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Regulation of plant water loss by manipulating the expression of phospholipase Dα

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Summary

Phospholipase D (PLD) has been implicated in various processes, including signal transduction, membrane trafficking, and membrane degradation. Multiple forms of PLD with distinct biochemical properties have been described in the cell. In *Arabidopsis,* PLDα and PLDγ, but not PLDβ, were detected in guard cells, and antisense suppression resulted in a specific loss of PLDα. The abrogation of PLDα rendered plants less sensitive to abscisic acid and impaired stomatal closure induced by water deficits. PLDα-depleted plants exhibited accelerated transpirational water loss and a decreased ability to tolerate drought stress. Overexpression of *PLDα* enhanced the leaf's sensitivity to abscisic acid. These findings provide molecular and physiological evidence that PLDα plays a crucial role in regulating stomatal movement and plant-water status.

Keywords: phospholipase, abscisic acid, drought, signal transduction, stress response.

Introduction

Phospholipids provide not only the structural base of cell membranes, but also rich resources for generating cellular regulators. Hydrolysis of phospholipids by phospholipases is often the first step in lipid hydrolysis and generation of lipid and lipid-derived messengers. Activation of phospholipase D (PLD) has been linked to various cellular processes, including signal transduction, membrane trafficking, cytoskeletal rearrangement, and membrane degradation in plants, animals and yeast (reviewed by Liscovitch *et al.*, 2000; Wang, 2000). PLD hydrolyzes phospholipids, generating phosphatidic acid (PA) and free head groups. Recent studies suggest that PLD mediates an important step in the abscisic acid (ABA) signal transduction. Genetic suppression of a *PLD* in *Arabidopsis* decreases the rate of ABA-promoted senescence in detached leaves (Fan *et al.*, 1997). Addition of PA to protoplasts of barley aleurone and *Vicia faba* guard cells partially mimics the effect of ABA (Jacob *et al.*, 1999; Ritchie and Gilroy, 1998). Activity and gene expression of PLD also increase in tissues treated with ABA (Jacob *et al.*, 1999; Xu *et al.*, 1997).

The involvement of PLD in ABA responses raises intriguing questions as to the role of PLD in plant–water relations. ABA is known to promote stomatal closure during drought stress, and this change is crucial to maintaining hydration status in leaves and to plant survival (Assmann and Shimazaki, 1999; Blatt, 2000; Leung and Giraudat, 1998). In guard cells, the added PA triggers events that lead to closure of the inward K^+ channel and stomatal aperture (Jacob *et al.*, 1999). In the resurrection plant, *Craterostigma plantagineum*, PLD is activated rapidly during dehydration, and this rise has been suggested to play a role in the early events leading to drought tolerance in the plant (Frank *et al.*, 2000). However, there has been no direct molecular or *in planta* evidence for the involvement of PLD in modulating plant-water status.

In addition, multiple forms of PLD have been identified in the cell recently, and they exhibit different biochemical properties in plants, animals, and microorganisms (Liscovitch *et al.*, 2000; Wang, 2000). Three types of PLD, PLDα, PLDβ, and PLDγ, have been characterized in *Arabidopsis* (Fan *et al.*, 1999; Pappan *et al.*, 1998; Qin *et al.*, 1997). PLDα is the common plant PLD that does not require phosphatidylinositol 4,5-bisphosphate (PIP_2) for activity, when assayed at millimolar concentrations of Ca^{2+}

(Pappan *et al.*, 1997). The newly identified PLDβ and PLDγ are PIP_2-dependent, and are most active at submicromolar levels of Ca^{2+} (Pappan *et al.*, 1997; Qin *et al.*, 1997). In addition, these PLDs differ in their substrate preferences, which are also modulated differently by substrate presentation (Pappan *et al.*, 1998). Furthermore, these PLDs have different patterns of subcellular distribution and tissue expression (Fan *et al.*, 1999; Wang *et al.*, 2000). For example, the expression of *PLDβ* and *PLDγ* genes increases in wounded *Arabidopsis* leaves, whereas PLDα is activated by increased association of the pre-existing enzyme with membranes (Wang *et al.*, 2000). Judged from the results of cloning, purification, activity distribution, and expression studies, PLDα is more prevalent and widespread than PLDβ and PLDγ in plant tissues (Fan *et al.*, 1999; Pappan *et al.*, 1997; Wang, 2000). These differences suggest that PLD isoforms are regulated differently and may have unique functions.

Therefore this study was undertaken to determine whether, and which, PLD is involved in regulating plant–water relations. We determined the occurrence of PLDα, β and γ proteins in guard cells, and the effect of genetic abrogation of PLDα on transpirational water loss in *Arabidopsis*. To verify the role of PLDα in plant water status, and also to test the potential use of *PLDα* in decreasing water loss in crop plants, we overexpressed *PLDα* in tobacco and examined the role of increased *PLDα* expression in regulating plant-water loss. These results demonstrate an important role of a specific PLD in regulating plant responses to water stress.

Results

Localization of PLD isoforms and suppression of PLDα in guard cells

The presence of PLDα, β and γ in *Arabidopsis* guard cells was determined using immunolabeling with antibodies raised against peptides of these isoforms (Fan *et al.*, 1999; Pappan *et al.*, 1997). PLDα was labeled with the PLDα antibody and was clearly detectable in guard cells (Figure 1a), whereas labeling with the PLDα pre-immune serum gave negligible background (Figure 1b). The presence of PLDα in guard cells was confirmed using fluorescence confocal imaging and immunogold electron microscopy (Y.Sang and X.Wang, unpublished results). The labeling specificity for PLDα was verified unequivocally by the absence of immunostaining in guard cells from PLDα-depleted plants (Figure 1c). Antisense suppression of PLDα resulted in a nearly complete loss of PLDα in *Arabidopsis* leaves, as indicated by the absence of PLDα activity (Figure 1g) and protein (Figure 1h). We have shown previously that, in addition to leaves, PLDα is expressed in flowers, roots, stems and silique, and that the residual PLDα of the antisense plants was slightly higher in roots and flowers than in leaves (Fan *et al.*, 1997). The expression and activities of the other PLDs were not altered in the PLDα-abrogated plants (Pappan *et al.*, 1997; Wang *et al.*, 2000).

