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Antisense Suppression of Phospholipase Dα Retards Abscisic Acid– and Ethylene-Promoted Senescence of Postharvest Arabidopsis Leaves

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Membrane disruption has been proposed to be a key event in plant senescence, and phospholipase D (PLD; EC 3.1.4.4) has been thought to play an important role in membrane deterioration. We recently cloned and biochemically characterized three different PLDs from Arabidopsis. In this study, we investigated the role of the most prevalent phospholipid-hydrolyzing enzyme, PLDα, in membrane degradation and senescence in Arabidopsis. The expression of PLDα was suppressed by introducing a PLDα antisense cDNA fragment into Arabidopsis. When incubated with abscisic acid and ethylene, leaves detached from the PLDα-deficient transgenic plants showed a slower rate of senescence than did those from wild-type and transgenic control plants. The retardation of senescence was demonstrated by delayed leaf yellowing, lower ion leakage, greater photosynthetic activity, and higher content of chlorophyll and phospholipids in the PLDα antisense leaves than in those of the wild type. Treatment of detached leaves with abscisic acid and ethylene stimulated PLDα expression, as indicated by increases in PLDα mRNA, protein, and activity. In the absence of abscisic acid and ethylene, however, detached leaves from the PLDα-deficient and wild-type plants showed a similar rate of senescence. In addition, the suppression of PLDα did not alter natural plant growth and development. These data suggest that PLDα is an important mediator in phytohormone-promoted senescence in detached leaves but is not a direct promoter of natural senescence. The physiological relevance of these findings is discussed.

INTRODUCTION

Senescence in plants is characterized by degradation of cellular components, leading to the loss of cellular compartmentalization and tissue structure and ultimately to plant death. Disruption of membrane integrity has been hypothesized to be a major contributing factor to senescence (Beutelman and Kender, 1977; Thompson, 1988; Paliyath and Droilard, 1992; Borochov et al., 1997). One of the most characteristic features in membrane deterioration is a progressive decline of phospholipid levels with a relative enrichment of free fatty acids and sterols in the membranes (Draper, 1969; Liljenberg and Kates, 1985; Manoharan et al., 1990; Paliyath and Droilard, 1992). The change in lipid composition may cause the localized transformation of a membrane lipid bilayer into destabilized bilayer and nonbilayer structures, such as gel, micellar, and hexagonal phases (Lafleur et al., 1990), leading to the loss of membrane integrity and functions of membrane-associated proteins during senescence.

How does the loss of membrane phospholipids occur during senescence? One model suggests that the breakdown of phospholipids results from a membrane lipid degradation pathway (Thompson, 1988; Paliyath and Droillard, 1992; Samama and Pearce, 1993; Voisine et al., 1993). In this process, phospholipase D (PLD) initiates the first reaction to produce phosphatidic acid (PA), which is rapidly converted to diacylglycerol by PA phosphatase. Diacylglycerol is deacylated by a nonspecific acyl hydrolase to release free fatty acids, some of which (i.e., polyunsaturated fatty acids) are peroxidized by lipoxygenase. Evidence supporting the operation of the PLD-initiated lipid degradation pathway has come primarily from kinetic studies on the release of various lipolytic products (i.e., PA → diacylglycerol → free fatty acids → oxidized fatty acids) in vitro and in vivo. Some studies have involved incubating isolated membrane fractions with a radiolabeled phospholipid and following its catabolic fate by measuring its metabolites at various time intervals (Paliyath et al., 1987; Duxbury et al., 1991). Other studies followed endogenous lipid catabolism during senescence or after exposure to stress (Yoshida, 1979; Willemot, 1983; Yapa et al., 1986; Voisine et al., 1993). Characterization of lipid-degrading enzymes has also provided some support for the PLD-initiated degradation pathway. For example, PLD activity increases during senescence and stress treatments in some plant systems (Ryu and Wang, 1995), nonspecific acyl hydrolase prefers diacylglycerol to phospholipids (Galliard, 1980), and lipoxygenase prefers free rather than esterified fatty acids (Todd et al., 1990).

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Although a number of studies have implicated PLD as a key enzyme in membrane degradation and senescence, the physiological role of this enzyme is not well defined. On the one hand, hydrolysis of phospholipids need not be enzymatic because deesterification of phospholipids could be mediated by free radicals (Niehaus, 1978; McKersie et al., 1988; Van Bilsen and Hoekstra, 1993). Similarly, lipid hydrolysis may not be essential for oxidative damage because membrane lipids are susceptible to nonenzymatic peroxidation. On the other hand, plant cells contain other phospholipid-degrading enzymes, such as C and A types of phospholipases, nonspecific acyl hydrolases, and lipoxygenases (reviewed in Huang, 1993; Vick, 1993; Wang, 1993). Those enzyme activities could result in a direct decrease of phospholipid content without requiring the concerted action of several enzymes. Indeed, one model proposes that activation of phospholipase A_2 (PLA_2) is involved in the initial stage of lipid degradation during senescence (Leshem, 1987). However, evidence supporting this hypothesis is limited because the characteristics of PLA_2 in plants are poorly understood.

During senescence, several processes of membrane damage may occur simultaneously. A central issue to understanding and controlling membrane deterioration is to discern the sequence of events leading to phospholipid degradation and membrane disruption. To determine the role of PLD in this sequence, an ideal approach is to suppress genetically the PLD gene expression in plants and then follow the effects of PLD alteration on membrane deterioration. If PLD plays an important role in membrane deterioration and senescence, changes in PLD gene expression may alter these cellular processes. We have recently reported cloning of PLD cDNAs that have enabled us to perform such studies (Wang et al., 1994). Three distinct PLDs, named PLDs α, β, and γ, have been cloned from Arabidopsis (Dyer et al., 1995; Pappan et al., 1997a; Qin et al., 1997). PLDα is more prevalent than are PLDs β and γ in Arabidopsis leaves (Pappan et al., 1997b). Previous biochemical and functional studies dealt mainly with PLDα because of its wide distribution and relative abundance in plant tissues (Wang, 1997). PLDα has been purified and characterized from a number of plant species, and it is present in soluble and membrane fractions (Heller, 1978; Wang, 1993; Dyer et al., 1994; Xu et al., 1996). An increase in membrane association of PLDα has been associated with increased phospholipid hydrolysis during abscisic acid (ABA)–promoted senescence and stress response (Voisine et al., 1993; Ryu and Wang, 1995; Ryu and Wang, 1996).

The pattern of PLDα gene expression during plant growth and development, however, argues against a general role of PLDα as a promoter of natural plant senescence. Results from a PLDα gene promoter and glucuronidase fusion study indicate that the PLDα promoter activity is higher in metabolically active tissues, such as meristematic and newly divided cells, than in mature and senescent ones (Xu et al., 1997). RNA gel blot analysis also showed higher amounts of PLDα mRNA in the early stages of leaf and seed development (Ryu et al., 1996; Xu et al., 1997). The same trends were found in the levels of PLDα protein and activity (Wang et al., 1993; Dyer et al., 1994; Xu et al., 1996).

