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The plasma membrane–bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*

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Freezing injury is a major environmental limitation on the productivity and geographical distribution of plants. Here we show that freezing tolerance can be manipulated in *Arabidopsis thaliana* by genetic alteration of the gene encoding phospholipase Dδ (PLDδ), which is involved in membrane lipid hydrolysis and cell signaling. Genetic knockout of the plasma membrane–associated PLDδ rendered *A. thaliana* plants more sensitive to freezing, whereas overexpression of PLDδ increased freezing tolerance. Lipid profiling revealed that PLDδ contributed approximately 20% of the phosphatidic acid produced in wild-type plants during freezing, and overexpression of PLDδ increased the production of phosphatidic acid species. The PLDδ alterations did not affect the expression of the cold-regulated genes COR47 or COR78 or alter cold-induced increases in proline or soluble sugars, suggesting that the PLD pathway is a unique determinant of the response to freezing and may present opportunities for improving plant freezing tolerance.

Freezing injury is one of the major environmental factors that limit plant growth, productivity and geographic distribution. In recent years, great progress has been made toward identifying genes involved in cold acclimation and freezing tolerance. This is exemplified by identification of the dehydration and cold response (DREB1) gene family as key transcriptional activators, the associated downstream cold-regulated (COR) genes and the upstream regulators of the DREB1 family[1–6]. Characterization of the *A. thaliana* mutation, eskimo1, suggests that increased freezing tolerance can also be acquired without altering the expression of COR genes[7]. Expression profiling indicates that the DREB1 activators do not regulate a majority of cold-inducible genes[8,9]. Freezing tolerance appears to be mediated by multiple pathways.

Research on the causes of freezing injury and tolerance has not kept pace with the identification of cold-regulated genes[1,2,10]. The cellular and metabolic function of most of the identified cold-regulated genes is unknown. Knowledge of the signaling processes leading to freezing injury and tolerance is even more scant. Changes in membrane phospholipid metabolism have been implicated in signaling cold responses in plants. Such alterations may regulate the cellular homeostasis of Ca^{2+}, which has been suggested to be involved in activating the cold acclimation process[11,12]. In *A. thaliana* suspension cells, cold exposure promotes rapid formation of phosphatidic acid. The phosphatidic acid increase has been attributed to the cold-induced activation of phospholipases C and D[13].

PLD hydrolyzes membrane phospholipids to produce phosphatidic acid. Recently, we showed that genetic suppression of the most abundant plant PLD, PLDα1, rendered *A. thaliana* plants more tolerant to freezing[14]. PLDα1-deficient plants displayed a decrease in freezing-induced hydrolysis of phosphatidylcholine with a concomitant decrease in the level of phosphatidic acid[14]. Phosphatidylcholine is a bilayer-stabilizing lipid, whereas phosphatidic acid has a tendency to form the non-bilayer hexagonal II phase in the presence of calcium[15,16]. The propensity of cellular membranes to form the hexagonal phase has been suggested to be a key event in freezing injury. The freezing-induced production of phosphatidic acid from PLDα1-catalyzed hydrolysis of phosphatidylcholine may contribute to freezing injury[14].

Besides lipid degradation, PLD and phosphatidic acid are involved in many cellular processes, including cell signaling, vesicular trafficking and membrane remodeling. *A. thaliana* has 12 genes encoding PLDs, which are grouped into five classes, *PLD*α, β, γ, δ and ζ[17,18]. The recently identified PLDδ has several properties that distinguish it from other PLDs. It is activated by oleic acid and associated with the plasma membrane[19] and microtubule cytoskeleton[20]. The expression of *PLD*δ increases in response to severe dehydration and high salts[21]. PLDδ is activated by the reactive oxygen species H_2O_2 in *A. thaliana*, and this activation enhances resistance to H_2O_2-induced cell death[22]. These properties raise the possibility that *PLD*δ may have an important function in cellular response to stress. In this study, we show that abrogation of *PLD*δ decreases *A. thaliana* freezing tolerance, whereas overexpression increases freezing tolerance.

RESULTS

Knockout of *PLD*δ decreases plant freezing tolerance

A *PLD*δ mutant was identified by screening lines of *A. thaliana* with T-DNA inserts from *Agrobacterium tumefaciens*. The T-DNA was inserted near the beginning of the 5′-coding region, 63 nucleotides downstream of the initiation codon (**Fig. 1a**). Homozygous knockout plants had no detectable PLDδ protein (**Fig. 1b**). Disruption of *PLD*δ gene function was further confirmed by the lack of PLDδ-specific, oleate-activated PLD activity in knockout plants[22]. The knockout of

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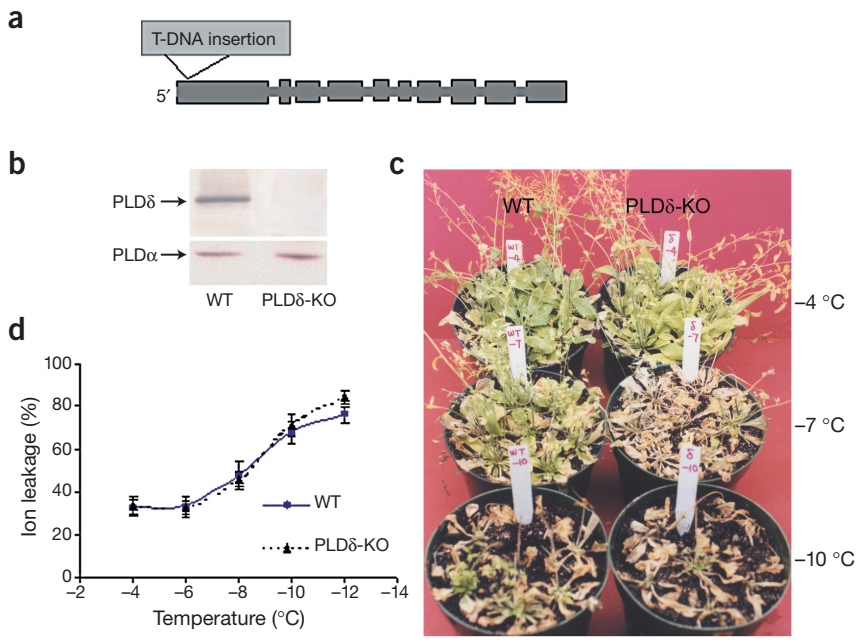