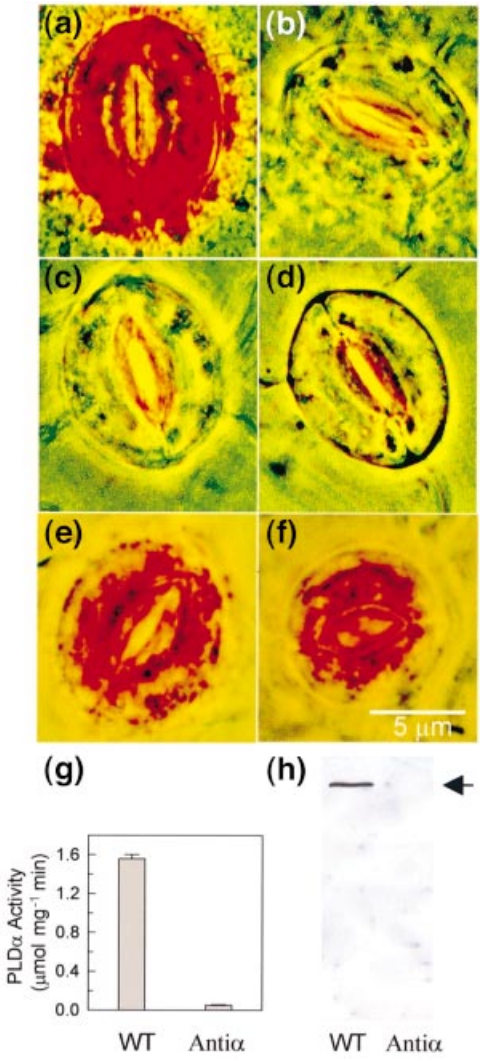


Figure 1. Immunolabeling of PLDα, β and γ and antisense depletion of PLD in *Arabidopsis* guard cells.
(a) *Arabidopsis* wild-type guard cell labeled with PLDα antibodies. Red color indicates positive labeling resulting from the activity of alkaline phosphatase conjugated to a second antibody.
(b) *Arabidopsis* wild-type guard cell labeled with PLDα pre-immune serum.
(c) Guard cell from PLDα-suppressed *Arabidopsis* labeled with PLDα antibodies.
(d) *Arabidopsis* wild-type guard cell labeled with PLDβ antibodies.
(e) *Arabidopsis* wild-type guard cell labeled with PLDγ antibodies.
(f) Guard cell from PLDα-suppressed *Arabidopsis* labeled with PLDγ antibodies.
(g) PLDα activity in PLDα-antisense (Antiα) and wild-type (WT) *Arabidopsis* leaf extracts.
(h) Protein immunoblotting of leaf proteins with PLDα-specific antibodies.
Both the activity and immunoblot assays used a 6000 *g* supernatant of total leaf extracts. Proteins (10 μg per lane) were separated on a 10% SDS–PAGE gel, and the PLDα band is marked by an arrow.

The suppression resulted from a single insertion (Fan *et al.*, 1997), and has been inherited for generations without variation (eight generations tested).

PLDγ protein was also detectable in guard cells labeled with the PLDγ antibody (Figure 1e), whereas labeling with the PLDγ pre-immune serum gave negligible staining (data not shown). In contrast to the labeling with PLDα antibody, positive staining was observed in both wild-type (Figure 1e) and PLDα-suppressed guard cells (Figure 1f). This shows that the loss of PLD in guard cells is specific to PLDα. The specific depletion of PLDα has been supported also by RNA gel-blotting assays, which showed the levels of *PLDγ* and *PLDβ* mRNA were similar between wild-type and PLDα-abrogated *Arabidopsis* (Wang *et al.*, 2000).

Under the same conditions, however, the labeling with the PLDβ antibody yielded no detectable signal in guard cells (Figure 1d). The PLDα and PLDβ antibodies both were raised against 12-amino-acid peptides corresponding to their respective C-termini. Titers for these antibodies are similar, based on their reactivity with bacterially expressed plant PLDα and β and with the synthetic peptides used for antibody production (Fan *et al.*, 1999). Using the same antibodies, PLDβ has been detected in the leaf vascular cells and young embryo cells (L. Fan and X. Wang, unpublished results). Thus the inability to detect PLDβ indicates the presence of a much lower level of PLDβ protein in guard cells than of PLDα. These results establish that *PLDα* and *PLDγ* are expressed in guard cells, and that only PLDα is abrogated in guard cells in *PLDα* antisense plants.

Decreased sensitivity to ABA-promoted stomatal closure in PLDα-depleted leaves

Terrestrial plants lose more than 90% of water through stomata, which are pores defined by pairs of guard cells. The genetic depletion of PLDα in guard cells provides an effective means to assess the role of this PLD in plant-water loss. Under normal growing conditions, PLDα-depleted and wild-type plants grew comparably; no differences occurred in plant size, development, and reproduction (Fan *et al.*, 1997), or the size and density of guard cells on leaves. The rate of water loss from leaves was determined by measuring diffusion resistance that increases as stomatal aperture decreases (Thimann and Satler, 1979). We also verified the changes in stomatal aperture using confocal microscopy. Incubation of leaves with 10 μM ABA induced stomatal closure, as indicated by an approximately twofold increase in diffusion resistance in wild-type leaves (Figure 2a). The same ABA treatment had a much smaller effect on stomatal closure in *PLDα*-suppressed leaves. The ABA-induced increase in diffusion resistance was approximately 50% of that observed in wild-type leaves, and returned to the basal level 20 min after ABA application. The ABA effect persisted for 30 min