In this study, we investigated the role of PLDα in senescence by using PLDα-deficient Arabidopsis. We genetically suppressed the expression of PLDα by introducing a PLDα antisense gene into Arabidopsis. The PLDα-deficient plants possessed regular PLDβ and PLDγ activities, as indicated by assays, in the presence of micromolar concentrations of Ca^{2+} and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Pappan et al., 1997b). Suppression of PLDα retarded membrane deterioration and senescence in detached leaves treated with ABA and ethylene but had little effect on natural plant growth and development. These data suggest that PLDα is an important mediator in phytohormone-promoted senescence and that suppression of PLDα expression may enhance plant resistance to stress-related damages during postharvest storage and handling.

RESULTS

Antisense Suppression of PLDα in Different Tissues of Arabidopsis

Putative domain structures of PLDα have been proposed recently based on sequence analysis (Figure 1A; Ponting and Kerr, 1996; Pappan et al., 1997b; Wang, 1997). The cDNA fragment for the PLDα antisense gene construction corresponds to the region coding for amino acids from positions 452 to 713 of Arabidopsis PLDα (Figures 1A and 1B). This region contains the second of two HKD motifs, which are likely to be involved in catalysis. The nucleotide sequence of this region is ~50% identical to that of PLDs β and γ.

The transgenic plants carrying a PLDα antisense cDNA were first identified by their resistance to kanamycin (Figure 1). The antibiotic-resistant plants were then screened for a decrease in PLDα activity in leaves. The PLDα activity assay measured the hydrolysis of phosphatidylcholine in the presence of 25 mM Ca^{2+} and 0.5 mM SDS (Wang et al., 1993). Under this condition, PLDα is fully active, whereas the PIP_2-dependent PLDs β and γ are inactive (Pappan et al., 1997a; Qin et al., 1997). One transgenic line that showed the most thorough suppression had lost almost all PLDα activity in leaves (Figure 2A). Immunoblotting with a PLDα-specific antibody detected PLDα protein in wild-type but not in antisense leaf extracts (Figure 2B). PLDα mRNA from the PLDα antisense leaves was undetected by RNA gel blotting using a PLDα cDNA probe (Figures 3A and 3B). These results indicate that the expression of the PLDα gene was suppressed in the antisense plants.

To evaluate the suppression of PLDα in different organs, we compared PLDα activity and protein levels in flowers, roots, stems, siliques, and leaves between the antisense and wild-type plants (Figure 2). PLDα was present in all of the organs examined in the wild type, and its specific activity

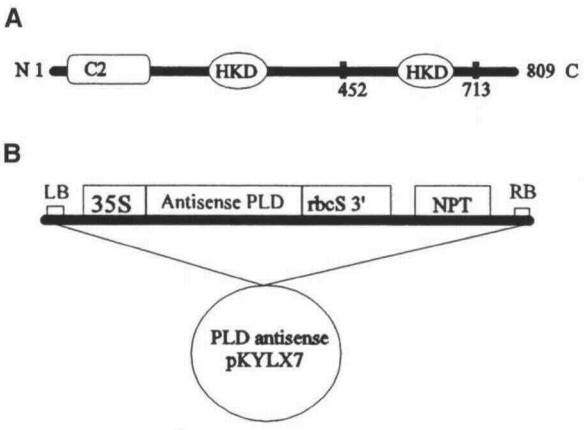

Figure 1. PLDα Domain Structure and the Antisense Construct.

(A) The regulatory C2 domain and catalytic HKD motifs. C2 is a Ca^{2+}/phospholipid binding domain, and the two HKD motifs represent the duplicated HXKXXXXD (where X indicates any amino acid) that is conserved in all cloned PLDs and potentially involved in catalysis. The numbers indicate the amino acid region to which a corresponding cDNA fragment was used to make the antisense construct.

(B) The vector used for the introduction of the inverted 780-bp Arabidopsis PLDα cDNA into Arabidopsis. The antisense PLD cDNA insert is flanked by the cauliflower mosaic virus 35S promoter at the 5′ end and by the transcriptional terminator (rbcS 3′) of the pea ribulose bisphosphate carboxylase small subunit *E9* gene at the 3′ end. LB, left border; NPT, neomycin phosphotransferase gene whose expression confers plant resistance to kanamycin; RB, right border.

in flowers and roots was approximately twofold higher than that in stems, siliques, and fully expanded leaves (Figure 2A). Transgenic plants showed suppression of PLDα in all organs; the percentages of decrease in PLD activity in leaves, stems, siliques, flowers, and roots were 97 ± 1.3, 93 ± 1.2, 94 ± 2.4, 90 ± 1.9, and 84 ± 7.2%, respectively. The residual PLDα protein in the antisense plants was detectable with a PLDα antibody in flowers and roots but not in leaves (Figure 2B).

The antisense suppression is specific to PLDα because the transgenic plants have normal PIP_2-dependent PLD activity when assayed in the presence of PIP_2 and micromolar concentrations of Ca^{2+} (Pappan et al., 1997b). The antisense suppression of PLDα was observed in T_1 plants and has been stably inherited for generations (four generations tested). T_2 plants with almost no PLDα activity, as well as those with normal PLDα activity, cosegregated with kanamycin resistance and susceptibility in a 3:1 ratio, indicating T-DNA insertion at a single locus in the genome.

Suppression of PLD Retards ABA-Promoted Senescence in Postharvest Leaves

Our previous studies have shown that ABA activates PLD gene expression in detached castor bean leaves (Ryu and Wang, 1995; Xu et al., 1997). ABA is a known promoter of senescence in detached organs. Thus, the effect of PLDα suppression on ABA-promoted senescence of postharvest leaves was examined. A common symptom of senescence is the yellowing of leaves. In the absence of ABA, detached leaves stayed green for >15 days. Leaves detached from control plants (wild type and Arabidopsis transformed with the vector) started yellowing 1 day after treatment and turned almost completely yellow 3 days after incubation in 50 μM ABA under light (Figure 4). In contrast, most parts of the PLDα-suppressed leaves were still green after the 3-day ABA treatment. It took nearly 10 days for the PLDα-suppressed leaves to appear the same as the ABA-treated control leaves, indicating a much slower senescence process in the PLDα-deficient leaves.

The differences in the rate of senescence between PLDα-suppressed and control leaves were further assessed by measuring chlorophyll level, photosynthetic activity, ion leakage, and phospholipid content (Figure 5). The degradation of chlorophyll in senescent leaves (Figure 5A) followed a trend similar to that of the changes in leaf color. After ABA incubation for 3 days, the chlorophyll levels in antisense and wild-type leaves were 62 and 17%, respectively, of their values before treatment. Photosynthetic activities decreased in the detached leaves during the ABA-promoted senescent process

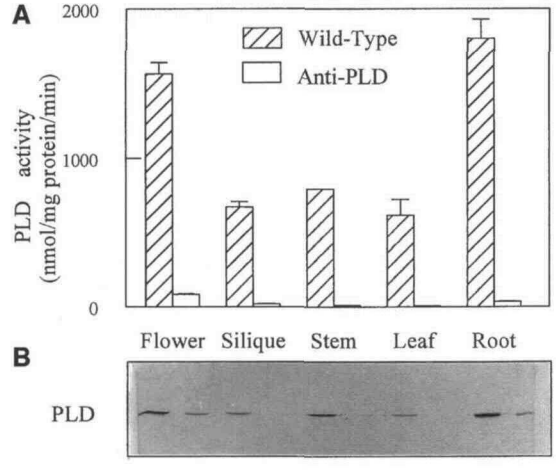

Figure 2. Suppression of PLDα in Different Arabidopsis Organs.

