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Phospholipase D δ knock-out mutants are tolerant to severe drought stress

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Abbreviations: PA, phosphatidic acid; PLD, phospholipase D; ROS, reactive oxygen species.

Phospholipase D (PLD) is involved in different plant processes, ranging from responses to abiotic and biotic stress to plant development. Phospholipase Dδ (PLDδ) is activated in dehydration and salt stress, producing the lipid second messenger phosphatidic acid. In this work we show that *pld*δ Arabidopsis mutants were more tolerant to severe drought than wild-type plants. PLDδ has been shown to be required for ABA regulation of stomatal closure of isolated epidermal peels. However, there was no significant difference in stomatal conductance at the whole plant level between wild-type and *pld*δ mutants. Since PLD hydrolyses structural phospholipids, then we looked at membrane integrity. Ion leakage measurements showed that during dehydration of leaf discs *pld*δ mutant has less membrane degradation compared to the wild-type. We further analyzed the mutants and showed that *pld*δ have higher mRNA levels of *RAB18* and *RD29A* compared to wild-type plants under normal growth conditions. Transient expression of AtPLDδ in *Nicotiana benthamiana* plants induced a wilting phenotype. These findings suggest that, in wt plants PLDδ disrupt membranes in severe drought stress and, in the absence of the protein (PLDδ knock-out) might drought-prime the plants, making them more tolerant to severe drought stress. The results are discussed in relation to PLDδ role in guard cell signaling and drought tolerance.

Introduction

Drought is a major stress factor that limits agricultural production worldwide. Plants are sessile and, to survive drought stress, respond via morphological modification and changes in physiological, biochemical, or molecular responses. The first drought response is to prevent water loss, which is mainly achieved by stomatal closure. In the long term, cuticle thickness can increase and the root/shoot growth ratio can be modified to increase water uptake. If the stress becomes severe, plants activate mechanisms to protect protein and membrane structure in order to avoid cell death such as the accumulation of proline, the cell wall hardening and the control of reactive oxygen homeostasis (summarized by[1]).

Phospholipase D (PLD) hydrolyses structural phospholipids to phosphatidic acid (PA) and free head groups.[2] A proposed function for PLD during senescence and wounding is to breakdown membrane lipids, which generates high levels of PA and as a consequence a loss of membrane bilayer phase, leading to loss of cell membrane integrity.[3,4] In addition PLD has been shown to be involved in vesicular trafficking during polar cell-expansion,[5,6] and to be associated with the microtubules.[7] The PA produced by PLD has been proposed to be a membranous second messenger molecule.[8] PLD activity has been shown to increase under water deficit and hyperosmotic conditions.[9,10,11] Under drought stress in cowpea and peanut plants, the activity and transcript levels of PLD increased faster in cultivars that are drought-sensitive compared to cultivars that are drought tolerant.[12,13] Furthermore, PLD has been suggested to be a negative regulator of the biosynthesis of the osmolite proline in Arabidopsis.[14]

Genetically modified plants have been used to address the role of some PLDs during a number of stress responses, demonstrating that there is a low functional redundancy. Arabidopsis has 12 PLD genes grouped into 6 types, PLDα (3), β (2), γ (3), δ, ε and ζ(2), based on sequence similarity and requirements for the *in vitro* activity such as Ca^{2+}, polyphosphoinositides and free fatty acids.[15] They have distinct patterns of temporal and spatial expression, and can be associated to membranes of different cell compartments.[16] PLDα and δ classes have been linked to high salinity and water-deficit stress as well as to the stress hormone abscisic acid (ABA).[4,11,17] ABA regulates many aspects of plant development during environmental stresses, allowing the plant to cope and survive in adverse conditions, such as drought, low or high temperature, or high salinity.[18]

Particularly, PLDδ is activated in response to H_2O_2,[19] dehydration,[11] freezing,[20] and salinity stress.[4] It is associated with the plasma membrane[2] and has been suggested to be the

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microtubule-binding PLD in Arabidopsis.[21] PLDδ mRNA levels of senescent tissue are higher than in young tissues, and the expression increases in response to severe dehydration and high salt concentrations.[11] *AtPLD*δ silencing indicated that the AtPLDδ isoform partially contributes to the dehydration induced PLD activity.[11] Cold-induced freezing tolerance is impaired in *Atpld*δ T–DNA knockout Arabidopsis plants and enhanced in *AtPLD*δ-overexpressing plants, which also display decreased and increased freezing-induced PA production, respectively.[20] AtPLDδ is also required for ABA-induced stomatal closure. Our recent report shows that PLDδ is downstream of H_2O_2 and NO during ABA-induced stomatal closure.[22] In order to gain insight into the role of PLDδ, we studied the response of PLDδ knockout mutants to drought stress.