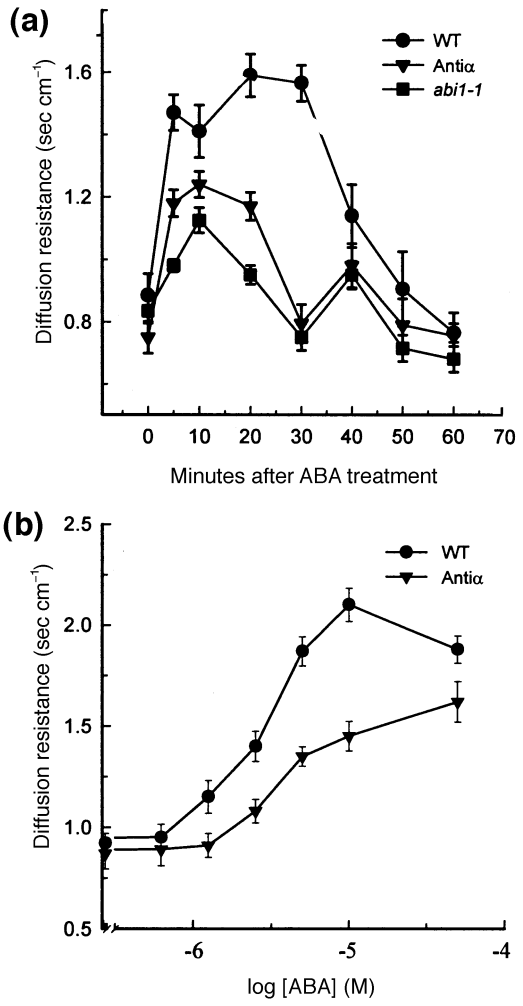


Figure 2. Decreased response to ABA-induced stomatal closure in PLDα-depleted *Arabidopsis* leaves.
(a) Temporal increases in diffusion resistance induced by 10 μM ABA in wild-type (WT), PLDα-depleted (Antiα), and *abi-1 Arabidopsis* leaves. (b) Effect of ABA concentrations on diffusion resistance in wild-type and PLDα-depleted *Arabidopsis*. Leaves were detached and incubated with the abaxial side down in solutions with different levels of ABA for 20 min. Values are means ± SE of two experiments.

in wild-type plants and then decreased. The greater stomatal aperture in *PLDα*-suppressed leaves than in the wild-type was confirmed by measuring directly on epidermal peels using confocal microscopy (data not shown). The response to ABA in PLDα-depleted leaves resembled that of the well characterized, ABA-insensitive mutant *abi-1,* which is defective in a protein phosphatase 2C that is involved in ABA signaling in *Arabidopsis* guard cells (Leung *et al.*, 1994; Meyer *et al.*, 1994). At the range of 0.5–50 μM ABA tested, the PLDα-depleted leaves exhibited a lower diffusion resistance than that of the wild type (Figure 2b). The 2 μM concentrations of ABA stimulated stomatal closure in wild-type leaves, but had no effect in

