in PLD-suppressed leaves to the level in wild-type leaves. Third, among all lipids tested in this system, PA was the most effective stimulator for the reactive oxygen generation. Fourth, infiltration of PA into leaves on plants augmented micro-oxidative burst at the sites of infiltration.

PLDα-deficient Arabidopsis leaves still produced substantial amounts (50%) of superoxide, which is indicated by the in vitro measurements of superoxide synthesis and in situ NBT staining. This activity could be supported by the residual PLDα (less than 5%) and other PLDs, such as PLDβ and PLDγ, which have been documented in Arabidopsis leaves. A previous study has shown that PA levels increase substantially, albeit less than in wild-type leaves, in PLDα-suppressed leaves in response to wounding (Wang et al., 2000). In addition, there may exist PLD-independent pathways that regulate superoxide synthesis. For example, induction of the oxidative burst in neutrophils involves activation of phospholipase C (PLC) and phospholipase A2, in addition to PLD

PA Produced in the Cell Is More Effective than Added PA in Promoting Superoxide Production

One marked difference between the PLDα-suppressed and wild-type leaves throughout this study was that added PA was much more effective in stimulating superoxide generation in the antisense leaves than in the wild-type leaves. One explanation for this differential effect is that PLDα in wild-type leaves can produce enough PA for stimulating superoxide production and this endogenous PA is much more effective than the added PA in regulating superoxide synthesis. Thus, added PA has a less effect on superoxide production in wild-type leaves. A higher level of PA was found in wild-type than PLDα-deficient leaves. The endogenously produced PA could be in a correct intracellular location and have the correct acyl composition and, thus, be more effective in stimulating reactive oxygen formation than exogenously supplied PA. In addition, PLDα is expressed consti-

DISCUSSION

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Figure 7. PA-promoted superoxide production in situ in Arabidopsis leaves. Leaves of wild-type (WT) and PLDα-deficient (anti-α) plants were infiltrated with approximately 10 μL of 10 mM potassium phosphate buffer (−), or the buffer containing 200 μM dioleoyl-PA (+). At indicated time intervals, the leaves were detached and stained with NBT. Multiple leaves were infiltrated for each treatment; a representative leaf is shown here.
tutively in leaves and becomes activated rapidly in response to environmental stresses such as wounding, ionizing irradiation, and other injuries (Ryu and Wang, 1996; Wang et al., 2000). Thus, perturbations such as tissue handling and homogenization can activate PLDα, resulting in production of enough PA, to mask or obscure the difference of PA levels caused by other cellular activation. On the other hand, the suppression of PLDα impaired the plant’s ability to produce PA, and, thus, the stimulation of superoxide synthesis by added PA became apparent.

A well-regulated and possibly localized production of PA has been indicated in some perturbations that potentially involve reactive oxygen generation. For example, in rice leaves undergoing hypersensitive interactions with Xanthomonas oryzae pv oryzae, PLDα clustered in the plasma membrane regions in contact with this bacterial pathogen (Young et al., 1996). In the susceptible interaction, however, PLDα was distributed evenly along the plasma membrane. This PLD clustering may produce more PA at the membrane region for the cell to use in battling against infection. Also, wounding triggers intracellular translocation of PLDα from cytosol to membrane and promotes PA formation on the membranes (Ryu and Wang, 1996, 1998). Such increased PA may be used for promoting the oxidative burst as a defense mechanism.

Using lipids extracted from leaf homogenates, the present data showed that PA constituted almost 10% of total phospholipids in wild-type leaf extracts. This PA to total phospholipid ratio was high, but it should not represent true levels of cellular PA. This was because in addition to the preexisting, cellular PA, a major portion of PA in the homogenate was generated by PLD during homogenization. In a separate study in which PLD activity was inhibited immediately after leaf excision by immobilizing leaves in 75°C isopropanol, PA accounted for about 2% of the total phospholipids (Zien et al., 2001). The PA levels were similar in non-wounded leaves of PLDα-deficient and wild-type plants. After wounding, however, PA levels in wild-type leaves increased to about 8% to 10% of total phospholipids (Zien et al., 2001), and the ratio is similar to that from the wild-type leaf homogenate in the present study. Thus, this high ratio of PA to total phospholipids resulted from increased PLD hydrolysis of phospholipids.

Potential Targets Activated by PLD/PA for Generating Superoxide

Although superoxide can be a by-product of metabolic processes such as respiration and photosynthesis, NADPH oxidase is believed to be a key enzyme for the stimulus-induced release of reactive oxygen (Lamb and Dixon, 1997; Potikha et al., 1999; Orozco-Cárdenas et al., 2001). Results of this study suggest that the lower level of superoxide synthesis in the leaf extracts of PLDα-depleted plants results from the PA modulation of an NADPH oxidase-like activity. This activity was NADPH dependent and sensitive to the presence of added SOD and DPI, a potent self-suicide inhibitor of neutrophil NADPH oxidase. Besides the LDC assay, the changes in superoxide synthesis also were confirmed by the SOD-inhibitory, NADPH-dependent reductions of cyt c and NBT.