Figure 1 Decreased freezing tolerance in *PLD*δ-KO *A. thaliana*. (**a**) *PLD*δ gene structure and the site of the T-DNA insertion. (**b**) Immunoblotting of PLDδ and PLDα protein in *PLD*δ-KO and wild-type plants. Proteins from *A. thaliana* leaves were separated by SDS-PAGE and transferred to PVDF membranes followed by blotting with PLDδ-specific or PLDα-specific antibodies. PLD bands were visualized using alkaline phosphatase that was conjugated to the second antibody. (**c**) Response of *PLD*δ-KO and wild-type plants to freezing. Soil-grown adult plants were cold-acclimated at 4 °C for 3 d before freezing. The freezing temperature was lowered gradually (1 °C per h), and plants stayed at the final temperature for 2 h. They were thawed at 4 °C overnight and then grown at 23 °C for 2 weeks. (**d**) Ion leakage after freezing at the indicated temperature and thawing at 4 °C overnight.

*PLD*δ did not alter expression of other *PLD*s, as indicated by the presence of PLDα (**Fig. 1b**) and transcripts of several other *PLD*s (data not shown). Under normal growth conditions, the *PLD*δ-knockout (*PLD*δ-KO) plants are indistinguishable from wild-type plants in terms of plant height, number of leaves, days required for flowering and seed maturation, and seed yield.

*PLD*δ-KO plants displayed increased sensitivity to freezing (**Fig. 1c**). After soil-grown adult plants were cold-acclimated for 3 d, exposure to –7 °C killed virtually all *PLD*δ-KO plants; most wild-type plants survived. Ionic leakage of leaves increased after exposure to –8 °C; however, there was no difference between *PLD*δ-KO and wild-type plants (**Fig. 1d**). *PLD*δ-KO and wild-type plants were also grown on the same agar plates and tested for freezing tolerance (**Fig. 2a**). Almost all *PLD*δ-KO seedlings were killed after 1 h at –12 °C, whereas about 50% of wild-type plants survived the treatment. In addition, the growth of *PLD*δ-KO plants after exposure to sublethal temperatures was retarded more than that of wild-type plants (**Fig. 2a**). Without cold acclimation, however, *PLD*δ-KO and wild-type plants behaved similarly in terms of freezing tolerance (**Fig. 2a,b**).

To confirm that the loss of *PLD*δ function is responsible for the decreased freezing tolerance, we genetically complemented the *PLD*δ–KO mutant by introducing the wild-type *PLD*δ gene. The complemented plants expressed PLDδ protein at a level comparable to that of wild-type plants[22]. Complementation restored the freezing sensitivity of the mutant to the same level as that of wild-type plants (**Fig. 2c**). In this experiment, plants were cold-acclimated at 4 °C for 7 d, instead of 3 d as in most of the study, to test whether *PLD*δ function was affected by the duration of cold acclimation. The longer cold-hardening enhanced *A. thaliana* freezing tolerance, as more than 80% of the wild-type and *PLD*δ-complemented plants survived the –12 °C assault. Still, exposure to –12 °C killed more than 60% of the *PLD*δ-KO plants.

Overexpression of *PLD*δ increases freezing tolerance

To further determine the role of *PLD*δ in freezing tolerance, we increased *PLD*δ expression in *A. thaliana* by placing the full-length *PLD*δ cDNA under the control of the 35S cauliflower mosaic virus

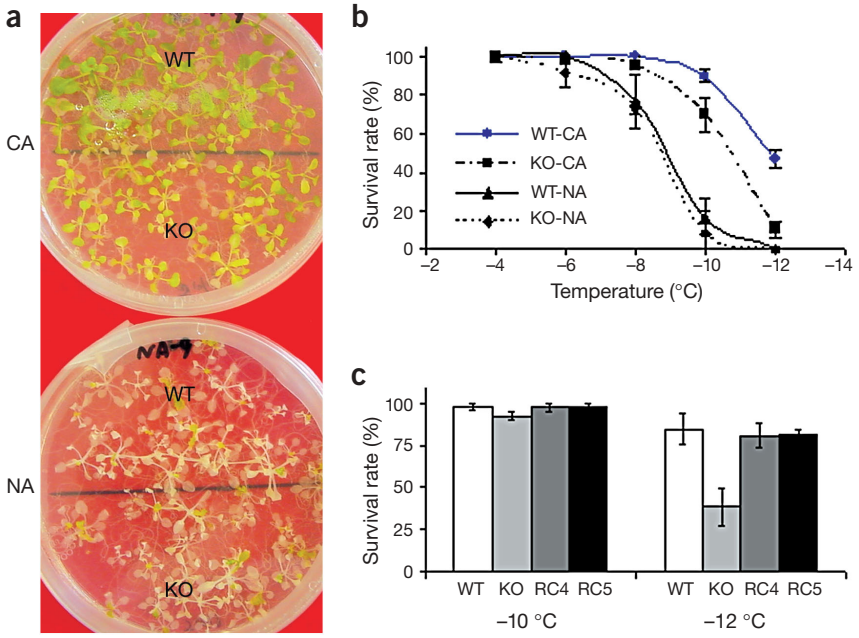

Figure 2 Increased freezing sensitivity of cold-acclimated *PLD*δ-KO seedlings of *A. thaliana*. (**a**) Phenotype of cold-acclimated (CA) and nonacclimated (NA) *PLD*δ-KO and wild-type seedlings after freezing at –10 °C for 2 h, followed by growth at 23 °C for 10 d. (**b**) Survival rate of cold-acclimated (4 °C for 2 d) and nonacclimated *PLD*δ-KO and wild-type seedlings after freezing and growth at 23 °C. (**c**) Introduction of the wild-type *PLD*δ into *PLD*δ-KO restored the freezing survival rate to the wild-type level. Plants were cold-acclimated at 4 °C for 7 d, subjected to freezing at –10 or –12 °C, and allowed to grow at 23 °C for 10 d. Two complementation lines (RC4-1 and RC5-2) were tested in four independent experiments.