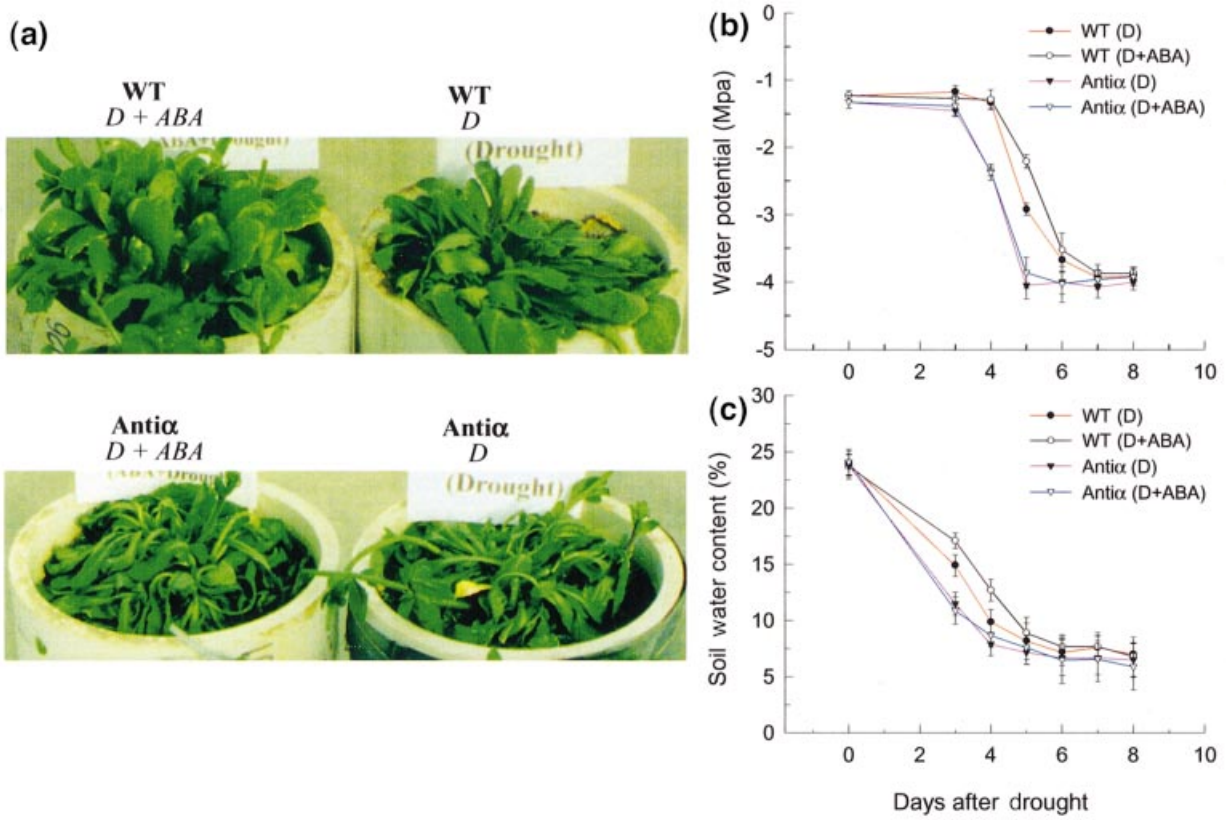


Figure 3. Impaired drought tolerance in PLDα-depleted *Arabidopsis*.
(a) Phenotype of PLDα-depleted (Antiα) and wild-type (WT) plants (6 weeks old, preflowering) after withholding irrigation for 5 days in a greenhouse. D, drought only; D + ABA, 10 μM ABA sprayed once a day on drought-stressed plants.
(b) Decrease in leaf water potential during drought. Leaf water potential of flowering plants was measured with a thermocouple psychrometer.
(c) Decrease in soil water content during drought treatment. Soil moisture in the 0–20 cm soil layer was monitored using a time-domain reflectometer. Values in (b) and (c) were measured on 8-week-old flowering plants. When ABA (10 μM) was sprayed on plants in some treatments, control groups of plants were sprayed with water in the same amount as for the ABA treatment. In (b) and (c), values are means ± SE ($n \geqslant 5$).

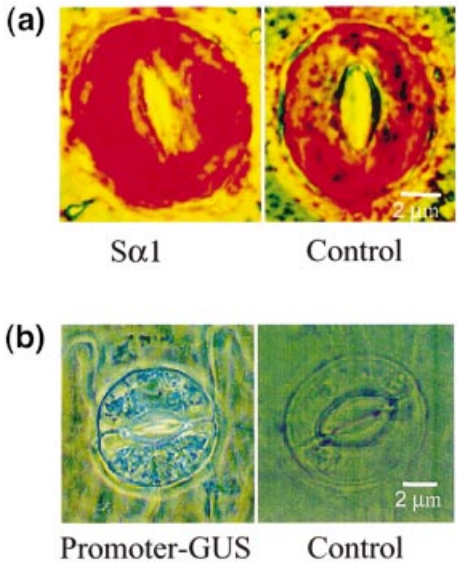


Figure 5. Localization of PLDα protein and promoter activity in tobacco guard cells.
(a) Immunolabeling of PLDα tobacco guard cells. Epidermal peels from PLD-overexpressing (Sα1, left) and empty-vector transformed (control, right) leaves were labeled with *Arabidopsis* PLDα-antibodies using a freeze-shattering method. Red color indicates positive labeling resulting from the activity of alkaline phosphatase conjugated to a second antibody.
(b) Histochemical detection of castor bean PLDα promoter activity in guard cells. The PLDα promoter was fused with β-glucuronidase, and the activity of β-glucuronidase in guard cells was detected with 5-bromo-4-chloro-3-indolyl β-glucuronide staining of leaf epidermal peels from plants transformed with the promoter construct (left) and without the promoter construct (right).

PLDα-suppressed leaves. The effect in wild-type leaves reached a plateau at 10 μM ABA, whereas such a plateau was not observed, even at 50 μM ABA, in PLDα-suppressed leaves. These results indicate that stomatal movements in PLDα-depleted leaves were less sensitive to ABA.

Increased transpirational water loss in PLDα-depleted plants

To determine whether the impaired stomatal closure compromises the plant's ability to cope with water stress, plants were subjected to progressive drought by withholding irrigation. Before drought stress was initiated, soil-water content in each pot was adjusted to the same level (24% soil-water content; Figure 3c). The soil surface was covered with a plastic wrap, so that the water loss from the soil came primarily from leaf transpiration. During drought, PLDα-depleted plants wilted earlier than wild-type plants (Figure 3a). A greater loss of water in leaves was indicated by lower leaf-water potentials in PLDα-deficient than in wild-type plants (Figure 3b). By 5 days of drought treatment, the decrease of water potential was twofold greater in PLDα-depleted than in wild-type leaves. The increased water loss was observed in PLDα-deficient plants at preflowering (Figure 3a) and flowering stages (Figure 3b,c). Measurement of soil-water content showed an accelerated decrease with PLDα-deficient plants (Figure 3c), indicating a greater transpirational loss of water in these plants.

Additionally, ABA (10 μM) was sprayed on a set of drought-stressed plants once a day, to test its effect on promoting drought resistance in PLDα-depleted and wild-type plants. This treatment enhanced resistance to drought in wild-type plants, as indicated by the maintenance of leaf turgidity during drought (Figure 3a, WT, D + ABA); increased leaf water potential (Figure 3b); and soil-water content (Figure 3c). The same ABA treatment had no detectable effect on water loss and drought resistance of PLDα-depleted plants (Figure 3a, Antiα, D + ABA). These data provide *in planta* evidence that suppression of *PLDα* decreased plant sensitivity to ABA. This reduction in ABA-induced stomatal closure resulted in increased transpirational water loss in PLDα-depleted plants.

Overexpression and promoter activity of PLDα *in tobacco*

To verify the role of *PLDα* in regulating plant-water status, and also to test the potential use of *PLDα* in decreasing water loss in crop plants, we overexpressed *PLDα* in plants and determined the effect of increased *PLDα* expression on ABA-induced stomatal closure and the rate of water loss. We attempted initially to overexpress *PLDα* in both *Arabidopsis* and tobacco (*Nicotiana tabacum*). But the overexpression of *PLDα* in *Arabidopsis* was not as successful as in tobacco, and thus tobacco plants overexpressing a castor bean *PLDα* were used in the following experiments. The castor bean *PLDα* is an ortholog of *Arabidopsis PLDα* based on their biochemical properties, gene structures and expression patterns, sequence similarity, and domain characteristics (Qin *et al.*, 1997; Wang, 2000; Xu *et al.*, 1997). Introduction of the *PLDα* to tobacco resulted in several-fold increases in PLDα activity (Figure 4a). The introduced *PLDα* was clearly detectable by immunoblotting with antibodies raised against *Arabidopsis* PLDα; the castor bean *PLDα* migrated slightly faster than the tobacco endogenous PLDα (Figure 4b). More than 20 tobacco lines overexpressing *PLDα* have been generated, and gene silencing of tobacco *PLDα* was not observed. The three transgenic lines used in this study all grew and developed normally to maturity, and showed