Results

PLDδ knockout mutants are tolerant to severe drought stress

Previously we showed that in isolated epidermal peels of PLDδ knock-out plants (*pld*δ) stomata do not close in response to ABA treatment.[22] Therefore, we analyzed the *pld*δ mutant's response to drought treatment. Four-week-old wild-type and mutant plants, watered for 3 days with water or 20 μM ABA, were subjected to drought by withholding water. Sensitivity to drought was evaluated by the observation of the stress symptoms and by measuring the leaf relative water content (RWC) 12 days after the last irrigation. **Figure 1** (A) shows images of representative wild-type and mutant plants after 12 days of water stress, where ABA treated wild-type plants were clearly not as wilted as non-treated plants. The wilting symptoms of each treatment were reflected in the RWC values that were 28% and 50% for non-treated or ABA treated plants, respectively (**Fig. 1(A)**). Interestingly, non-treated *pld*δ*-1* and *pld*δ*-3* mutant plants were turgid and showed a higher RWC than wild-type (55% and 57% of RWC, respectively) (**Fig. 1(A)**). To ensure that the observed phenotype was not due to differences in soil water content, the experiment was performed with wild-type and *pld*δ plants grown in the same pot. **Figure 1(B)** shows that both *pld*δ mutants are more tolerant to drought than wild-type plants.

Since *pld*δ mutants do not close the stomata in response to ABA,[22] we analyzed the stomatal behavior at the whole plant level. Wild-type and *pld*δ plants were sprayed with water or 20 μM ABA and the stomatal conductance was measured after 3 hours using an infrared gas analyzer (IRGA). **Figure 2** shows no differences between water sprayed wild-type and *pld*δ mutants. In addition, ABA treatment induced a significant reduction of stomatal conductance in both wild-type and *pld*δ mutants plants (**Fig. 2**).

Analyses of parameters that confers drought tolerance in PLDδ knockout plants

We evaluated a number of parameters in order to explain the increased tolerance. First, we analyzed stomatal index (number of guard cells/number of epidermal cells including guard cells) in *pld*δ mutant plants. No differences were found compared to wild-type (data not shown). Cuticle thickness and proline accumulation under normal growth conditions did not show differences between wild-type and mutants plants (data not shown).