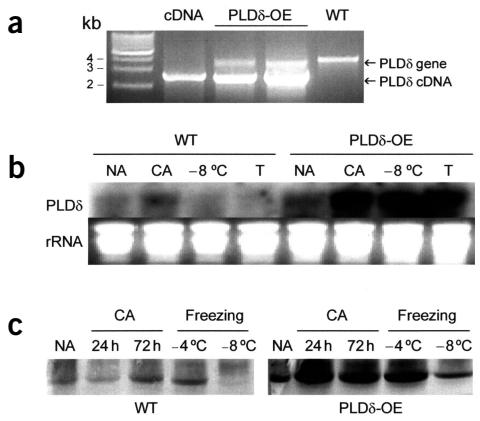

Figure 3 Overexpression of *PLD*δ (*PLD*δ-OE) and the effect of cold acclimation and freezing on the *PLD*δ expression in *A. thaliana*. (**a**) PCR detection of the *PLD*δ cDNA transforming *A. thaliana* lines. *PLD*δ-OE and wild-type plants also contain the 4.1 kb *PLD*δ gene. (**b**) RNA blotting of PLDδ mRNA in *PLD*δ-OE and wild-type *A. thaliana* during cold acclimation, freezing and thawing. Each lane was loaded with an equal amount of total RNA (15 μg/lane) isolated from rosettes of wild-type and *PLD*δ-OE plants with no acclimation (NA), after cold-acclimation for 2 d (CA), after freezing at –8 °C and after thawing (T) at 4 °C for 4 h. (**c**) Immunoblotting of PLDδ with a PLDδ-specific antibody. Each lane was loaded with 15 μg total protein isolated from wild-type and *PLD*δ-OE plants during cold acclimation at the indicated times, followed by freezing at the indicated temperatures.

promoter. The introduced *PLD*δ cDNA was detectable in the transformed plants (**Fig. 3a**); one line was characterized in detail. The increased expression of *PLD*δ was shown by detection of greater levels of *PLD*δ mRNA and protein in the transgenic plants than in wild-type plants (**Fig. 3b,c**). The level of *PLD*δ mRNA increased further in *PLD*δ-overexpressing (*PLD*δ-OE) plants during cold acclimation. Immunoblotting with PLDδ-specific antibody showed that the increased transcript level was accompanied by an increased protein level of PLDδ in the *PLD*δ-OE plants (**Fig. 3c**).

When subjected to cold acclimation, freezing and a 10- to 14-d recovery period, *PLD*δ-OE plants grew better and were more tolerant of freezing damage than wild-type plants. Exposure of *A. thaliana* to –10 °C killed almost all soil-grown, wild-type plants, but none of the *PLD*δ-OE plants (**Fig. 4a**). At –8 °C, wild-type plants were injured and growth was severely retarded. But freezing injury and growth retardation of *PLD*δ-OE plants were slight or absent at this temperature. Although the killing temperatures varied with plants of different ages and growth conditions, *PLD*δ-OE plants were consistently more resistant to freezing than wild-type whereas *PLD*δ-KO plants were more sensitive than wild-type (**Fig. 4b**). Without cold acclimation, however, *PLD*δ-OE plants did not exhibit enhanced freezing tolerance; both wild-type and *PLD*δ-OE plants were killed at –8 °C.

Overexpression of the transcriptional factor DREB1A results in an increase in expression of cold-regulated genes and an increase in freezing tolerance, but overexpression is accompanied by growth retardation under normal growing conditions[6]. Growth retardation is also observed for *eskimo1*, which overaccumulates the compatible solute proline[7]. In contrast, *PLD*δ-OE plants grew and developed indistinguishably from wild-type plants under regular growing conditions (**Fig. 4c**). When wild-type, *PLD*δ-KO and *PLD*δ-OE plants were tested by repeated chilling at 4 °C for 6 h every morning, *PLD*δ-OE plants produced a greater number of siliques and were taller than wild-type plants (**Fig. 4d**). In addition, the *PLD*δ-altered and wild-type seedlings were grown in the presence of 150 mM NaCl to test salt tolerance. No significant difference was observed among wild-type, *PLD*δ-OE, *PLD*δ-KO and *PLD*δ-complemented plants in the rate of seed germination, root elongation and fresh weight (see **Supplementary Fig. 1** online).

Alterations in *PLD*δ do not change cold-induced expression of two *COR* genes

To gain insight into the function of *PLD*δ in freezing tolerance, we measured expression levels of *PLD*δ and the cold-regulated genes