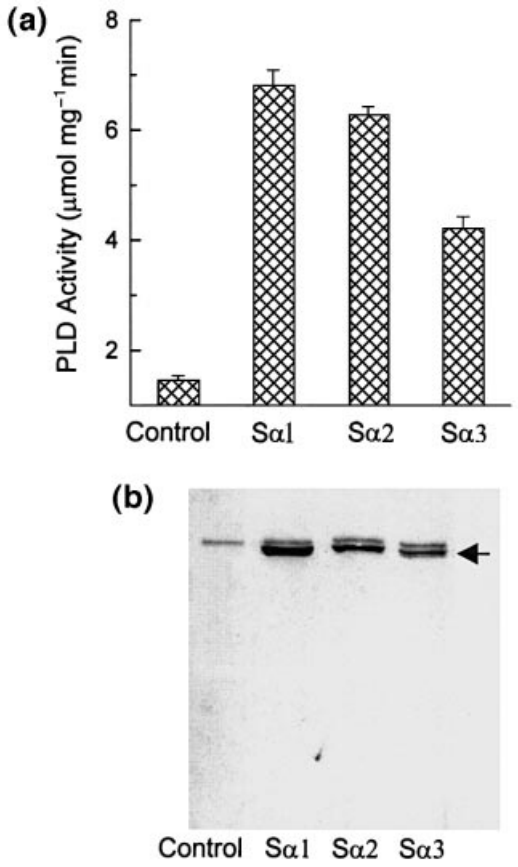
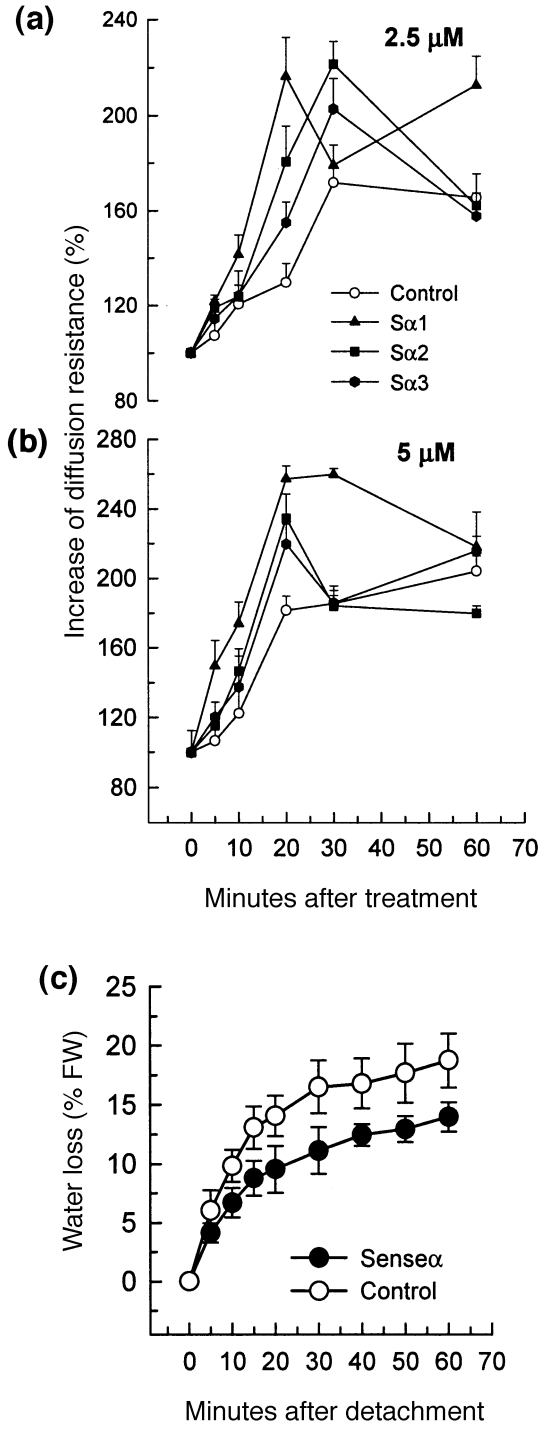


Figure 4. Overexpression of *PLDα* in tobacco plants.
(a) PLDα activity in leaves of tobacco transformed with a sense *PLDα* cDNA (Sα) or with an empty vector (control).
(b) Immunoblot with a PLDα-specific antibody of proteins from PLDα-overexpressing and control leaves. Three independent transgenic lines, Sα1, Sα2 and Sα3, were used. Proteins (10 μg per lane) were separated on an 8% SDS–PAGE gel, and PLDα was made visible by staining with alkaline phosphatase. The arrow marks the overexpressed *PLDα*. Both the activity and immunoblot assays used 6000 *g* supernatant of total leaf extracts.

no difference in the leaf numbers, plant heights, date of flower, and seed yield under normal growth conditions. Cellular fractionation showed that the introduced *PLDα* had the same intracellular association as the endogenous PLDα, being present in both soluble and microsomal membrane fractions. These observations support a previous proposal that PLDα activity is tightly regulated after translation (Wang *et al.*, 2000). They also indicate that overexpression of *PLDα* does not perturb overall cellular metabolism.