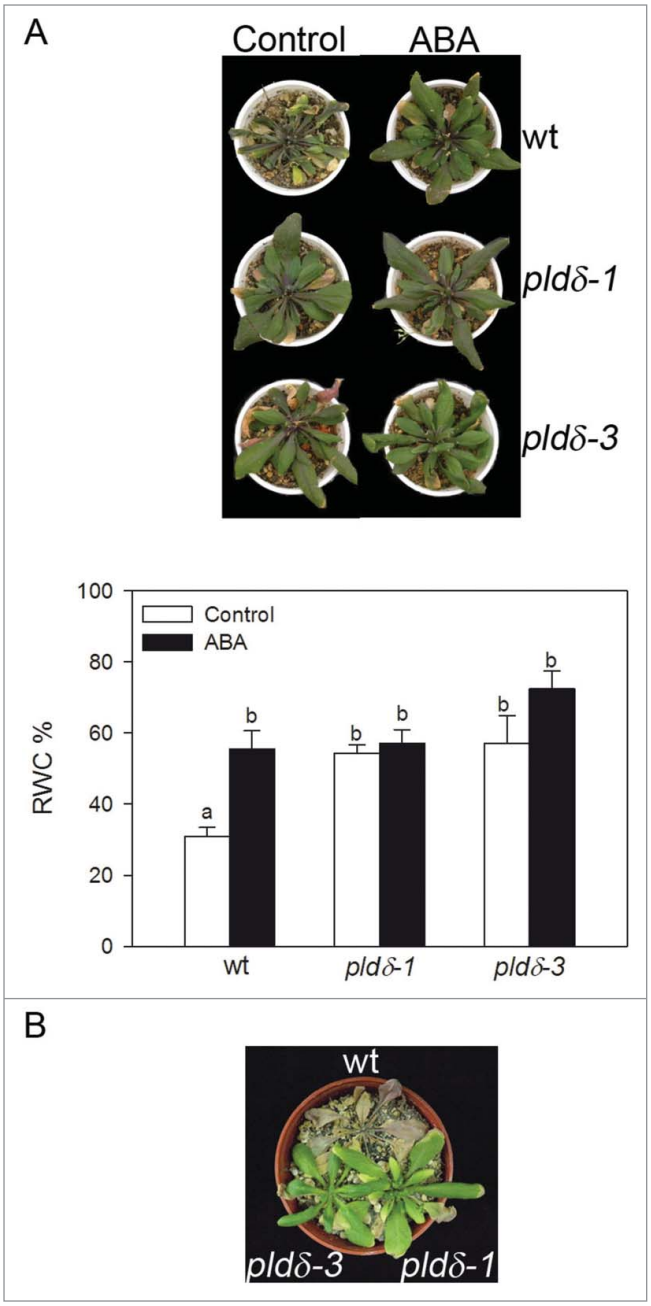

Figure 1. Drought tolerance. Four week old wild-type (wt) and PLDδ knock-out (*pld*δ*-1* and *pld*δ*-3*) Arabidopsis plants were watered for 3 days with 20 μM ABA or water (control) and subsequently subjected to drought by withholding water. (**A**) Upper panel shows the phenotype of representative plants. The lower panel shows the relative water content (RWC) after 12 days of withholding water. Values are expressed as percentages and represent the mean of 3 independent experiments. Error bars correspond to SE and letters denote statistical difference (One way ANOVA on ranks test, $P < 0.05$). (**B**) A representative picture showing the phenotype of wild-type and mutant plants grown in the same pot ($n = 3$).

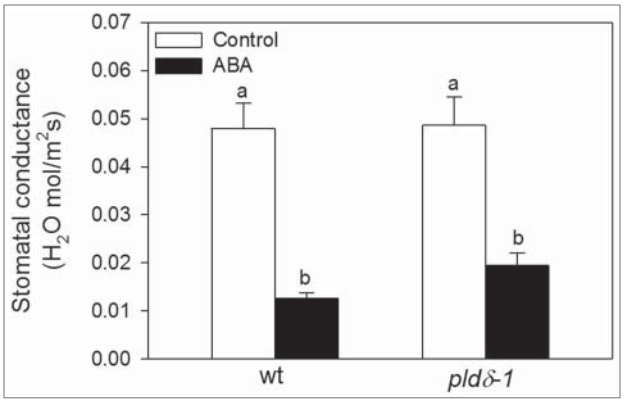

Figure 2. Stomatal conductance upon ABA treatment. Stomatal conductance of 4-week old wild-type (wt) and PLDδ knockout (*pld*δ*-1)* plants, treated with 20 μM ABA or water (control) for 3 h, was determined with an exchange gas system. Values represent the mean of 3 independent experiments. Error bars correspond to SE and letters denote statistical difference (One way ANOVA on ranks test, $P < 0.05$).