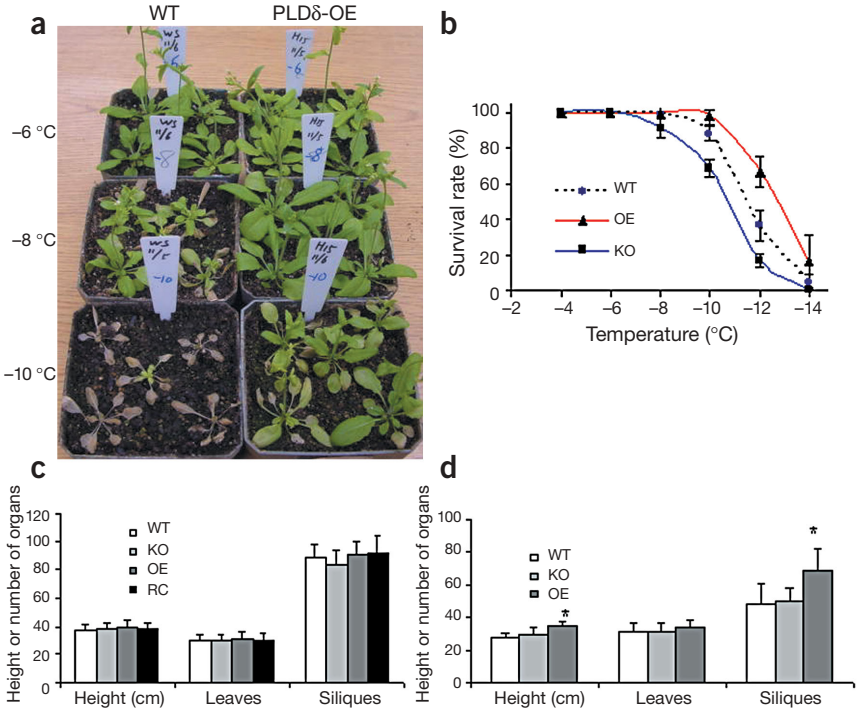

Figure 4 Increased freezing tolerance in *PLD*δ-overexpressing plants (*PLD*δ-OE). (**a**) Phenotypes of soil-grown *PLD*δ-OE and wild-type plants after freezing followed by growth at 23 °C for 2 weeks. (**b**) Survival rate of cold-acclimated (4 °C for 2 d) *PLD*δ-OE, *PLD*δ-KO and wild-type seedlings after freezing at indicated temperature, followed by growth at 23 °C for 10 d. (**c**) Growth performance of *PLD*δ-OE, *PLD*δ-KO, WT (wild-type) and *PLD*δ-RC (complemented) *A. thaliana* under normal growth conditions (12 h, 120 μmol m^{-2} s^{-1} light; 22 °C/day and 19 °C/night) for 8 weeks. Values are means ± s.d. ($n = 10$). Height refers to the stem length. (**d**) Growth performance of *PLD*δ-OE, *PLD*δ-KO and wild type under cold stress. Plants were grown under 12 h light (120 μmol m^{-2} s^{-1}, 22 °C) and exposed to 4 °C daily from 2:00 am to 8:00 am during the dark period for 8 weeks. An asterisk denotes significant difference ($P < 0.05$) from wild-type plants. Values are means ± s.d. ($n = 6$).

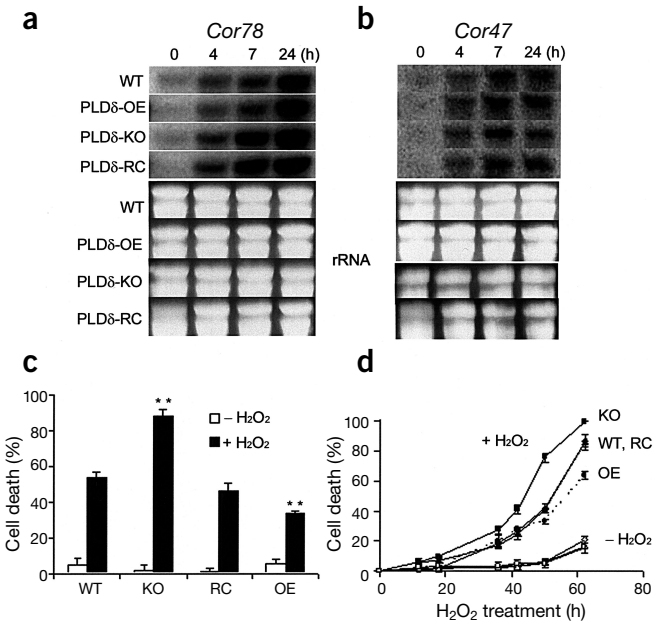

Figure 5 Effect of *PLD*δ-KO and *PLD*δ-OE on the expression of cold-regulated genes and H_2O_2-induced cell death. (**a**,**b**) RNA blotting of *COR47* (**a**) and *COR78* (**b**) transcripts. We loaded equal amounts of RNA (20 μg/lane) from rosettes of *PLD*δ-KO, *PLD*δ-OE, WT (wild-type) and *PLD*δ-RC (complemented) plants. Plants were cold-acclimated at 4 °C for the indicated time. (**c**) H_2O_2-induced cell death in leaf protoplasts isolated from non-cold-acclimated WT, *PLD*δ-KO, *PLD*δ-RC and *PLD*δ-OE plants. Cells were treated with 1.5 mM H_2O_2 for 18 h. Asterisks indicate that the mean value is significantly different from that of wild-type ($P < 0.01$). (**d**) H_2O_2-induced cell death in leaf protoplasts isolated from cold-acclimated plants. Protoplasts were incubated at 23 °C with 1.5 mM H_2O_2 for the first 36 h and then an additional 0.5 mM H_2O_2 was added. Lines clustered near the baseline (–H_2O_2) indicate no difference in cell death among the four genotypes in the absence of added H_2O_2. Cells were counted at the indicated time intervals after H_2O_2 treatment, and cell death is expressed as a percentage of total cells.

COR47 and *COR78*. *PLD*δ mRNA and protein were detectable in wild-type plants (**Fig. 3**), but absent in the *PLD*δ-KO mutant[22]. The level of *PLD*δ mRNA showed some increase when soil-grown wild-type plants were exposed to 4 °C for 3 d (**Fig. 3b**). Immunoblotting with a PLDδ-specific antibody showed that PLDδ protein level was low before cold acclimation and increased somewhat after 3 d of acclimation (**Fig. 3c**).

The mRNA levels of the cold-regulated genes *COR47* and *COR78* increased during cold-acclimation (**Fig. 5a**,**b**). The cold induction of *COR47* plateaued at 7 h; *COR78* mRNA levels continued to increase after 24 h at 4 °C. The patterns of cold induction of the two *COR* genes were similar among wild-type, *PLD*δ-KO, *PLD*δ-OE and *PLD*δ-complemented plants.