PLD was detected in tobacco guard cells using antibodies against *Arabidopsis* PLDα (Figure 5), whereas preimmune serum gave negligible background, as shown in *Arabidopsis* (Figure 1b). The *Arabidopsis* PLDα antibodies recognized PLDα from castor bean and tobacco because the C-terminal sequence used for raising the PLDα antibodies was highly conserved in all plant PLDαs. The introduced *PLDα* was expressed in tobacco guard cells because the labeling intensity in the *PLDα*-overexpressing guard cells (Figure 5a, *Sα1*) was greater than that of the control leaves (Figure 5a, control). Because the overexpression was driven under the control of the cauliflower mosaic virus 35S promoter, we further verified whether the castor bean *PLDα* was expressed in guard cells with its own promoter (Figure 5b). The castor bean PLD promoter was fused with β-glucuronidase (GUS) and transformed to tobacco (Xu *et al.*, 1997). The PLD promoter activity has been shown previously to occur in various tissues, including leaves, flowers, roots, stems and silique (Xu *et al.*, 1997). Histochemical localization of the GUS activity showed blue staining in guard cells from the tobacco leaves carrying the promoter–GUS fusion (Figure 5b, left), but not from the leaves without the fusion (Figure 5b, right). These results demonstrate that like *Arabidopsis*, *PLDα* from tobacco and castor bean is also expressed in guard cells.



Increased leaf sensitivity to ABA and decreased water loss in PLD*α-overexpressing plants*

The large size of tobacco leaves allowed direct measurement of transpirational water loss on plants after ABA treatments. When leaves were sprayed with 2.5 and 5 μM ABA, stomata closed more quickly and to a greater extent in all the *PLDα*-overexpressing plants than in control

Figure 6. Increased sensitivity to ABA-promoted stomatal closure and decreased water loss in *PLDα*-overexpressing tobacco leaves.
(a) Increase in diffusion resistance after spraying 2.5 M ABA to three *PLDα*-overexpressing lines and control tobacco leaves.
(b) Increase in diffusion resistance after spraying 5 μM ABA to three *PLDα*-overexpressing lines and control tobacco leaves. Leaf diffusion resistance was measured directly on plants that were sprayed with ABA and was expressed as a percentage of that of plants sprayed with water. Values are percentages of means ± SE of three experiments. The diffusion resistance (sec cm^{-1}) prior to the treatment for wild-type and three *PLDα*-overexpressing lines were: control = 2.12 ± 0.30; Sα1 = 2.22 ± 0.13; Sα2 = 2.29 ± 0.26; Sα3 = 2.10 ± 0.72 ($n \geqslant 10$).
(c) Water loss from detached leaves of three *PLDα*-overexpressing lines and control tobacco plants. Leaves were detached and left in ambient conditions. The percentages of decreases in fresh weight were expressed as percentage water loss. Values are percentages of means ± SE of three leaves each from Sα1, Sα2 and Sα3 *PLD*-overexpressing lines.

plants (tobacco transformed with an empty vector; Figure 6a). Leaf diffusion resistance increased approximately 50–100% in the three *PLDα*-overexpressing lines, and only 30% in control leaves, 20 min after ABA application. The differences in diffusion resistance between the two genotypes were most noticeable within the first 20 min after ABA application, and diminished afterwards (Figure 6a). These differences were also more pronounced at a lower (Figure 6a) than a higher ABA concentration (Figure 6b). These data indicate that *PLDα*-overexpressing plants respond more rapidly and are more sensitive to ABA than control plants. This enhanced ABA responsiveness also suggests that PLDα occupies a limiting step in the early stages of stomatal movement induced by ABA.

To assess water loss from leaves without exogenously added ABA, leaves of similar size, age and positions on *PLDα*-overexpressing and control plants were detached and measured for decreases in fresh weight, as described in other studies (Tan *et al.*, 1997). Leaves from control and *PLDα*-overexpressing plants were similar in size and fresh weight, and there were also no differences in the number of stomata per leaf area or the size of stomatal aperture before detachment (Figure 6a). After detachment, however, leaves from the *PLDα*- overexpressing lines exhibited lower loss of fresh weight than those from control plants under ambient conditions (Figure 6c). The differences occurred within 5 min, and became more apparent between 20 and 30 min following detachments. This decreased rate of water loss is consistent with the above results (Figure 5a,b), suggesting that stomata on *PLDα*-overexpressing leaves close more rapidly than control leaves in response to water stress.

Discussion

PLD has emerged recently as a new family of phospholipases that are involved in signal transduction and cell regulation, but the cellular and physiological function of PLD has remained elusive (Liscovitch *et al.*, 2000; Wang, 2000). With PLDα-depleted *Arabidopsis* and PLDα-overexpressing tobacco, this study provides molecular and physiological evidence for a crucial role of PLD in regulating transpirational water loss. Genetic depletion of PLDα results in increased transpirational water loss, and this increase is caused by impaired stomatal closure and reduced response to ABA. The PLD step leading to the stomatal closure can be enhanced by increasing PLDα expression. These results show that PLDα occupies a critical step in controlling stomatal movements and plant response to water stress.

In addition, this study provides insights into the role of different PLD isoforms in plant cells. PLDα, β and γ exhibit distinctive activities. Although PLDα *in vitro* is known to be most active at millimolar concentrations of Ca^{2+}, a recent study has shown that the requirement for Ca^{2+} and PIP_2 by PLDα is strongly influenced by pH and substrate lipid composition (Pappan and Wang, 1999). PLDα is stimulated greatly at near-physiological, micromolar levels of Ca^{2+} under acidic conditions, and this activity requires phosphoinositides in mixed lipid vesicles. In contrast, the need for Ca^{2+} and PIP_2 by PLDβ and γ is not affected by pH, but the activity requires high amounts of a non-lamellar lipid phosphatidylethanolamine in substrate vesicles (Pappan *et al.*, 1998). Recently, we have identified another novel *Arabidopsis* PLD, PLDδ, that is activated by oleic acid (Wang, C. and Wang, X. unpublished results). These differences suggest that changes in cellular levels of Ca^{2+}, PIP_2 and pH, and in membrane composition and conformation, may activate PLD isoforms differentially. The activation of different PLD isoforms may also result in selective hydrolysis of membrane phospholipids (Pappan *et al.*, 1998). The PLDα-depleted *Arabidopsis* used in this study showed no alteration in the activity and expression of *PLDβ*, *PLDγ* (Pappan *et al.*, 1997; Wang *et al.*, 2000; Figure 1), and *PLDδ* (Wang, C. and Wang, X. unpublished results). But the presence of the other PLDs cannot compensate for the loss of PLDα in regulating water loss. Localization results show that the level of PLDα is much higher than that of PLDβ in guard cells (Figure 1). The differential expression of *PLD* isoforms in the cell may underlie one of the mechanisms for their specific functions. It is possible that the constitutively expressed *PLDα* is activated first in response to water stress to initiate or prime signaling and metabolic events, such as perturbing membranes and increasing the production of PIP_2, which then activate other PLD isoforms.