In addition, the PA effect on superoxide generation in Arabidopsis resembled the PA activation of neutrophil NADPH oxidase in several aspects (Qualliotine-Mann et al., 1993). In cell-free systems, PA induced approximately a 2-fold increase of NADPH oxidase in both neutrophils and PLDα-deficient Arabidopsis. The stimulated activity was associated with membranes. The effective concentration of PA in Arabidopsis was also in a range similar to that used for the neutrophil enzyme. PA and DAG were stimulators for both plant and neutrophil oxidases. Neutrophil NADPH oxidase is stimulated synergistically by PA and DAG, and PA, in combination with DAG, induced a more than 10-fold activation of neutrophil oxidase (Qualliotine-Mann et al., 1993). However, little synergistic stimulation by PA and DAG was noted in the Arabidopsis enzyme. The optimum stimulation by PA and DAG occurred at approximately 0.1 mM. These relatively high concentrations seem to make it questionable that such concentrations could be achieved in vivo. However, interpretation of effective lipid concentrations for enzymes that act on or regulated by lipids is complicated because the interfacing concentrations, rather than total concentrations of PA, are critical to the activity of an enzyme. It might be possible that when PA is mixed with other lipids in cellular environments, the effect concentrations required for activating superoxide production would be much lower than 0.1 mM.

Recent studies in mammalian systems have indicated that PA increased NADPH oxidase activity by activating a PA-selective protein kinase that phosphorylates the cytosolic p47-phox and the membrane p22-phox subunits of NADPH oxidase (McPhail et al., 1999; Regier et al., 1999, 2000). The phosphorylation increases the interaction of the two subunits with other components of the oxidase. In plants, the presence of p47-phox- and p22-phox-like subunits in plants has been suggested based on the crossreactivity of plant proteins with antibodies against mammalian p47-phox and p22-phox (Dwyer et al., 1996; Xing et al., 1997). Phosphorylation of p47-phox was indicated also in elicitor-treated tomato cells. Although genes homologous to mammalian p47-phox- and p22-phox have not been identified, plant homologs of gp91-phox, the catalytic unit of animal NADPH oxidase, have been cloned (Keller et al., 1998; Torres et al., 1998). How NADPH oxidase is regulated is not understood in plants. In this study, we have found that PA added during homogeniza-
tion is more effective than adding PA after homogenization. One possible explanation is that the superoxide-generating enzyme complex might be unstable, and added PA could help stabilize the NADPH oxidase complex during extraction. Although the detailed mechanisms await further studies, the present results show that PLD and PA play a role in mediating the NADPH-dependent production of superoxide in plants, and that the NADPH oxidase is a likely target of PLD activation. Further studies are under way to identify the targets that PLD and PA interact with in regulating plant oxidative stress responses.

MATERIALS AND METHODS

Plant Material and Confirmation of Transgenic Plants

Seeds of wild-type and PLDα-suppressed Arabidopsis ecotype Columbia were sown in soil and cold treated at 4°C overnight. Plants were grown under 14-h-light/10-h-dark cycles with cool-white fluorescent light of 100 μmol m⁻² s⁻¹ at 23 ± 3°C. Generation of the PLDα-deficient line was described previously (Fan et al., 1997). Before each treatment, the PLDα deficiency of the transgenic plants was confirmed by assaying extracts for PLDα activity (Wang et al., 1993) and sometimes by immunoblot analysis using PLDα-specific antibodies (Fan et al., 1997).

Phospholipid Treatment and Protein Extraction

Synthetic PA and DAG were purchased from Avanti Polar Lipids (Alabaster, AL), and other phospholipids were products of Sigma (St. Louis). Lipids were stored at −20°C in chloroform and were dried with a stream of N₂ prior to use. Water-soluble dioleoyl-PA was dissolved directly in water, and other lipids were emulsified by sonication in water. For in vitro lipid treatment, phospholipids or DAG were added into extraction buffer just before homogenization at the concentration of 200 μM or otherwise as specified. Leaves from plants grown for 4 to 6 weeks were used in all lipid treatments. One part of leaves was ground with a tissue grinding pestle directly in a 1.5-ml microcentrifuge tube in three parts of homogenization buffer (with or without lipids) containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mg mL⁻¹ bovine serum albumin, 0.5 mM phenylmethanesulfonyl fluoride, and 10 mM β-mercaptoethanol at 4°C. As a homogenate was placed on ice for 30 min, and the supernatant was collected by centrifugation at 10,000g at 4°C for 5 min. In some experiments, the supernatant was centrifuged further at 100,000g to obtain the cytosolic (100,000g supernatant) and microsomal (100,000g pellet) fractions. Protein content was determined by the Bradford method, according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). These fractions were used immediately or stored at −80°C until use.