Alterations in *PLD*δ levels affect H_2O_2-induced cell death

The level of the reactive oxygen species hydrogen peroxide (H_2O_2) increases in plant cells in response to various stress conditions, including freezing[2,23]. Ablation of *PLD*δ renders *A. thaliana* cells more sensitive to H_2O_2 without altering stress-induced H_2O_2 production[22]. We speculated that *PLD*δ-OE plants might show increased tolerance to oxidative stress and thus enhanced freezing tolerance. To test this, we compared the effect of H_2O_2 on cell death of *PLD*δ-OE to that of wild-type and *PLD*δ-KO plants, using leaf protoplasts isolated from nonacclimated and cold-acclimated plants. Without acclimation, approximately 90% of *PLD*δ-KO and 50% of wild-type cells died after incubation in 1.5 mM H_2O_2 for 18 h (**Fig. 5c**). This increased sensitivity in *PLD*δ-KO cells is consistent with that reported previously[22]. *PLD*δ-OE cells displayed the least sensitivity to H_2O_2; compared with wild-type cells, overexpression of *PLD*δ decreased H_2O_2-induced cell death by 30%. The difference between wild-type and *PLD*δ-OE was transient; the percentage of cell death was similar in the two genotypes after H_2O_2 treatment for 24 h.

Cold acclimation greatly increased tolerance to H_2O_2 (**Fig. 5d**). After cold acclimation, it took approximately three times longer for the same fraction of cells of all four genotypes to undergo cell death than for nonacclimated cells, even at a higher concentration of H_2O_2 (2 mM versus 1.5 mM). Among cold-acclimated cells, there was no difference between wild-type and *PLD*δ-complemented cells, whereas more cell death occurred for *PLD*δ-KO cells. *PLD*δ-OE were no different from wild-type cells in the first 40 h but had a lower rate of cell death at longer incubation times. After H_2O_2 treatment for 65 h, the rate of cell death in *PLD*δ-OE protoplasts was about 25% lower than that of wild-type.

Changes in phosphatidic acid molecular species during cold acclimation and freezing

PLD activity hydrolyzes phospholipids to phosphatidic acid. To determine the effect of *PLD*δ alterations on the freezing-induced lipid changes, we analyzed the molecular species of phosphatidic acid using electrospray ionization tandem mass spectrometry (ESI MS/MS)[14]. Phosphatidic acid levels tended to be lower in *PLD*δ-KO and higher in *PLD*δ-OE than in wild-type plants both before and after cold acclimation. The distribution of phosphatidic acid molecular species was similar among the three genotypes in cold-acclimated and nonacclimated plants. Within each genotype, there was no significant difference in the level and molecular species of phosphatidic acid between cold-acclimated and nonacclimated plants (**Fig. 6a**).

Significant differences in phosphatidic acid levels occurred during freezing within each genotype and also among wild-type, *PLD*δ-KO and *PLD*δ-OE plants ($P < 0.05$). The total amounts of phosphatidic acid in each genotype increased about fourfold, but *PLD*δ-KO and *PLD*δ-OE plants accumulated 80% and 125%, respectively, as much phosphatidic acid as wild-type plants. The same phosphatidic acid species were increased in *PLD*δ-KO and wild-type plants. Overexpression of *PLD*δ significantly increased phosphatidic acid production during freezing ($P < 0.05$). In particular, the polyunsaturated species, 36:5 phosphatidic acid, 36:6 phosphatidic acid, 34:3 phosphatidic acid and 34:2 phosphatidic acid in *PLD*δ-KO plants were significantly higher than those in wild-type plants ($P < 0.05$). *PLD*δ-KO and *PLD*δ-OE plants were not significantly different from wild-type plants in fatty acid composition during cold acclimation and freezing (data not shown).

No change in cold-induced accumulation of proline and soluble sugars

Increases in osmolytes, such as proline and soluble sugars, occur in many plant species during cold acclimation. Such increases are believed to ameliorate the impact of dehydration associated with freezing[1,2]. After cold acclimation for 3 and 5 d, the proline content in wild-type plants increased by 10-fold and 15-fold, respectively (**Fig. 6b**). Such increases are comparable to those observed in other studies of cold acclimation[7]. The proline contents of *PLD*δ-KO, *PLD*δ-OE and wild-type plants increased to a similar extent. The cold-induced increase in soluble sugars occurred more quickly than that of proline. Cold acclimation for 1 d resulted in an ~50% increase in soluble sugars