An examination of the biochemical properties of PLD sheds light on the question of how the PLD function is related to some of the signaling components previously identified in the ABA-promoted stomatal closing. ABA is known to promote Ca^{2+} oscillations and increases in guard cells (Allen *et al.*, 1999; Hamilton *et al.*, 2000; Leckie *et al.*, 1998; Staxen *et al.*, 1999; Wu *et al.*, 1997); mutation or inhibition of the ABA signaling components, such as protein phosphatase 2C, cADP ribose, or phospholipase C impedes ABA-induced Ca^{2+} increases and impairs stomatal closure. However, little is known about the immediate targets of Ca^{2+}. Ca^{2+} is a regulator of plant PLD; it is required for PLD activity and also promotes PLDα association with membranes (Ryu and Wang, 1996). A recent study has shown that PLDα and β bind Ca^{2+} at their N-terminal C2 domain, and that this binding induces a conformational change which promotes the protein's association with phospholipids (Zheng *et al.*, 2000). ABA has been shown to increase PLD activity in guard cells (Jacob *et al.*, 1999). Thus PLD could be a target of Ca^{2+} changes that are induced by water deficit and increased ABA levels in guard cells.

PLD activation generates the lipid product PA which, when applied to guard cell protoplasts, resulted in an inhibition of the activity of the inward potassium channel (Jacob *et al.*, 1999). PLD-derived PA may mediate cellular effects by activating protein kinases and lipid kinases, as shown in animal systems (Liscovitch *et al.*, 2000; Regier *et al.*, 2000; Rizzo *et al.*, 2000). In particular, PA is a potent stimulator of the phosphatidylinositol 4-phosphate 5-kinase needed for the production of phosphatidylinositol 4,5-bisphosphate (PIP_2). In addition to being an activator of PLDs and the substrate of PLC, PIP_2 also serves as a membrane-attachment site for various proteins with pleckstrin homology domains involved in membrane trafficking (Liscovitch *et al.*, 2000). Increasing evidence has been obtained for the participation of PLD in membrane trafficking and secretion in animal systems, and one way in which PLD does so is through regulating the synthesis of PIP_2. In plants, recent results suggest that stomatal movement may involve active membrane trafficking (reviewed by Blatt, 2000). It would be of great interest to use the PLD-altered plants in future studies to investigate the role of PLD in regulating membrane trafficking during stomatal movements.

Presently, it is unclear how the position of PLD is related to the previously identified enzymes, such as protein phosphatase 2C (Leung *et al.*, 1994); protein kinase (Li *et al.*, 2000); and protein farnesyltransferase (Pei *et al.*, 1998), in ABA signal-transduction pathways. Depletion of PLDα has a similar effect on ABA promoted stomatal closure as the phosphatase 2C, ABA-insensitive mutant *abi-1*. It has been proposed that the phosphatase 2C regulates both a Ca^{2+}-dependent and a Ca^{2+}-independent pathway leading to stomatal closure (Allen *et al.*, 1999). Recent results in animal cells show that PA interacts directly with some protein kinases and protein phosphatases (Liscovitch *et al.*, 2000; Regier *et al.*, 2000; Rizzo *et al.*, 2000). Mammalian PLD is also regulated by protein phosphorylation and dephosphorylation (Kim *et al.*, 2000; Xie *et al.*, 2000). Thus PLD can be a target and regulator of protein kinases and phosphatases, which may form a complex network leading to the change in ionic channel activities and cell volumes in guard cells (Assmann and Shimazaki, 1999; Blatt, 2000; Leung and Giraudat, 1998). Further studies on the mechanism of PLD function may reveal important links in the cascades that mediate plant-stress responses.

Experimental procedures

Transgenic plants and plant growth

The *Agrobacterium tumefaciens* vector pKYLX7 was used for introducing the *PLDα* cDNA into *Arabidopsis thaliana* (Columbia) and tobacco (*Nicotiana tabacum*). The antisense vector was constructed using a 783 bp fragment from the *Arabidopsis PLDα* cDNA (Fan *et al.*, 1997), and the overexpression vector used a 2.8 kb cDNA encoding the full-length castor bean *PLDα* (Wang *et al.*, 1994). Expression of both the antisense and sense cDNAs was under the control of the cauliflower mosaic virus 35S promoter. Plasmids with the inserts were transferred into the *A. tumefaciens* strain EHA105. *Arabidopsis* was transformed with the T-DNA via infiltrating plants with agrobacteria, and tobacco was transformed through leaf-disc inoculation. Overexpression and suppression of *PLDα* were confirmed by assaying PLDα activity and blotting with PLD-isoform-specific antibodies and gene probes according to published procedures (Fan *et al.*, 1999; Pappan *et al.*, 1997). Transgenic lines containing the empty vector only were also produced and used as controls. The ABA-insensitive mutant *abi1-1* was provided by the Ohio State University *Arabidopsis* Resource Center, and its identity was confirmed by its hypersensitivity to water stress and insensitivity to ABA (Figure 2a). Unless stated otherwise, plants were grown in a growth chamber under 12 h light/dark cycles with cool-white fluorescent light of 100 μmol m^{-2} sec^{-1} at 22±1°C and 60% relative humidity.