SDS-PAGE and Immunoblotting

Proteins in the 10,000g supernatant were separated by 10% (w/v) SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blotted with PLDα antibodies that was raised in rabbit against the 13-amino acid peptide of the Arabidopsis PLDα C terminus (Pappan et al., 1997a). The proteins recognized by antibodies were visualized by staining alkaline phosphatase conjugated to a second antibody with a Bio-Rad immunoblotting kit (Wang et al., 1993).

Assays of NADPH-Dependent Superoxide Synthesis

Three assays were used to determine the activity of NADPH-dependent superoxide synthesis, LDC- and SOD-inhibitable, NADPH-dependent reductions of cyt c and NBT. LDC was assayed in the reaction mixture containing 10 μg protein, 80 μM NADPH, 0.02% (w/v) Triton X-100, 0.2 mM lucigenin, and 1 mM EDTA in 0.1 M Gly-NaOH buffer (pH 9.0) in a final volume of 2 mL. The SOD inhibitor N,N-diethyldithiocarbamate (1 mM) was added to the reaction mixture to block the dismutation of O₂⁻ to H₂O₂ by SOD. LDC was detected in a scintillation spectrometer with the counts reported every 6 s for 30 s, and the last two values were averaged (Auh and Murphy, 1995). The lucigenin stock (10×) was stored for 2 weeks at 4°C before use because LDC from this aged stock was less variable than that of the freshly prepared lucigenin. To determine the specificity of the assay for NADPH oxidase, SOD (45 units; Sigma), varied concentrations of DPI, or 10 mM H₂O₂ were added to the reaction mixture. In addition, NADPH was replaced by NADH or omitted from the standard mixture to test the NADPH dependence of the LDC.

A standard mixture to assay the SOD-inhibitory, NADPH-dependent reduction of cyt c contained 100 μM cyt c, 20 μM ATP, 3 μM GTP(γ) S, 0.02% (w/v) Triton X-100, 20 mM Tris-HCl (pH 7.5), and 20 to 40 μg protein in a total volume of 1 mL (Xing et al., 1997; Murphy et al., 1998). The reaction was initiated in a disposable microcuvette at 23°C by addition of 80 μM NADPH, and the reduction of cyt c was scanned by the change in A₅₅₀ over the 1st min in a dual-beam spectrophotometer. SOD (40–60 units) was added to the mixture in a duplicated cuvette as the reference to monitor the SOD-dependent changes of absorption. In assaying the SOD-inhibitory, NADPH-dependent reduction of NBT, the reaction components and conditions were the same as for the reduction of cyt c, except that NBT was used, instead of cyt c, and the reduction of NBT was monitored by the change at A₅₃₀. The specific activity for superoxide production was expressed as nmol O₂⁻ min⁻¹ μg⁻¹ with the reported coefficients (Murphy et al., 1998).

NBT Detection of Superoxide Generation in Situ

All the reagents in the NBT staining procedure were prepared in a 10-mm potassium phosphate buffer (P-K) at pH 7.5. A 1-mL syringe with no needle was used to pressure infiltrate a spot area on one leaf half. The extracellular superoxide generation system of xanthine and xanthine oxidase and SOD (30 units) were used to titrate the sensitivity of the staining procedure (data not shown). About 10 μL of dioleoyl-PA (200 μM) was infiltrated into the test
leaves, and the control leaves were infiltrated with the P-K buffer only. For NBT staining, leaves were detached at 5, 60, and 120 min after infiltration and then were vacuum infiltrated in 10 mM NaNO₃ in 10 mM P-K buffer and immersed in 2 mL of the same buffer containing 0.1% (w/v) NBT at room temperature for 30 min. The blue precipitates of reduced NBT were visualized after boiling the leaves in 96% (v/v) ethanol for 10 min (Thordal-Christensen et al., 1997).

Phospholipid Extraction and Determination

Leaves were homogenized as described earlier and incubated on ice for 30 min. The homogenates then were extracted for lipid using a procedure described previously (Ryu and Wang, 1996). In brief, hot isopropanol (75°C) was added to leaf extracts and incubated at 75°C for 15 min to inactivate PLD and other lipolytic enzymes. Chloroform then was added, the chloroform phase was dried under N₂, and lipids were resolved in 200 μL chloroform. A portion of lipids (20 μL) was used for assaying total phospholipid content. PA was separated from other lipid by thin layer chromatography (silica gel 60) and identified by comparing the Rₚₛ (relative mobility of the component to the solvent front) with an authentic PA standard. PA was collected by scraping the spot, and phospholipid content was assayed and calculated based on determination of phosphorus content in the lipids (Rouser et al., 1970).

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