(A) PLD activity in flowers, siliques, stems, leaves, and roots of wild-type and PLDα antisense plants as assayed by the release of choline from dipalmitoyl glycero-3-phosphate-(methyl-^{3}H)-choline in the presence of 25 mM Ca^{2+}, 0.5 mM SDS, 100 mM Mes–NaOH, pH 6.5, and 2 mM egg yolk phosphatidylcholine. Values are means ±SE of three experiments.

(B) Immunoblot of PLDα in different organs in wild-type and PLDα-antisense plants, using the polyclonal antibody raised against the synthetic 13–amino acid peptide of the C-terminal sequence of PLDα. Each lane was loaded with 20 μg of protein from the 10,000*g* supernatant.

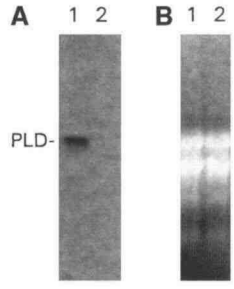

Figure 3. PLDα mRNA Level in Antisense and Wild-Type Arabidopsis.

(A) Autoradiography of a gel blot of PLDα, using total leaf RNA (20 μg per lane) isolated from wild-type (lane 1) and antisense (lane 2) plants. The probe used was specific for the sense PLDα mRNA.
(B) Ethidium bromide staining of the gel used for RNA blotting, which indicates equal loading of total RNA from wild-type (lane 1) and antisense (lane 2) plants.

(Figure 5B). The photosynthetic activities in the detached leaves were estimated by measuring chlorophyll fluorescence, which can provide information about many aspects of photosynthetic performance and is a sensitive indicator for the effects of environmental conditions (Krause and Weis, 1991). The measured chlorophyll fluorescence was expressed as the ratio of variable fluorescence (F_v) to maximum yield of fluorescence (F_m). This ratio of F_v/F_m is considered to be an indicator of photosystem II activity because it is related to the efficiency of electron transport through photosystem II and nonphotochemical quenching processes associated with photosystem II (Hipkins and Baker, 1987). Consistent with the decreased loss of chlorophyll, the PLDα-suppressed leaves retained a higher photosynthetic activity than that of control leaves. After a 3-day treatment with ABA, the ratios of F_v/F_m in antisense and wild-type leaves were ~85 and 35% of their respective initial values (Figure 5B).

The membrane integrity of the senescent leaves was assessed by measuring ion leakage and phospholipid content. Loss of membrane integrity was expressed as the percentage of electrolytes leaked from leaves (initial conductivity/total conductivity × 100%). In wild-type leaves, the leakage increased 1 day after ABA treatment, and the leakage rate was 20-fold higher than that of freshly excised leaves after incubation with ABA for 3 days. However, the leakage in PLDα-suppressed leaves increased only slightly after 3-day ABA treatment (Figure 5C). The phospholipid content in the PLDα-suppressed leaves was consistently higher than that of wild-type leaves after ABA treatment. The total phospholipid level decreased 55% in wild-type leaves treated with ABA for 3 days, whereas the decrease in PLDα-suppressed leaves was 15% under the same conditions (Figure 5D). Those differences demonstrate that the process of phospholipid degradation and membrane deterioration is much slower in the PLDα-suppressed leaves than in wild-type leaves.

Changes of PLDα mRNA, Protein, and Enzymatic Activity during ABA Treatment

The attenuation of ABA-accelerated senescence in PLDα-deficient plants suggests that PLDα is modulated by the ABA treatment. To examine how ABA affected PLDα, we analyzed the changes in PLDα activity, mRNA, and protein after ABA treatment (Figures 6 and 7). PLDα activity increased approximately threefold in wild-type leaves after 1 day of treatment with ABA and remained relatively high after 3 days (Figure 6A). PLDα activity in antisense leaves was very low without ABA treatment and increased fivefold 1 day after ABA treatment. However, the total PLDα activity in antisense leaves was still less than 5% of that in the ABA-treated control leaves. In the absence of ABA, a small increase in PLDα activity (~40%) was observed 1 day after incubation of the detached leaves (Figure 6B). These results indicate that most of the increased PLDα activity observed was due to exogenous addition of ABA and was not merely the consequence of leaf detachment.

The higher PLDα activity in ABA-treated leaves was correlated with greater amounts of PLDα protein and mRNA (Figure 7). Hybridization with PLDα cDNA probes specifically detected PLDα mRNA under high-stringency conditions (Pappan et al., 1997a; Qin et al., 1997). PLDα mRNA in the wild-type leaves increased 1 day after treatment and decreased afterward (Figure 7A). These results suggest that ABA induces a transient increase in PLDα gene expression. PLDα protein increased 1 day after ABA treatment and remained relatively high in the following 2 days examined (Figure 7B). No PLDα protein or mRNA was detected in the antisense leaves (Figure 7). Leaves from the vector-transformed control plants showed the same pattern as that of wild-type plants in the activity, protein, and mRNA (data not shown).

Suppression of PLDα Attenuates Ethylene-Promoted Senescence in Detached Leaves

Both the retardation of leaf senescence in PLDα-deficient plants and the increase of PLDα gene expression by ABA suggest that PLDα is a key mediator of the ABA-accelerated senescence in detached organs. To test whether PLDα is involved specifically in the ABA response, we analyzed the effect of ethylene, a well-known senescence hormone, on senescence of PLDα-suppressed and control leaves. Ethylene was given in the form of ethephon, which is water soluble and releases ethylene in the cell. As with the ABA treatment, the PLDα-suppressed leaves showed a slower rate of senescence than did control leaves (Figure 8). Four days after incubation with 50 μM ethephon, the leaves of wild-type plants were almost completely yellow, whereas the antisense leaves were mostly green (Figure 8A). The retardation of senescence in the PLDα-deficient plant leaves was also clearly indicated by their higher levels of chloro-

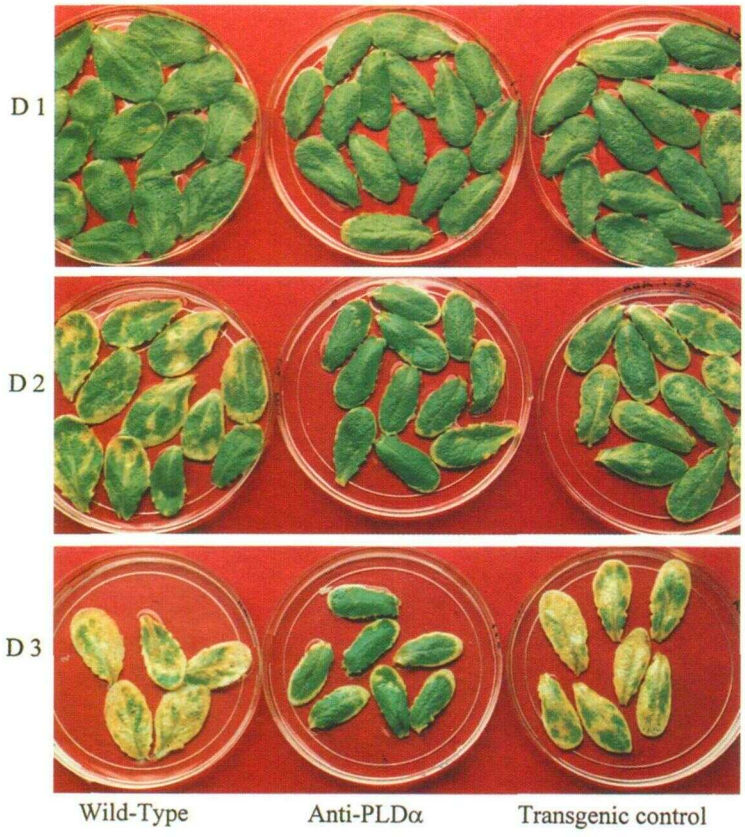

Figure 4. Retardation of ABA-Promoted Senescence in PLDα-Suppressed Leaves.