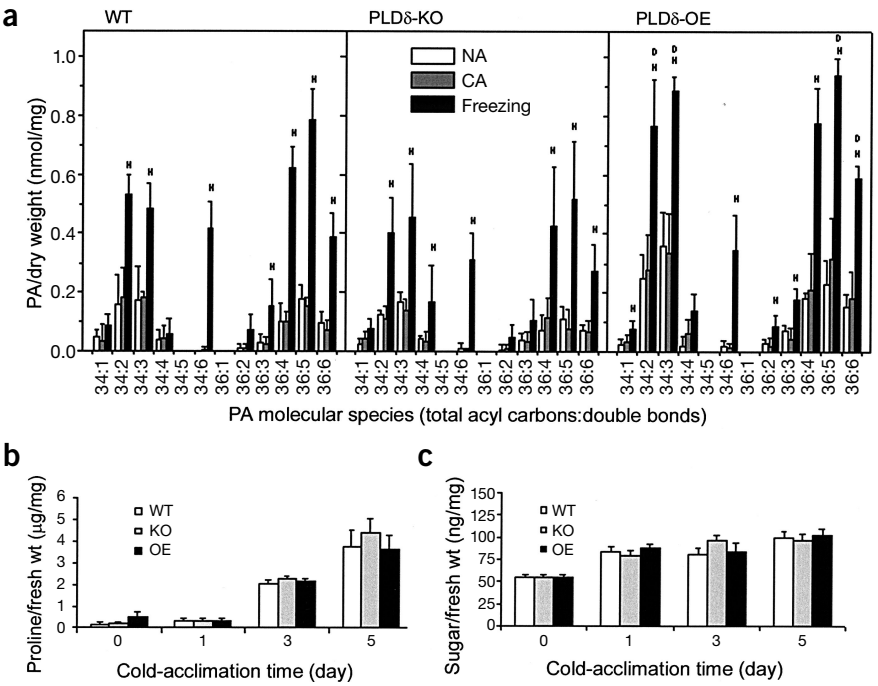

Figure 6 Changes in phosphatidic acid (PA) species and proline and soluble sugar content after cold treatments of *PLD*δ-KO, *PLD*δ-OE and wild-type *A. thaliana*. (**a**) Phosphatidic acid molecular species in *A. thaliana* were determined by ESI-MS/MS. The white, gray and black bars represent nonacclimated (NA), cold-acclimated (CA) and freezing treatments, respectively. An 'H' indicates that the value is higher than that of cold-acclimated plants of the same genotype; $P < 0.05$. A 'D' indicates that the value is significantly different from that of wild-type plants under the same treatment; $P < 0.05$. (**b**,**c**) Changes in proline and soluble sugar contents, respectively, during cold-acclimation in *PLD*δ-KO, *PLD*δ-OE and wild-type *A. thaliana*. Values are means ± s.d. ($n = 4$).

in wild-type plants, but as with proline increases, no significant difference occurred among the three genotypes (**Fig. 6c**).

DISCUSSION

Although freezing injury has been an area of intensive research for many years, progress in developing cultivars with the desired freezing tolerance has been slow. A number of genes have been manipulated to increase freezing tolerance; these include genes involved in production of compatible solutes, detoxification of reactive oxygen species and transcriptional activation of cold- and abscisic acid–regulated genes[2]. The induction of glycinebetaine, a compatible solute that is not produced in wild-type *A. thaliana*, by introducing a choline oxidase gene, increased freezing tolerance in *A. thaliana* before, but not after, cold acclimation[24]. Results of overexpressing superoxide dismutase to better freezing tolerance have been variable[25], and the conflicting results may reflect the multifaceted function of reactive oxygen in plant growth and stress responses. Overexpression of transcriptional activators, such as *DREB1A*, in cold-regulated gene expression has led to increases in freezing tolerance[5,6], but this promising approach is plagued by growth retardation when expression is driven by a strong constitutive 35S promoter[6,26].

By comparison, *PLD*δ is constitutively expressed in plants[19], and this study shows that it plays a positive role in plant freezing tolerance. The altered freezing tolerance resulting from genetic knockout and overexpression of *PLD*δ is not accompanied by corresponding changes in expression of the cold-regulated genes *COR47* and *COR78*, or in levels of the compatible solutes, proline and sugar. These data suggest that *PLD*δ and its associated membrane lipid hydrolysis are unique determinants in mediating plant response to freezing. Moreover, *PLD*δ-induced freezing tolerance is not accompanied by growth retardation under normal plant growing conditions.

The altered freezing tolerance occurs only in cold-acclimated plants, indicating that cold acclimation is required for *PLD*δ function during freezing. This requirement could result from cold regulation of *PLD*δ expression, other cellular factors, or a combination of both, brought about during the acclimation process. RNA blot analysis indicates that *PLD*δ mRNA levels increased slightly during cold acclimation, but the extent of the *PLD*δ increase was far smaller than that of the examined *COR* genes. The upstream region of *PLD*δ does not contain the cold- and dehydration-responsive cis-acting DNA element *DRE/CRT*. The levels of *PLD*δ RNA and protein in *PLD*δ-OE plants are similar to those of cold-acclimated wild-type plants. However, without cold acclimation, *PLD*δ-OE plants failed to reach the level of freezing tolerance attained by cold-acclimated wild-type plants. Therefore, increased expression of *PLD*δ alone is not sufficient to explain the requirement for cold acclimation in *PLD*δ action in freezing tolerance. It is likely that *PLD*δ enhances freezing tolerance in coordination with other cold-induced processes, including expression of cold-regulated genes, osmolytes and lipid composition.

The effect of PLDδ-deficiency on decreasing freezing tolerance contrasts to that observed previously for PLDα1-deficient plants that exhibited increased freezing tolerance[14]. Lipid profiling revealed that PLDα1 is responsible for most of the phosphatidic acid produced during freezing. The high activity of PLDα1 during freezing is believed to promote lipid degradation and membrane deterioration. The present results suggest that PLDδ contributes about 20% of the phosphatidic acid produced in the cell during freezing. In addition to the different amounts of phosphatidic acid, the location and timing of phosphatidic acid produced by PLDα1 and PLDδ may play an important role in phosphatidic acid's cellular function. PLDδ is associated with the plasma membrane, whereas PLDα1 is associated with both the plasma and intracellular membranes. The regulated production of phosphatidic acid by PLDδ on the plasma membrane may mediate the cell's response to freezing.

The lack of an increased rate of ion leakage and the decreased freezing tolerance in PLDδ-KO plants (**Fig. 1**) seem to point to a discrepancy. It is possible, however, that PLDδ increases freezing tolerance through its role in post-freezing recovery, in which cells restore membrane and cell shapes after freezing-induced dehydration[1,2]. These changes would require extensive cytoskeletal reorganization and membrane trafficking activities in which roles for PLD and phosphatidic acid have been suggested[17,20]. For example, PLDδ has been shown to bind to microtubule cytoskeleton, and this observation led to speculations that PLDδ may bridge plasma membrane and cytoskeletal networks to transduce stress signals[20]. The decreased or increased expression of *PLD*δ may decrease or increase, respectively, the activities

of proteins mediating membrane trafficking and cytoskeletal rearrangements and, thus, the cell's freezing tolerance.

Oleate stimulation of PLD activity is another distinct property of PLDδ[19]. Oleate has been implicated in decreased cell death in both plants and animals[27,28]. Our recent study has indicated an anti-death function of *PLD*δ; *PLD*δ-KO *A. thaliana* cells displayed increased sensitivity to H_2O_2 and PLDδ-derived phosphatidic acid mitigated H_2O_2-promoted cell death[22]. That work also showed that knockout of *PLD*δ did not alter stress-induced H_2O_2 production. The present results show that overexpression of *PLD*δ increased the cell's tolerance of oxidative stress. The level of H_2O_2 increases in plant cells in response to various stress conditions, including freezing[2,23]. Thus, the impaired response to oxidative stress in *PLD*δ-KO plants may be a basis for the decreased freezing tolerance.