Immunocytochemical labeling of PLD

After 4–5 weeks' growth, fully expanded *Arabidopsis* leaves were detached. Epidermal peels were collected from the abaxial side of *Arabidopsis* leaves immediately following detachment, and incubated for 1 h in a solution containing 5 mM MES–KOH pH 6.1, 22 mM KCl and 1 mM $CaCl_2$. The peels were then fixed in 1.5% formaldehyde, 0.5% glutaraldehyde, 0.1 M PIPES pH 6.9, 5 mM EGTA, 2 mM $MgCl_2$ and 0.05% Triton X-100 for 35 min with gentle shaking. The fixed peels were washed in phosphate-buffered saline (PBS) for 30 min with three changes of solution. Then they were spread onto microscope slides, blotted to remove excess solution, and freeze-shattered according to a published procedure (Wasteneys *et al.*, 1997). The peels adhered to slides were incubated with an enzyme mixture of 1% cellulase, 1% pectinase and 2% driselase in PBS for 30 min at 37°C, followed by incubating with proteinase K (5 mg ml^{-1}) for 10 min at 37°C. The peels were permeabilized with PBS containing 1% Triton X-100 for 1.5 h, incubated in PBS containing 50 mM glycine for 30 min, and blocked in PBS containing 3% BSA for 30 min Then they were incubated with antibodies to PLDα, β or γ or their respective pre-immune sera at 4°C overnight, followed by incubation for 20 min at room temperature. 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.*, 1997). PLDγ antibodies were raised against a 13-amino-acid peptide near its C-terminus and affinity-purified using *Arabidopsis* PLDγ expressed in *Escherichia coli* (Fan *et al.*, 1999). All antibodies were diluted 1 : 100 in the blocking solution. The slides were rinsed and then incubated for 2 h with a second antibody (1 : 50 dilution) which was conjugated to an alkaline phosphatase (Sigma, St Louis, MO, USA). After rinsing, slides were incubated at room temperature for 20 min with the phosphatase substrate fast red/naphthol which contained 0.6 mM levamisole, to block endogenous AP activity from tissues. The slides were rinsed three times with PBS and sealed for observation and photographing using a microscope.

Stomatal aperture and drought treatments

Detached *Arabidopsis* leaves were floated with the abaxial side downward in a solution containing 5 mM MES–KOH pH 6.1, 22 mM KCl, and 1 mM $CaCl_2$ for 1 h under the same light conditions used for growing plants. Leaves then were incubated

without or with ABA at the indicated concentrations. ABA was made as a 10 mM stock solution in 5% dimethyl sulfoxide (DMSO), and the same amount of DMSO (0.005%) was also added to the control solution in all treatments. Stomatal aperture of detached leaves was measured as diffusion resistance with a steady-state porometer (Feild *et al.*, 1998; Thimann and Satler, 1979). For tobacco plants, leaf diffusion resistance was also measured in leaves attached to approximately 2-month-old plants following foliar spraying of ABA at the indicated concentrations. Changes in diffusion resistance in response to ABA in both detached and intact leaves were monitored at the indicated time intervals. In addition, stomatal aperture was measured directly on epidermal peels using confocal microscopy.

Before drought treatment was imposed, *Arabidopsis* plants were grown in a greenhouse for 6–8 weeks and watered regularly. Soil water content in each pot was adjusted to 24% before drought treatment. Plants were subjected to drought by withholding irrigation. The soil surface in each pot was covered with plastic wrap to minimize evaporation. Soil moisture in the 0–20 cm soil layer was monitored during drought using a time-domain reflectometer. In experiments when ABA (10 μM) was sprayed on plants, control groups of plants were sprayed with water in the same amount as for the ABA treatment. Leaves were collected at various times of drought treatment, and leaf water potential (Ψ_w) was measured with a thermocouple psychrometer.

Tobacco plants were grown in equal amounts of a growth medium containing peat, vermiculite and processed bark ash (Hummert International, Earth City, Missouri, USA). The medium water content for all plants was adjusted to the same level prior to treatments. To measure the rates of water loss from tobacco, leaves of similar size, age and position on PLDα-overexpressing and empty vector-transformed control plants were detached and left in ambient conditions (Tan *et al.*, 1997). Decreases in fresh weight were recorded as a function of time, and the percentages of decreases were expressed as percentage water loss.

PLD activity assay and immunoblotting

Total protein from *Arabidopsis* or tobacco leaves 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 PMSF, 2 mM DTT). The homogenate was centrifuged at 6000 *g* for 10 min at 4°C to remove tissue debris, and the resulting supernatant was used for activity assays and immunoblotting. PLD activity was determined based on procedures described previously (Pappan *et al.*, 1997). Briefly, the PLDα 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 2–10 g protein in a total volume of 200 l. The release of [^{3}H]choline into the aqueous phase was quantified by scintillation counting. For immunoblotting, protein extracts were separated by 8 or 10% SDS–PAGE and transferred onto polyvinylidene difluoride filters. The membranes were blotted with *Arabidopsis* PLD antibodies raised against its C-terminal peptide, followed by incubation with a second antibody conjugated to alkaline phosphatase, according to a published procedure (Fan *et al.*, 1999). The proteins recognized by antibodies were made visible by staining the phosphatase activity with a Bio-Rad (Hercules, CA, USA) immunoblotting kit.

PLD promoter–β-glucuronidase fusion and detection

A 1.2 kb 5′-untranslated region of the castor bean PLDα gene was fused to GUS as described previously (Xu *et al.*, 1997). The promoter–GUS cassette was excised by *Eco*RI and *Hin*dIII digestion, and cloned into a binary agrobacterial transfer vector. The vector was introduced into *A. tumefaciens* and transferred into tobacco using a leaf-disc inoculation method. GUS activity in guard cells was histochemically localized with 1 mM 5-bromo-4-chloro-3-indolyl β-glucuronide staining of epidermal peels of tobacco leaves, as described (Xu *et al.*, 1997).

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