Leaves detached from wild-type (left), PLDα-suppressed (center), and vector-transformed transgenic plants (right) were treated with 50 μM ABA under 80 μmol m^{-2} sec^{-1} at 23 ± 2°C for 1, 2, and 3 days (D1, D2, and D3, respectively). The progressive decrease in the number of leaves at days 2 and 3 was because some of the leaves were removed for determination of the senescence parameters, as shown in Figure 5.

phyll (Figure 8B) and photosynthetic activity (Figure 8C), lower ion leakage (Figure 8D), and higher phospholipid content (Figure 8E) than control leaves after 4 days of treatment. The ethephon-treated leaves showed more than a twofold increase in PLDα activity (Figure 9A), which was correlated with an increase in PLDα protein as indicated by the immunoblotting with a PLDα antibody (Figure 9B).

Deficiency in PLDα Does Not Alter Normal Plant Development or Natural Leaf Senescence

To examine the role of PLDα in natural plant senescence, we compared the time required to complete a life cycle between the PLDα-suppressed and control plants (wild type and plants transformed with vector) grown under two different growth conditions: continuous light and a 14-hr photoperiod. In both cases, the PLDα-deficient plants grew and developed normally to maturity. There were no significant differences between wild-type and antisense plants in terms of plant height, number of leaves, date of flowering, number of siliques, number of seeds per silique, and weight per seed (Table 1). When the seeds of wild type and antisense plants were incubated on moist filter papers at room temperature, they germinated at a similar rate (Table 1). To evaluate the rate of leaf senescence on plants, we measured the overall photosynthetic capacity, F_v/F_m, and ion leakage of fully expanded and aged leaves and found no differences between wild-type and antisense plants (Table 1). The duration of leaves was also the same on both wild-type and PLDα-suppressed plants. These results indicate that the rate of natural growth and senescence of the PLDα-suppressed plants is the same as the rate of the control plants.

The senescence rates of the leaves detached from PLDα-suppressed and control plants in the absence of any phytohormone were compared. Fully expanded leaves were placed on wetted filter papers under light at room temperature or stored at 4°C in the dark. Under both conditions, PLD antisense and control plants underwent senescence at a similar pace. No significant difference was observed between

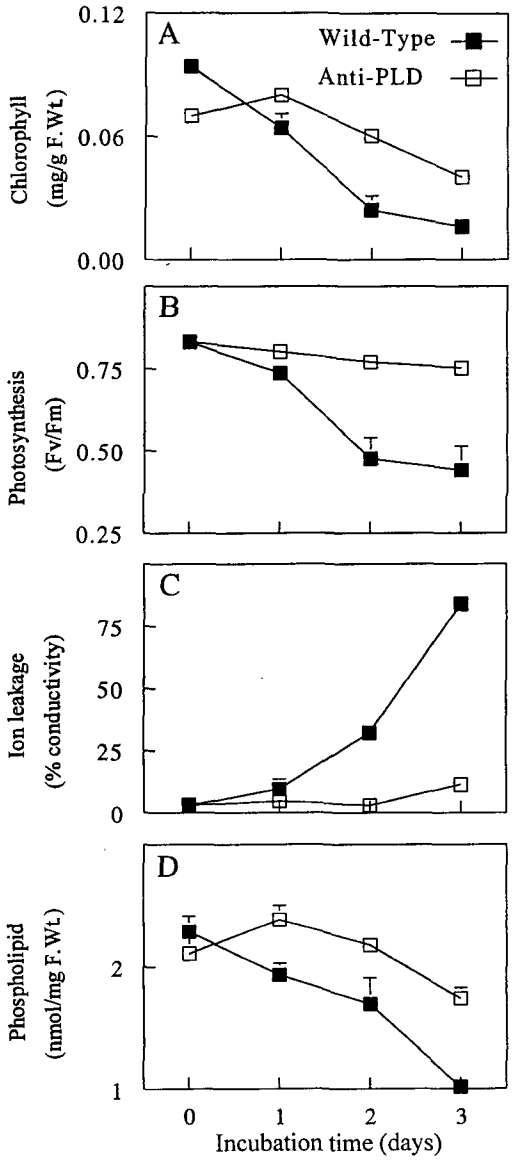

Figure 5. Attenuation of ABA-Promoted Losses of Chlorophyll, Photosynthetic Activity, Membrane Integrity, and Phospholipids in PLDα-Suppressed Leaves.

(A) Chlorophyll content, which was calculated based on the absorbance at 666 and 650 nm.

(B) Photosynthetic activity as measured by chlorophyll fluorescence (F_v/F_m).

(C) Membrane ion leakage as measured by the percentage of conductivity (*initial conductivity/total conductivity* × 100).

(D) Phospholipid content, which was determined from the total phosphorus content of total lipids.

Leaves detached from wild-type and PLDα-suppressed plants were incubated in 50 μM ABA and sampled at the indicated time intervals for measuring these parameters. Values are means ±SE of three experiments. F.Wt., fresh weight.

the antisense and control plants in terms of leaf yellowing, ion leakage, loss of chlorophyll (Figure 10A), and decreases in photosynthetic activity (Figure 10B) and phospholipid content (Figure 10C).

DISCUSSION

PLDα as an Important Mediator of Phytohormone-Promoted Senescence in Detached Leaves

ABA and ethylene are well-documented promoters of senescence in detached organs (Nooden and Leopold, 1978; Sacher, 1983). This study has demonstrated that the phytohormone-promoted senescence is retarded in detached leaves when the expression of the PLDα gene is suppressed. This retardation in PLDα-suppressed leaves has been clearly indicated by delayed leaf yellowing, higher content of chloro-

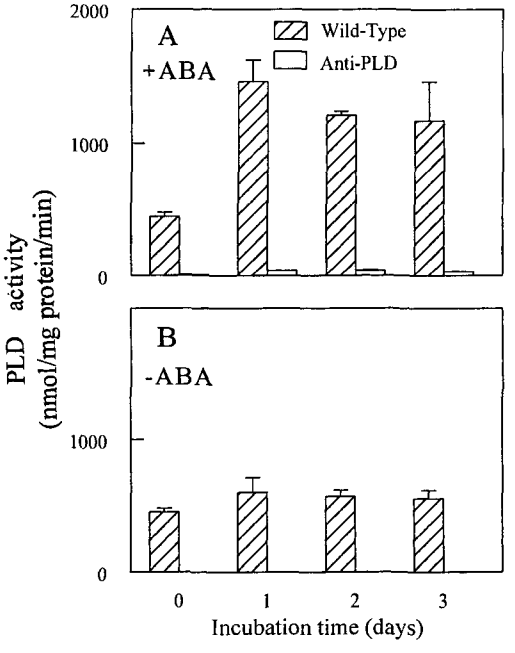

Figure 6. Increase of PLDα Activity in Detached Leaves Treated with ABA.