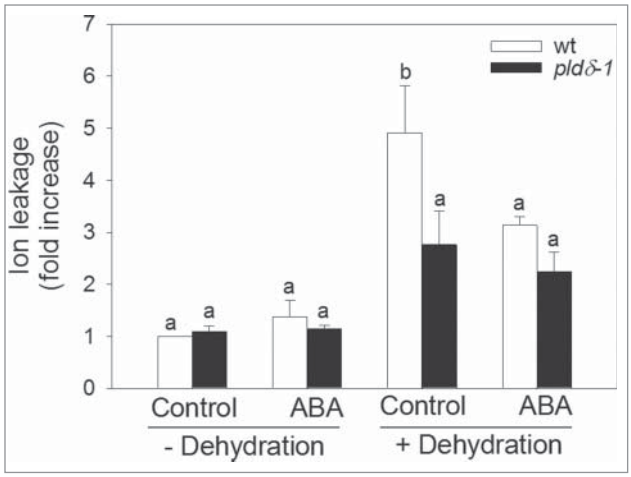

Figure 3. Membrane integrity upon dehydration stress. Leaf discs of 4-week old wild-type (wt) and PLDδ knock-out (*pld*δ*-1*) plants were incubated in buffer over night and treated with 20 μM ABA or buffer (Control) for 2.5 h. Then, they were submitted to 1 h of dehydration stress. Ion leakage is expressed as fold increase with respect to the untreated wild-type control. Values represent the mean of 3 independent experiments. Error bars correspond to SE and letters denote statistical difference (One way ANOVA on ranks test, $P < 0.05$).

Finally we analyzed the expression of the rate-limiting ABA biosynthetic genes NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) and ALDEHYDE OXIDASE 3 (AAO3), as indicators of ABA levels. Real time PCR analysis of these 2 marker genes showed no differences between wt and the *pld*δ mutant plants (**Suplemental Fig. 1**).

During drought stress, membranes are subject to degradative processes, and there is an increment on lipolytic and peroxidative activities.[12,23,24,25] Based on this, we hypothesized that the loss of PLDδ activity involves a reduction in membrane degradation upon drought stress, being the reason for the enhanced drought tolerance of the mutant plants. To test this hypothesis, we measured ion leakage as an indicator of membrane integrity. Thus, *Arabidopsis* leaf disc of wild-type or *pld*δ plants were pre-treated with water or 20 μM ABA, submitted to dehydration stress and then ion leakage was measured. **Figure 3** showed that the increase of the ion leakage upon the dehydration stress was significantly higher in wild-type plants than in *pld*δ plants. Moreover, the drastic ion leakage observed in wild-type plants was partially prevented by ABA treatment. In accordance to the whole plant symptoms observed in **Figure 1**, there was no differences in the ion leakage of control or ABA treated dehydrated *pld*δ plants and in both cases the ion leakage was as low as ABA treated wild-type plants.

Transcript levels of *RAB18* and *RD29A,* which are widely used as genes markers of drought response, were analyzed by means of qPCR. Interestingly, *RAB18* and *RD29A* transcript levels were higher in *pld*δ than in wild-type plants, when grown under normal conditions (**Fig. 4**).

Transient expression of AtPLDδ induces wilting

The role of PLDδ was further studied by transient expression of *AtPLD*δ in *Nicotiana benthamiana* plants. After 4 days of expression and simultaneous water withhold, transgenic leaves show strong wilting symptoms (**Fig. 5(A)**). RT-PCR analysis confirmed the presence of *AtPLD*δ transcripts in agroinfiltrated leaves while no amplification product was detected in the leaves agroinfiltrated with the empty vector (**Fig. 5(B)**). The AtPLDδ transgenic leaf also had an increased PLD activity with respect to the control (**Fig. 5(C)**).

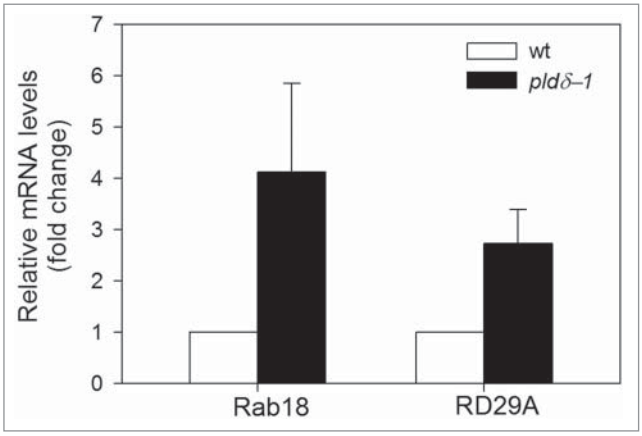

Figure 4. *RD29A and Rab18* mRNA levels. Total RNA was extracted from leaves of 4-week old wild-type (wt) and PLDδ knockout (*pld*δ*-1*) plants. The mRNA was analyzed by qPCR. The transcript levels of *RD29A* and *Rab18* for every sample were normalized against the transcript level of actin. *mRNA* levels in the mutant are expressed as a fold-change with respect to the wild-type. Bars show the quantification of 3 independent experiments. Error bars correspond to SE.