The finding that *A. thaliana* freezing tolerance can be manipulated by depletion or overexpression of *PLD*δ indicates that *PLD*δ plays an important, positive role in freezing tolerance. Manipulation of *PLD*s and thus phospholipid hydrolysis may present an avenue for improving plant freezing tolerance.

METHODS

***PLD*δ-KO mutant and genetic complementation of *PLD*δ.** The *PLD*δ-KO mutant was isolated from the Wassilewskija (WS) ecotype of *A. thaliana*, as described previously[22]. The loss of *PLD*δ function was confirmed by the absence of *PLD*δ's protein, enzymatic activity (**Fig. 1**) and transcript (data not shown). To complement for the loss, we cloned the *PLD*δ gene with its own 1.5 kilobase, 5′-untranslated region from the wild-type WS ecotype of *A. thaliana* by PCR. The gene with its own promoter was ligated to an agrobacterial binary vector pBin 19 and introduced into homozygous *PLD*δ-KO plants by *A. tumefaciens*-mediated transformation using floral dipping. The T-DNAs used in *PLD*δ-KO and pBin19 carried the selection marker for kanamycin- and hygromycin-resistance, respectively. Thus, the *PLD*δ-complemented plants were selected by their resistance to both the antibiotics. The complementation was verified by PCR that indicated the presence of the original T-DNA insertion allele and the introduced wild-type *PLD*δ gene, as well as by detection of the production of PLDδ protein.

35S:*PLD*δ construction and overexpression. The *A. tumefaciens* vector pKYLX7 was used to introduce the *PLD*δ cDNA into the WS ecotype of *A. thaliana*. The overexpression vector used a full-length 2.6 kb *PLD*δ cDNA[19] under the control of the cauliflower mosaic virus 35S promoter. Plasmids with the insert were transferred into the *A. tumefaciens* strain EHA105. *A. thaliana* was transformed with the T-DNA by infiltrating plants with agrobacteria[29]. Overexpression of *PLD*δ was confirmed by blotting with PLDδ-specific antibodies and gene probes according to published procedures[29,30].

Plant growth and treatments. Wild-type (WS), *PLD*δ-KO and *PLD*δ-OE *A. thaliana* plants were sown in Scott's Metromix soil. *PLD*δ-KO and *PLD*δ-OE plants were confirmed by PCR and immunoblotting with a PLDδ-specific antibody, following the procedure described previously[26]. The pots were kept at 4 °C for 2 d, then moved to a growth chamber at 23 °C (day) and 19 °C (night) with a 12-h day length, daytime fluorescent lighting at 120 μmol m^{-2} s^{-1}, and 58% relative humidity.

For lipid profiling, 35-d-old, pre-flowering plants were placed at 4 °C for 3 d in a growth chamber with a continuous light at 30 μmol m^{-2} s^{-1}. Nonacclimated plants remained in the 23 °C growth chamber until they were harvested on the same day that the cold-acclimated plants were harvested. For freezing, cold-acclimated plants at 4 °C were subjected to a temperature drop from 4 °C to –2 °C at 3 °C per h in the growth chamber. When the temperature reached –2 °C, ice crystals were placed on the soil to induce crystallization and prevent super-cooling. After 2 h at –2 °C, the temperature was lowered to –8 °C at 1 °C per h. After 2 h at –8 °C, the plants were harvested for lipid analysis.

Freezing tolerance was tested on *A. thaliana* plants grown in soil pots and agar plates. Soil-grown plants were subjected to cold acclimation and freezing as described above. To measure ionic leakage, we collected *A. thaliana* rosettes one h after freezing at the indicated temperatures and then thawed at 4 °C overnight. Leaked ions were measured as described previously[14]. To observe freezing damage, we thawed frozen plants at 4 °C overnight and grown under normal conditions for 14 d. To test freezing tolerance using seedlings on agar plates, we germinated seeds on 0.7%-agar-solidified plates containing 1× Murashige and Skoog basal medium and 3% sucrose. After 2 d at 4 °C, plants were germinated and grown at 23 °C/19 °C (day/night) under 120 μmol m^{-2} s^{-1} light for 11 d. Seedlings were acclimated at 4 °C under 30 μmol m^{-2} s^{-1} light for 3 d and then incubated with ice chips at –2 °C for 2 h for nucleation. Temperature was lowered by 2 °C per h. After 1 h at the final temperature, the plates were thawed at 4 °C overnight. Pictures were taken after recovery for 10 d under normal growth conditions described above.

ESI-MS/MS analysis of lipid molecular species. The processes of lipid extraction, ESI-MS/MS analysis and quantification followed the procedure detailed previously[14]. Briefly, the above-ground rosette of two or three plants was cut at the sampling time and transferred immediately to 3 ml isopropanol with 0.01% butylated hydroxytoluene at 75 °C to inhibit lipolytic activities. The tissues were extracted with chloroform/methanol five times with 30 min agitation each time. The remaining plant tissue was heated overnight at 105 °C and weighed. The weights of these dried, extracted tissues are the 'dry weights' of the plants. Lipid samples were analyzed on a 'triple' quadrupole MS/MS (Micromass Ultima, Micromass) equipped for ESI. Data processing was done using Masslynx software (Micromass). The lipids in each class were quantified in comparison to the two internal standards of that class using a correction curve determined between standards. Five replicates of each treatment for each phenotype were carried out and analyzed. The Q test for discordant data was done on the replicates of the total amount of lipid in each head group class[14]. Paired values were subjected to the *t*-test to determine statistical significance.