(A) PLDα activity in ABA-treated (+ABA) leaves detached from wild-type and PLDα-suppressed plants.

(B) PLDα activity in detached leaves without ABA (−ABA) treatment. Leaves were incubated in 50 μM ABA under 80 μmol m^{-2} sec^{-1} at 23 ± 2°C for 1 to 3 days. PLD activity was assayed in the presence of 25 mM Ca^{2+}, 0.5 M SDS, 100 mM Mes–NaOH, pH 6.5, and 2 mM egg yolk phosphatidylcholine with 0.02 μCi dipalmitoyl glycero-3-phosphate-(methyl-^{3}H)-choline. Values are means ±SE of three experiments.

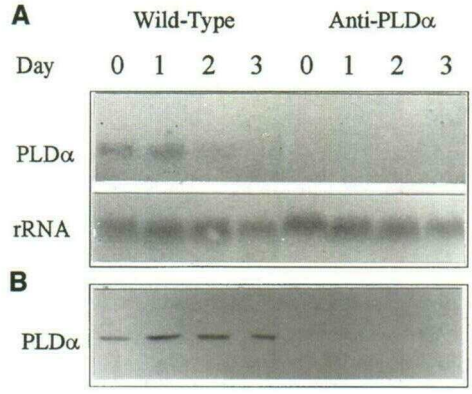

Figure 7. Changes in PLDα mRNA and Protein Levels in ABA-Treated Leaves.

(A) Autoradiography of a gel blot of PLDα and the control rRNA, using total RNA (20 μg per lane) isolated from wild-type and PLDα-suppressed leaves treated with 50 μM ABA.
(B) Immunoblot of PLDα from wild-type and PLDα-suppressed leaves treated with 50 μM ABA.
Protein was resolved by 8% SDS-PAGE, and each lane was loaded with 20 μg of protein from the 10,000*g* supernatant. The polyclonal antibody of PLDα was raised against a synthetic 13–amino acid peptide at the C terminus of PLDα. The PLDα antibody complex was visualized by alkaline phosphatase staining.

phyll and phospholipids, greater photosynthetic activity, and lower ion leakage when compared with the leaves from control plants. Such effects can only be attributed to the deficiency in PLDα because plants transformed with the same vector but without the PLDα antisense fragment behaved like the wild type. These results suggest that PLDα is a major mediator in phytohormone-promoted senescence (discussed below). Therefore, suppression of PLDα gene expression is able to retard the ABA- and ethylene-promoted deterioration in detached Arabidopsis leaves.

Involvement of PLDα in Phytohormone-Promoted Senescence

It is generally accepted that the breakdown of membranes is an early stage of plant senescence and that the decline in membrane phospholipid is characteristic of the whole process (Thompson, 1988). Many reports have indicated that ABA and ethylene alter membrane structure, cause a decline in all cellular glycerolipids, and induce membrane leakiness (Suttle and Kender, 1980; Thompson et al., 1982; Ho, 1983; Thompson, 1988; Paliyath and Droillard, 1992). In this study, suppression of PLDα decreased the phytohormone-promoted loss of total phospholipids and led to prolonged membrane integrity. This suggests that PLDα is a key component contributing to membrane degradation in phytohormone-promoted senescence. One way for this to occur is through PLDα-catalyzed lipid

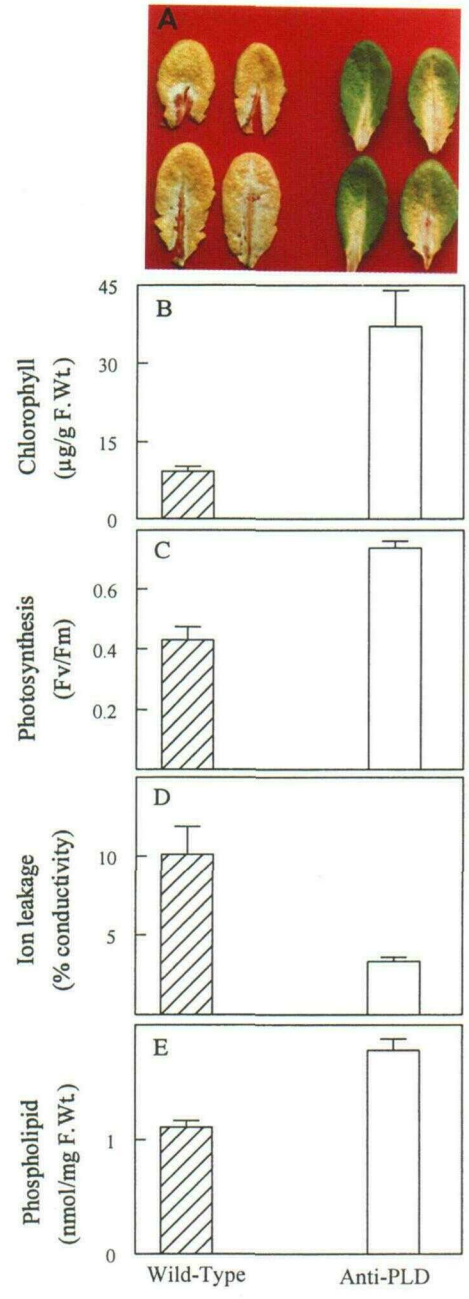

Figure 8. Delayed Ethylene-Promoted Senescence in Detached Leaves of PLDα-Suppressed Arabidopsis Plants.

(A) Morphological difference of leaves from PLDα-suppressed (right) and wild-type (left) plants.
(B) to **(E)** Chlorophyll content, photosynthetic activity, ion leakage, and phospholipid content, respectively, from ethephon-treated leaves of PLDα-suppressed and wild-type plants.
Leaves were incubated in 50 μM ethephon under 80 μmol m^{-2} sec^{-1} at 23 ± 2°C for 4 days. Values are means ±SE of two experiments. F.Wt., fresh weight.

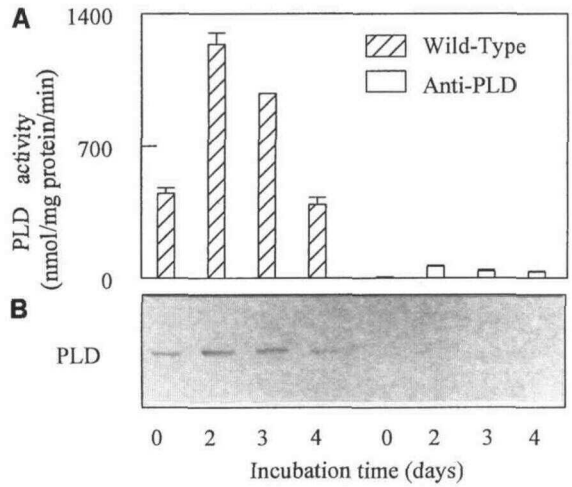

Figure 9. Changes in PLDα Expression in Ethylene-Treated Leaves of PLDα-Suppressed and Wild-Type Arabidopsis Plants.