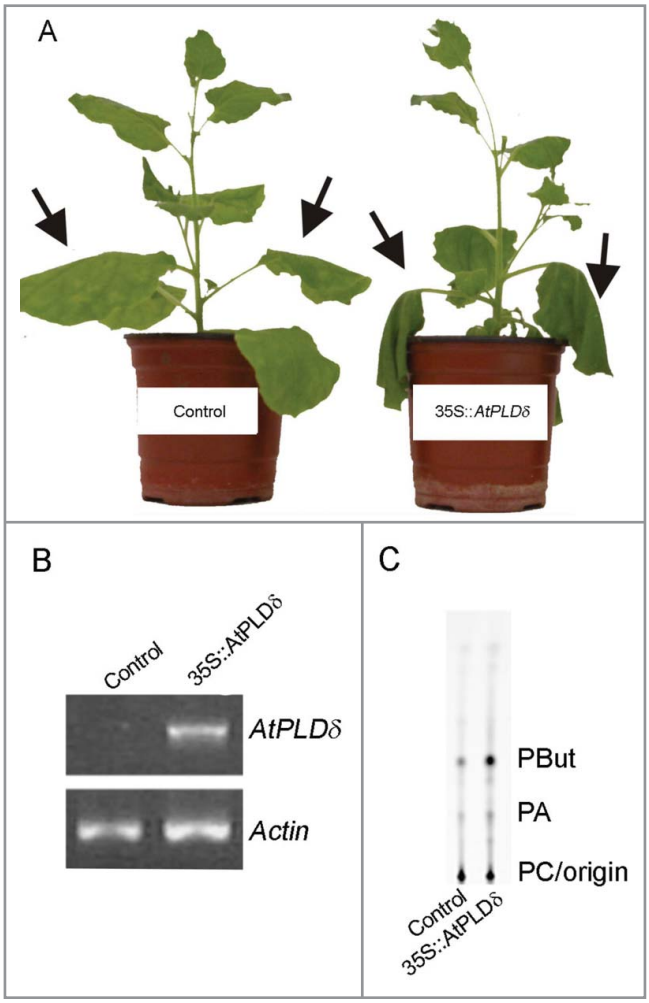

Figure 5. Transient expression of *AtPLD*δ in *Nicotiana benthamiana*. Five weeks-old *N. benthamiana* plants were agroinfiltrated with *Agrobacterium tumefaciens* harbouring a vector containing 35S: *AtPLD*δ or the empty vector (control). Thereafter water was withheld for 4 days. (**A**) Phenotype of representative plants ($n = 3$). The arrows indicate the agroinfiltrated leaves. (**B**) At*PLD*δ expression analyzed by RT-PCR 3 days after *Agrobacterium* infiltration (picture showed different parts of the same gel). (**C**) Protein extracts from leaves 3 days after *Agrobacterium* infiltration were assayed for in vitro δ-class PLD activity. The transfosfatidylation of BODIPY-phosphatidilcholine (PC) to BODYPY-phosphatidylbutanol (PBut) was visualized by separation of the lipids on a TLC plate.

Discussion

At an early stage of drought, the control of transpirational water loss through stomata is a major factor in water balance. When drought stress persists it leads to a massive breakdown of membrane lipids, the degradation being more severe in drought sensitive plants.[26] In this report, we show that *pld*δ plants are tolerant to drought stress. After severe stress conditions, the wild-type plants are completely wilted showing a characteristic brown coloration of a dry phenotype, while *pld*δ mutant plants are still green. Since all plants were grown in the same pot, we ruled out any effect of the soil composition. Guo and co-workers[17] recently reported that under mild drought stress conditions *pld*δ mutant plants had higher stomatal conductance, higher cumulative water transpiration, and higher photosynthetic rate than the wild-type. However, at severe water deficit the mutants accumulated more dry weight than the wild-type, suggesting a decreased growth inhibition of the mutant, but the stomatal conductance, the cumulative water transpiration and the photosynthetic rate are the same as the wild-type. They also reported that *pld*δ mutant plants wilted faster than the wild-type, however, at the end of their drought period both wild-type and mutant plants still look green.[17] This discrepancy can be explained by differences in drought conditions.