(A) PLDα activity from the 10,000*g* supernatant of detached leaves.
(B) Immunoblot of PLDα of the 100,000*g* protein extracted from leaves detached from wild-type and PLDα-suppressed plants.
Leaves were incubated in 50 μM ethephon under 80 μmol m^{-2} sec^{-1} at 23 ± 2°C for 0, 2, 3, and 4 days. Values are means ±SE of two experiments.

degradation. PLDα cleaves major membrane phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylglycerol, into PA and free head groups (Dyer et al., 1994). PA is a nonbilayer lipid and a potent promoter for the formation of hexagonal phase. Thus, an increase in PA may destabilize membranes; furthermore, this perturbation may also activate other lipid-degrading enzymes. These include PA phosphatase, acyl hydrolase, and lipoxygenase, which have been proposed to form a PLD-initiated phospholipid degradation pathway (Thompson, 1988; Paliyath and Droillard, 1992). Such changes may lead to the loss of membrane integrity and functions of membrane-associated proteins, thereby promoting senescence.

In addition, the retardation of senescence may be caused by the participation of PLDα in phytohormone perception and signal transduction pathways. PLD has emerged as an important enzyme regulating various cellular processes (Munnik et al., 1995; Ryu and Wang, 1996; Young et al., 1996; Wang, 1997). Activation of PLD produces PA, which has been shown to stimulate a number of signal transducing enzymes, including PIP_2–phospholipase C, phosphatidylinositol 4-phosphate kinase, and protein kinase C. In addition, PA can be further metabolized by PA phosphatase into diacylglycerol, a known activator of protein kinase C and a proposed enhancer for flower senescence (Borochov et al., 1997). PA can also be converted to lysophosphatidic acid and free fatty acid, both of which are biologically active compounds. It is also conceivable that a rapid PA generation upon cell stimulation may result in an increase of negative charge at the cytoplasmic leaflet of the plasma membrane, which could facilitate movement to the membrane of certain cytosolic proteins with net positive charge as well as altering cation interaction with membranes. Cations, such as Ca^{2+} and K^+, and protein (de)phosphorylation have been proposed as mediators in ABA signaling (McAinsh et al., 1991). Therefore, a deficiency in PLDα may decrease the efficiency to transduce the phytohormone signals into physiological responses.

The retardation of senescence has been observed in the PLDα-suppressed leaves treated with either ABA or ethylene. These effects could result from PLDα functioning in the convergent or/and independent pathways of the two senescence-promoting hormones. In addition, it has been reported that the increase of ABA is accompanied or preceded by an elevation in ethylene evolution in detached organs (Nooden and Leopold, 1978). Thus, it may also be possible that treatment of ethylene may increase endogenous ABA concentration or vice versa, thus stimulating PLD activity.

Regulation of PLDα by ABA and Ethylene

The ability of PLDα to mediate ABA- and ethylene-promoted senescence indicates that PLDα is regulated by the phyto-

Table 1. Growth and Development Parameters of Wild-Type and PLDα-Suppressed Plants

Characteristics	Wild Type	PLDα Suppressed
Plant height (cm)[a]	35.9 ± 4.6[b]	35.8 ± 6.3
Number of leaves per plant		
4-week-old plants	8.8 ± 0.5	8.7 ± 0.4
8-week-old plants	21.1 ± 0.6	20.6 ± 0.5
Number of siliques per plant	43.7 ± 6.7	42.5 ± 6.4
Number of seeds per silique	40.7 ± 2.9	41.7 ± 2.1
Seed weight (mg per 1000)	18.8 ± 0.7	19.2 ± 0.6
Seed germination rate (%)[c]	96.5 ± 1.7	97.4 ± 1.3
Photosynthetic activity of leaves (F_v/F_m)[d]	0.832 ± 0.01	0.833 ± 0.01
Ion leakage (% conductivity)[d]	4.2 ± 0.25	4.0 ± 0.21

[a] Plants (8 weeks old) were grown under 14-hr-light/10-hr-dark cycles with a cool-white fluorescent light of 100 μmol m^{-2} sec^{-1} at 23 ± 3°C.
[b] The values are means ±SE of five to 10 independent samples.
[c] Seeds were germinated on moist filter papers in Petri dishes under continuous light.
[d] Six-week-old leaves were measured.

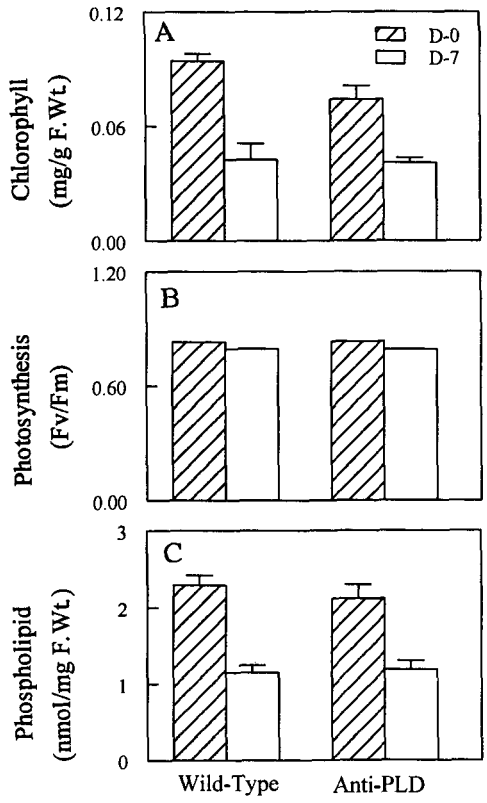

Figure 10. Similar Rate of Senescence in Detached Leaves of Wild-Type and PLDα-Suppressed Arabidopsis Plants in the Absence of Any Phytohormone Treatment.

(A) Chlorophyll content.
(B) Photosynthetic activity as measured by F_v/F_m.
(C) Total phospholipid content.
Measurements were taken on leaves newly detached (D-0) and those incubated for 7 days (D-7). Leaves from PLDα-suppressed and wild-type plants were placed on wet filter papers in Petri dishes under 80 μmol m^{-2} sec^{-1} at 23 ± 2°C. Values are means ±SE of two experiments. F.Wt., fresh weight.

hormones. PLDα could be stimulated by factors involved in the phytohormone signal transduction pathways, such as the stimulators of PLDα protein and gene expression. Our current and previous results have provided evidence for the ABA-induced expression of the PLDα gene (Ryu and Wang, 1995; Xu et al., 1997). The PLDα mRNA level increased in ABA-treated detached leaves from Arabidopsis and castor bean leaves. The PLDα gene promoter from castor bean has been characterized, and the promoter activity was found to increase in response to ABA treatment (Xu et al., 1997). Our data have also shown increases in PLDα protein and activity in ethylene-treated leaves. The increase in gene expression is likely to give rise to higher levels of PLDα protein and activity in the phytohormone-treated detached leaves. Elevation of PLDα activity will lead to increased lipid degradation, membrane deterioration, and thus senescence.

In addition to the above long-term regulation of PLDα, the phytohormone treatments could increase PLDα activity by affecting the preexisting PLDα protein. ABA increases intracellular Ca^{2+} (reviewed in McAinsh et al., 1991), and PLDα is known to be regulated by Ca^{2+} (reviewed in Heller, 1978; Wang, 1993, 1997). Recent studies have indicated that an increase in cytoplasmic Ca^{2+} may promote binding of PLDα to membranes (Ryu and Wang, 1996). Sequence analysis has identified a regulatory domain, C2/CalB, within PLDα (Ponting and Parker, 1996). This domain has been found in >50 different proteins, including phospholipase C and PLA_2. One main function identified for this domain is to mediate Ca^{2+}-dependent phospholipid binding, and this function is involved in intracellular translocation of proteins between soluble and membrane fractions (Shao et al., 1996). Indeed, the translocation of PLDα from soluble to membrane fractions has also been observed in castor bean leaves upon wounding, and this change was associated with an increase in wound-induced lipid hydrolysis by PLD (Ryu and Wang, 1996). Inclusion of physiological concentrations of Ca^{2+} in vitro also stimulated the association of castor bean PLDα with membranes (Ryu and Wang, 1996). In addition, ABA treatment of detached castor bean leaves increased membrane-associated PLD, which was positively correlated with the rate of detached leaf senescence (Ryu and Wang, 1995). Thus, an ABA-triggered influx of Ca^{2+} can be one mechanism that activates PLDα.

We also tested the possibility that phytohormones might increase PLDα activity by physically interacting with this protein or by directly altering membrane lipid bilayers. It has been reported that ABA modulates membrane bilayer properties, such as increasing membrane electrical conductivity and permeability to ions and neutral solutes, inducing lipid vesicle fusion, and altering microheterogeneity of lipid bilayers (Burner et al., 1993). PLDα is located on plasma and microsomal membranes (Xu et al., 1996; Young et al., 1996). Previous studies have documented that PLDα activity can be modulated by the physical state of lipid vesicles (Heller, 1978), and rigidification of microsomes activates PLD activity (McCormac et al., 1993). In this study, we measured the effect of ABA on in vitro PLDα activity by adding different concentrations of ABA (0, 5, 50, and 500 μM) to substrate vesicles and found no stimulation of PLD activity (data not shown). This result indicates that the PLDα activity in vitro is not modulated by direct contact with ABA or by inclusion of ABA in phosphatidylcholine vesicles.

PLDα Is Not a Direct Senescence Promoter during Natural Senescence

Although the results have clearly demonstrated that suppression of PLDα retards phytohormone-promoted senescence in detached leaves, the loss of PLDα activity did not alter the

rate of natural senescence in the plant. The PLDα-deficient plants grew and developed normally, and the lack of retardation of senescence was also observed in detached leaves without hormone treatment. In addition, we also generated a number of PLDα-overexpressing tobacco and observed no significant difference in leaf senescence (X. Wang, unpublished data). Leaves of these plants had at least 20-fold higher PLDα activity than did those of control plants transformed with the vector alone (Wang et al., 1997). This suggests that PLDα is not a direct promoter of senescence during regular plant growth and development.

The lack of apparent effects of the PLDα suppression on senesence in untreated plants is likely to result from a decreased level of PLDα gene expression in senescent tissues. We examined the pattern of PLDα gene expression by using different systems. A study of the PLDα gene promoter and glucuronidase fusion indicated that the PLDα promoter is more active in meristematic and newly divided cells than in naturally senescent leaves (Xu et al., 1997). RNA gel blot analysis showed higher amounts of PLDα mRNA in the early stages of leaf and seed development (Ryu et al., 1996; Xu et al., 1997). The levels of PLDα protein and activity also exhibited the same trends as did the levels of PLDα gene expression (Wang et al., 1993; Dyer et al., 1994; Xu et al., 1996). Therefore, the low PLDα activity in naturally senescing leaves may decrease its effect on membrane degradation and its role in natural senescence. On the other hand, when nonsenescent leaves were treated with ABA and ethylene, these treatments increased PLDα gene expression and its activity in the leaves that were already high in PLDα levels (Figures 6, 7, and 9). Such increases promoted the loss of phospholipid content and membrane integrity and senescence (Figures 5 and 8).

The differences between hormone-promoted and natural senescence exhibited by the PLDα-deficient leaves may indicate that PLDα activity does not initiate senescence, but when a premature senescence is initated, a high PLDα activity is detrimetal to membranes and accelerates the senescence process. This intepretation suggests that PLDα-mediated membrane degradation is an important but not a causal factor in senescence. Another intepretation of our results is that distinct pathways leading to senescence may be operating under different physiological conditions. We have shown that PLDα is an important mediator in hormone-induced senescence, but our results provide no support for such a role of PLDα in mediating natural senescence.

Roles of PLDα in Stress-Related Lipid Turnover

One important function, as implied by the results of this study, is that PLDα-catalyzed hydrolysis is involved in stress-related membrane lipid turnover. Both ABA and ethylene are involved in plant responses to stress. Endogenous ABA levels increase under adverse conditions (e.g., in response to drought and cold temperatures). Ethylene release in plants also increases under stress, including mechanical wounding, freeze injuries, and pathogen infection. Meanwhile, it has been demonstrated that PLDα activity and/or gene expression increase in response to wounding, stresses, and pathogen infection (Ryu and Wang, 1996; Young et al., 1996). Most of the early studies that suggested a PLD-initiated lipid degradation pathway in membrane deterioration used stress-inducing systems. Increased PA formation has been observed after exposure of plant tissues to frost injuries (Yoshida, 1979; Willemot, 1983), drought (Svenningsson and Liljenberg, 1986), Ca^{2+} starvation (Yapa et al., 1986), and γ-irradiation (Voisine et al., 1993); it has also been observed in senescing callus (Manoharan et al., 1990) and aging seeds (Samama and Pearce, 1993). It is conceivable that the increase in stress-related phytohormones in the cell activates PLDα. PLD hydrolysis generates PA that, along with its metabolites, such as diacylglycerol and polyunsaturated fatty acids, can be used for signaling and membrane lipid remodeling in stress adaptation. On the other hand, the lipid metabolites of PLD may promote membrane deterioration, cell death, and senescence. Whether PLDα acts in stress adaptation or damage is likely to be influenced by the nature and severity of the stresses and the physiological conditions of plants. As shown in phytohormone-promoted senescence in this study, an increase in PLDα activity contributes to the degradative and senescence process in detached leaves.

Molecular and Functional Heterogeneity of PLD

Many processes, such as signal transduction, cell proliferation, membrane trafficking, and reproduction, have been proposed to involve PLD (Rose et al., 1995; Wang, 1997). One role defined for an eukaryotic PLD comes from recent studies that revealed that PLD activity is essential in yeast for sporulation. Based on its sequence similarity with PLDα (Wang et al., 1994), the yeast *SPO14* gene, which is required for meiosis and sporulation (Honigberg et al., 1992), has been identified as encoding a PLD (Rose et al., 1995; Ella et al., 1996; Waksman et al., 1996). In this study, we have found no significant differences in the number of siliques and seeds produced by wild-type and PLDα-suppressed plants. Although it remains possible that the residual PLDα (~10% of wild type) in the PLDα-antisense plants may be sufficient for normal flower development, the lack of any apparent effect on regular seed production in the PLDα-suppressed plants suggests that a high level of PLDα is not required for meiotic signaling and reproduction.