We have previously shown that, in isolated epidermal peels, *pld*δ mutants do not close the stomata in response to ABA, NO or H_2O_2, however the ABA-dependent accumulation of NO or H_2O_2 was not affected, indicating that PLDδ has a role in guard cell signaling and is downstream of ABA, NO and H_2O_2.[22] Here we present data of stomatal conductance measurements showing that *in planta pld*δ mutant plants response to ABA was only marginally affected. The differenes found between stomatal responses in isolated epidermal peels compared to intact leaves could result from the presence of the mesophyll in intact leaves.[27,28,29] In addition, wild-type and mutant plants showed the same stomatal index and stomatal density (data not shown). Thus, the drought tolerant phenotype of *pld*δ mutants cannot be explained by means of water balance through stomatal pore width regulation.

We analyzed a number of parameters that might explain the drought tolerant phenotype. Previously, it has been described that PLDs are negative regulators of the biosynthesis of proline, a solute that accumulates during drought stress and functions as an osmoprotectant.[14] Wild-type and *pld*δ plants have similar levels of proline and transcripts of the enzymes involved in key steps on proline biosynthesis (data not shown). Other phenotypic changes that could confer drought tolerance such as primary root growth and cuticle thickness are not different in *pld*δ mutants compared to wild-type plants (data not shown). The drought tolerant phenotype could be explained due to a higher content of ABA in the mutants compared to the wild-type. Thus, we measured the expression of the rate-limiting ABA biosynthetic genes NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) and ALDEHYDE OXIDASE 3 (AAO3), as indicators of ABA levels. Again, no differences were found between *pld*δ mutants and wild-type, suggesting that the ABA levels are not affected in the mutants. Other data supporting this evidence is the fact that no differences were found on: germination, number of lateral roots, growth of primary root, growth rate and development throughout the life cycle and stomata aperture measured as transpiration rate with an infrared gas exchange analyzer.

Some reports showed that drought tolerant plants have high levels of *RD29A* and *RAB18,* which are used as drought response markers.[12,30,31] Non-stressed *pld*δ plants have *RD29A* and *RAB18* transcript levels 3 to 4 times as high as found in the wild-type, which correlates with the observed tolerant phenotype. The fact that *pld*δ mutant plants presented higher mRNA levels of these genes suggests that they are primed, and therefore, more resistant to stress. Arabidopsis plants subjected to cycles of

dehydration/water recovery treatments maintained higher relative water content than plants experiencing dehydration stress for the first time.[32] During recurring dehydration stresses, Arabidopsis plants display transcriptional stress memory, showing that dehydration stress 'memory' also affected gene expression. *RD29A*, *RAB18* or *PLD*δ were not within the memory stress genes.[32] However, we could not discard that the *pld*δ mutant is primed.

PLDs might play important roles in the major membrane-rearrangement and -remodelling events that occurred during osmotic stress. PA has a conical shape compared to the cylindrical shape of structural phospholipids. Therefore a high PA content induces loss of the lipid bilayer phase and incites the formation of hexagonal-phase lipid particles.[33] This process would cause loss of cell-membrane integrity and thus a loss of cell viability.[8] Dehydration stimulates PA accumulation which is significantly suppressed in *PLD*δ silenced and knock-out plants.[4,11,12] Jia and coworkers[34] showed that AtPLDδ knockout plants display retarded ABA-induced senescence, by suppression of membrane lipid degradation. We showed that *pld*δ plants have less ion leakage upon dehydration stress, and that the transient expression of AtPLDδ in *Nicotiana benthamiana* provokes a wilting phenotype. Thus, the drought tolerant phenotype of *pld*δ could be due to increased membrane stability. Another PLD proposed to be related with membrane remodelling and metabolism of lipids is the PLDα1. Hong and co-workers[35] reported that tobacco plants overexpressing AtPLDα1 were more susceptible to drought than the control. They also showed this was due to membrane degradation. Yet, in the early stage of drought PLDα1-overexpressing plants decreased transpiration water loss. The authors hypothesized that PLDα1 has different effects on plant response to water deficits depending on the timing of the stress. As it was proposed for PLDα1, PLDδ would be involved in the promotion of stomatal closure at early stages of drought, but would disrupt membranes in prolonged drought stress. However, antisense *pldα1* plants experienced severe water stress compared to wild-type plants.[36,35] Interestingly, these plants presented high level of *PLD*δ gene expression under water deficit.[37] Altogether, our results suggests that PLDδ mediates early events in signaling in stomatal response to ABA and in severe drought has catabolic function impacting negatively on drought tolerance, showing that these enzymes have a dual role in drought response.

Material and Methods

Plant material and growth conditions

The knock-out phospholipase D mutants were isolated from *Arabidopsis thaliana* Columbia-0 (wild-type) ecotype. PLDδ knockout T-DNA insertion line SALK_023247(*pld*δ*-1*), was kindly provided by Dr. Munnik. An independent Col-0 T-DNA insertion line for *pld*δ, SALK_092469 *(pld*δ*-3*), was obtained from the Ohio State University Arabidopsis Biological Resources Center (ABRC). Homozygosity of the T-DNA insertions was verified by PCR, and confirmed by RT-PCR. Seeds were germinated in soil (soil:vermiculite:perlite (1:1:1)) and kept at 4°C for 2 d. Then, they were grown at 25°C using a 16-h photoperiod. Four-weeks-old plants were used for the experiments.

For *AtPLD*δ transient expression assays, 5 weeks-old *Nicotiana benthamiana* plants were used.

Stomatal conductance and relative water content measurements

For stomatal conductance assays, 4-week-old plants were sprayed with water (control) or 20 μM ABA. After 3 h of treatment, the leaf gas-exchange was measured *in planta* using a S151 infrared gas analyzer (IRGA; Qubit System, Kingston, Canada); the leaf temperature was measured using a S171 Leaf Chamber Thermistor (QUBIT, Kingston, Canada) according to the manufacturer's instructions.

For determination of relative water content (RWC), 4-week-old plants were watered for 3 days with water (control) or 20 μM ABA, and then subjected to drought by withholding water. RWC was determined according to the equation: ((FW-DW)/(TW-DW))*100. Fresh weight (FW) was determined at the end of the drought period. Then, turgor weight (TW) was determined by subjecting leaves to rehydration for 2 h. Finally, dry weight (DW) was determined after drying the samples at 75°C.

Ion leakage

Leaf discs from 4-week-old plants were incubated in buffer 10 mMKCl, 10 mM MES, pH 6.1, overnight. Then, they were treated with ABA or buffer for 2.5 h and then placed in a filter paper for 1 h, afterwards, they were placed in Petri dishes with deionized water at room temperature for 2 h. The conductivity was determined (Xi) with an HI8733 conductivity meter (Hanna Instruments, Sigma). Then, the discs were heated at 80°C for 2 h and conductivity was determined (Xt). Ion leakage was expressed as a percentage of the total conductivity after heating at 80°C [(Xi/Xt) × 100′.

RNA isolation and qRT-PCR

Total RNA was extracted by Trizol method as described by the manufacturer (Invitrogen, NY, USA). cDNA (cDNA) was synthesized on 1 μg of total RNA by MMLV reverse transcriptase (RT) from Promega (Madison, USA) using oligo-dT primer and used for quantitative PCR (qPCR). The Fast Universal SYBR Green Master mix from Roche (Mannheim, Germany) was employed, using a Step-one Real-time PCR machine from Applied Biosystems (California, USA). The expression levels of the gene of interest were normalized to that of the constitutive ACT2 gene by subtracting the cycle threshold value of ACT2 from the CT value of the gene (ΔCT). The gene expression level was calculated as 2-(ΔCT).[38] Primer sequences: RD29A (AT5G52310) Fw:5′-GAATGGTGCGACTAAGATGTTTAG-GAA-3′ and Rv: 5′-GCTCATGCTCATTGCTTTGT-3′. Rab18 (AT5G66400) Fw: 5′-GAACATGGCGTCTTAC-CAGA-3′ and Rv: 5′-ATCGGATTTCCGTACTCGTC-3′. ACT2 (AT3G18780) Fw ′CTATGATGCACTTGTGTGTGA-3′ and Rv: 5′-ATCAATTCGATCACTCAGAGC-3′. NCED3