Genetic redundancy may be another possible explanation for the lack of apparent alteration of normal growth and development in the PLDα-suppressed plants. Our recent results have demonstrated that PLD is a family of heterogenous enzymes (Qin et al., 1997; Wang, 1997). Three PLDs have been cloned from Arabidopsis, and they are distinct gene products. PLDα is the conventional PLD, which is characterized by its requirement for millimolar calcium for optimal activity

in vitro. PLDs β and γ are novel plant PLDs that require micromolar concentrations of calcium and polyphosphoinositides for activity (Pappan et al., 1997a, 1997b). The three PLDs differ in size, phospholipid activation, calcium concentration dependence, and PIP_2 binding ability. These differences suggest that PLDα is regulated very differently from PLDs β and γ. The antisense suppressed plants used in this study are specific to PLDα because these plants possess normal PIP_2-dependent PLD activity that is caused by PLDs β and γ (Pappan et al., 1997b). The delayed senescence in PLDα-suppressed plants indicates that the loss of PLDα cannot be completely compensated by other PLDs present in the antisense plants and that PLDα has unique functions in phytohormone-promoted lipid degradation and senescence. Our results also suggest that suppression of PLDα may enhance plant resistance to stress losses, such as during postharvest storage and handling of vegetables and fruits, without an adverse effect on regular plant growth and development.

METHODS

Plant Material and Generation of Transgenic Plants

Arabidopsis thaliana ecotype Columbia was used throughout this study. Seeds were sown in soil and cold-treated at 4°C overnight. Plants were grown 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 plants at flowering stages were transformed with T-DNAs through *Agrobacterium tumefaciens*–mediated gene transfer. A DNA fragment corresponding to nucleotides 1446 to 2231 of the full-length Arabidopsis phospholipase Dα (PLDα) cDNA was cloned into the T-DNA transfer vector pKYLX7 (Schardl et al., 1987). The DNA was inserted in the antisense orientation under the control of the cauliflower mosaic virus 35S promoter (Figure 1). The plasmid was transformed into Agrobacterium and then was transferred into Arabidopsis via a vacuum infiltration method (Bechtold et al., 1993).

To screen transgenic plants, seeds from the vacuum-infiltrated plants were surface sterilized in 0.5% sodium hypochlorite–0.05% Tween 20 and rinsed three times with sterile water. The seeds (~5000 per 85-mm Petri dish) were plated on Murashige and Skoog medium (Sigma) containing 70 μg/mL kanamycin. After growth on the plates for 10 days, kanamycin-resistant seedlings were transferred to soil and grown to maturity. Leaves of the transgenic plants were assayed for PLDα activity (described below). The presence of the introduced antisense DNA in PLDα-suppressed plants was verified by DNA gel blot analysis. Transgenic plants with the transfer vector were produced as control plants.

RNA Gel Blot Analysis

Total RNA was isolated from 4-week-old Arabidopsis leaves by using a cetyltrimethylammonium bromide extraction method described previously (Wang et al., 1994). RNA (20 μg per lane) was subjected to denaturing 1% formaldehyde–agarose gel electrophoresis and transferred onto a nylon membrane. The membrane was prehybridized in a solution of 6 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 100 μg/mL salmon sperm DNA at 65°C. The PLDα-specific probe, a 1.2-kb fragment near the 5′ end of the cloned Arabidopsis PLD cDNA (Dyer et al., 1995), was labeled with α-^{32}P-dATP by random priming. The probe made from this region detected only sense PLDα RNA. Hybridization was performed in the same solution at 65°C overnight. The blots were washed with 1 × SSC and 0.1% SDS at 65°C and exposed to x-ray film. As a control, the hybridized blots were stripped and hybridized with a radiolabeled fragment of the constitutively expressed Arabidopsis rRNA gene.

Protein Extraction, SDS-PAGE, and Immunoblotting

The total proteins from different organs of flowering Arabidopsis were extracted using a previously described procedure (Wang et al., 1993). Briefly, one part of a tissue was ground with a chilled mortar and pestle in three parts of a homogenization buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM DTT at 4°C. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was collected, and its protein content was determined by the Bradford method, according to manufacturer's instructions (Bio-Rad). The supernatant was used directly or stored at −80°C until use.

For immunoblotting analysis, the protein extracts were separated by 8% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were blotted with anti-PLDα antibodies raised in rabbit against a peptide corresponding to the 13 amino acids of the Arabidopsis PLDα C terminus (Pappan et al., 1997b). The PLDα–antibody complex was visualized by staining alkaline phosphatase conjugated to a second antibody (Wang et al., 1993).

PLDα Activity Assay

The reaction mixture contained 100 mM Mes, pH 6.5, 25 mM $CaCl_2$, 0.5 mM SDS, 1% (v/v) ethanol, 5 to 15 μg of protein, and 2 mM phosphatidylcholine (egg yolk) containing dipalmitoyl glycero-3-phosphate-(methyl-^{3}H)-choline in a final volume of 200 μL. Substrate preparation, reaction conditions, and product separation were based on a previously described procedure (Wang et al., 1993). The release of ^{3}H-choline into the aqueous phase was quantitated by scintillation counting.

Phytohormone Treatments

Fully expanded leaves were collected from ~6-week-old PLDα antisense and control plants. The detached leaves were rinsed briefly with sterile water and placed adaxial side up in Petri dishes containing 50 μM abscisic acid (ABA), ethephon, or H_2O. ABA was first dissolved in ethanol and then diluted in sterile H_2O to the final concentration. The ABA treatment contained 0.005% ethanol, and thus, this concentration of ethanol was included in all of the treatments. The leaves were incubated at 23 ± 3°C under a 14-hr photoperiod and light of 80 μmol m^{-2} sec^{-1}.

Measurements of Membrane Leakage, Phospholipids, Chlorophyll, and Photosynthetic Activity

Leaves newly detached from plants or leaves after treatment were washed briefly in deionized water before measurements. Membrane leakage was determined by measurement of electrolyte leaked from leaves. Three leaves of each treatment group were immersed in 3 to 5 mL of 0.4 M mannitol at 23°C with gentle shaking for 3 hr, and the bathing solution was measured for conductivity. The total conductivity was determined after boiling the sample for 10 min. The conductivity due to leakage was expressed as the percentage of the initial conductivity versus the total conductivity.

Total lipids were extracted as described previously (Ryu and Wang 1996). For each sampling, three leaves were immersed in 2 mL of hot isopropanol (75°C) for 15 min to inactivate lipolytic enzymes. Phospholipid content was calculated based on the phosphorus content of the total lipids (Rouser et al., 1970). A portion of the total lipid extract was used for chlorophyll determination. Chlorophyll content was measured based on the absorbance of the extract at 650 and 666 nm (Crafts-Brandner et al., 1984).

Photosynthetic activities of leaves were assessed by use of the ratio of variable chlorophyll fluorescence (F_v) to maximum yield of chlorophyll fluorescence (F_m), expressed as F_v/F_m. The ratio of F_v/F_m indicates potential quantum efficiency (Hipkins and Baker, 1987) and was monitored with a Plant Efficiency Analyzer (Hansatech, Norfolk, UK). Leaves were adapted to the dark for 10 min at room temperature before measurements were made. Three readings were obtained from one leaf, and at least three leaves were measured for each treatment (Hipkins and Baker, 1987).

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