(AT3G14440) Fw: 5′-CGGTGGTTTACGACAAGAACAA-3′ and Rv: 5′-CAGAAGCAATCTGGAGCATCAA-3′. AAO3 Fw: 5′GGAGTCAGCGAGGTGGGAAGT-3′ and Rv: 5′-TGCTCCTTCGGTCTGTCCTAA-3′. Stepone Software v2.1 (Applied Biosystems) was used to analyze the transcript levels of the analyzed genes.

AtPLDδ transient expression in Nicotiana benthamiana

All DNA manipulations were performed by standard protocols.[39] PCR was performed with Pfx polymerase (Invitrogen). Restriction enzymes (Promega) and T4 ligase (Invitrogen) were used according to the manufacturer's instructions. The authenticity of cloned PCR fragment was confirmed by sequencing.

The binary vector pMOG-AtPLDδ was constructed as follows: the ORF of the *AtPLD*δ gene was amplified from the clone U14432 provided by TAIR (The Arabidopsis Information Resource), with primers GTAAACATGTTTCTACACGGTGACCTC and TCACCTGCAGTGGTTAAAGTGTCAGG (*Psc*I and *Pst*I sites are underlined, respectively). The PCR product was digested with *Psc*I and *Pst*I and cloned into pAT1 between 35S CaMV promoter and PI-II terminator, previously digested with *Nco*I and *Pst*I, generating pAT1-PLDδ. The promoter-ORF-terminator cassette of pAT1-PLDδ was subsequently transferred to pMOG800 with *XbaI* and *Sma*I restriction sites, creating the binary plasmid pMOG-AtPLDδ.

Agrobacterium tumefaciens-mediated transient expression was carried out as described in Van der Hoorn et al.[40]

At*PLD*δ overexpression was analyzed by RT-PCR. Total RNA from leaves of *N. benthamiana* transformed plants was isolated with Trizol reagent. cDNA was synthesized from 2 μg of total RNA in a total volume of 20 μL using MLV REVERSE TRANSCRIPTASE (Invitrogen) according to the manufacturer's instructions; 2 μl of cDNA was used in subsequent RT-PCRs. The primers used were D-BamDown (5′-CCGTTCTATCGACTCAGGGTCCGTGAAAGGA-3′) and D-Stop (5′-TCACCTGCAGTGGTTAAAGTGTCAGG-3′). Optimized PCR conditions were 94°C for 5 min, 38 cycles of 94°C for 30 s, 45°C for 50 s, and 72°C for 1 min, followed by a final extension of 72°C for 10 min. Actin was used as an internal standard with the primers actin Down (5′-ATCCACAT(tc)TG(ct)TGGAA-3′) and actin Up (5′-TGGGA(tc)GA(tc)ATGGA(ga)AA(ga)AT-3′). PCR products were analyzed by electrophoresis of 10-μl aliquots of reactions on 0.7% agarose gels in Tris/Borate/EDTA buffer and visualized with SYBRSafe - DNA Gel Stain (Invitrogen).

AtPLDδexpression was analyzed by*in vitro* PLD activity assays. PLDδ activity was assayed using the protocol published by Wang and Wang, 2001.[2] Briefly, 10 μg of total protein extracted from leaves of *N. benthamiana* transformed plants was incubated with 50 μM BODIPY-PC as a substrate in a buffer containing 100 mM MES pH 6.8, 1 mM $MgCl_2$, 80 mMKCl, 200 μM $CaCl_2$, and 1% (v/v) n-butanol, for 30 minutes at 30°C. Lipids were extracted as described in Distéfano et al., 2008 [41] and separated by ethyl acetate TLC. BODIPY-lipids were visualized by fluoroimaging.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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