Immunoblotting of PLDs and RNA blotting. Total protein from wild-type, *PLD*δ-KO, and *PLD*δ-complemented leaves was isolated as described previously. Protein contents in the supernatants were determined using a dye-binding assay. Equal amounts of the supernatant protein (20 μg/lane) were separated by SDS-PAGE analysis and then transferred onto polyvinylidene difluoride filters. The filters were blotted with PLDδ- or PLDα-specific antibodies, followed by incubation with a second antibody conjugated to an alkaline phosphatase. The PLDδ was made visible by staining the blot with the phosphatase activity.

Total RNA was isolated from *A. thaliana* leaves and separated by 1% formaldehyde-agarose denaturing gel electrophoresis. cDNAs for *PLD*δ, *COR47* and *COR78* were labeled with [α-^{32}P]dCTP by random priming. The RNA isolation and hybridization were done as described previously.

Protoplast preparation and cell death assay. The expanded leaves from 4-week-old cold-acclimated or nonacclimated plants were used to isolate protoplasts using a protocol described previously[22]. Cold acclimation was done at 4 °C for 3 d with continuous light at 30 μmol m^{-2} s^{-1}. Freshly isolated protoplasts (5×10^5) were incubated in the dark with 1.5 mM or 2.0 mM H_2O_2. Cells were stained with 0.5 mg/ml fluorescein diacetate for 15 min, and total and live cells were counted under a fluorescent microscope.

Proline and sugar measurements. Four-week-old plants were placed at 4 °C under continuous light for the indicated time intervals. Rosettes were harvested, weighed and ground into powder in liquid nitrogen. The tissue powder was incubated with 75% ethanol overnight with shaking and then centrifuged at 20,000*g*[7]. An aliquot of each extract (100 μl) was incubated with 900 μl ninhydrin reagent (1% (wt/vol) ninhydrin, 60% (vol/vol) glacial acetic acid, 40% H_2O) at 100 °C for 1 h. Toluene (3 ml) was added, followed by vortexing and incubation at 23 °C for 24 h. The absorbance was measured at 520 nm[31]. A second aliquot of each extract was also used to measure the soluble sugars. Twenty μl of extract was incubated with 1,000 μl anthrone reagents (0.15% (wt/vol) anthrone, 72% (vol/vol) H_2SO_4, 28% (vol/vol) H_2O) at 100 °C for 1 h. The absorbance was measured at 625 nm. Sugar value was expressed as glucose equivalents[32].

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Thomashow, M.F. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 571–599 (1999).
2. Iba, K. Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. *Annu. Rev. Plant Biol.* **53**, 225–245 (2002).
3. Gong, Z. *et al.* RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. *Proc. Natl. Acad. Sci. USA* **99**, 11507–11512 (2002).
4. Chinnusamy, V. *et al.* ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* **17**, 1043–1054 (2003).
5. Jaglo-Ottosen, K.R., Gilmour, S.J., Zarka, D.G., Schabenberger, O. & Thomashow, M.F. *Arabidopsis* CBF1 overexpression induces COR genes and enhances freezing tolerance. *Science* **280**, 104–106 (1998).
6. Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K. & Shinozaki, K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat. Biotechnol.* **17**, 287–291 (1999).
7. Xin, Z. & Browse, J. *eskimo1* mutants of *Arabidopsis* are constitutively freezing-tolerant. *Proc. Natl. Acad. Sci. USA* **95**, 7799–7804 (1998).
8. Seki, M. *et al.* Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* **13**, 61–72 (2001).
9. Fowler, S. & Thomashow, M.F. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**, 1675–1690 (2002).
10. Thomashow, M.F. So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* **125**, 89–93 (2001).
11. Knight. H., Trewavas, A.J. & Knight, M. Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *Plant Cell* **8**, 489–503 (1996).
12. Plieth, C., Hansen, U.P., Knight, H. & Knight, M. Temperature sensing by plants: the primary characteristics of signal perception and calcium response. *Plant J.* **18**, 491–497 (1999).
13. Ruelland, E., Cantrel, C., Gawer, M., Kader, J.C. & Zachowski, A. Activation of phospholipases C and D is an early response to a cold exposure in *Arabidopsis* suspension cells. *Plant Physiol.* **130**, 999–1007 (2002).
14. Welti, R. *et al.* Profiling membrane lipids in plant stress responses. Role of phospholipase Dα in freezing-induced lipid changes in Arabidopsis. *J. Biol. Chem.* **277**, 31994–32002 (2002).
15. Cullis, P.R. & DeKruijff, B. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* **559**, 399–420 (1979).
16. Verleij, A.J., DeMaagd, R., Leunissen-Bijvelt, J. & DeKruijff, B. Divalent cations and chlorpromazine can induce non-bilayer structures in phosphatidic acid-containing membranes. *Biochim. Biophys. Acta* **684**, 255–262 (1982).
17. Wang, X. Phospholipase D in hormonal and stress signaling. *Curr. Opin. Plant Biol.* **5**, 408–414 (2002).
18. Qin, C. & Wang, X. The *Arabidopsis* phospholipase D family. Characterization of a calcium-independent and phosphatidylcholine-selective PLDζ1 with distinct regulatory domains. *Plant Physiol.* **128**, 1057–1068 (2002).
19. Wang, C. & Wang, X. A novel phospholipase D of *Arabidopsis* that is activated by oleic acid and associated with the plasma membrane. *Plant Physiol.* **127**, 1102–1112 (2001).
20. Gardiner, J.C. *et al.* 90-kD phospholipase D from tobacco binds to microtubules and the plasma membrane. *Plant Cell* **13**, 2143–2158 (2001).
21. Katagiri, T., Takahashi, S. & Shinozaki, K. Involvement of a novel *Arabidopsis* phospholipases D, AtPLD, in dehydration-inducible accumulation of phosphatidic acid in stress signaling. *Plant J.* **26**, 595–605 (2001).
22. Zhang, W. *et al.* Phospholipase Dδ and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*. *Plant Cell* **15**, 2285–2295 (2003).
23. Prasad, T.K., Anderson, M.D., Martin, B.A. & Stewart, C.R. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *Plant Cell* **6**, 65–74 (1994).
24. Sakamoto, A., Valverde, R., Alia, R., Chen, T.H.H. & Murata, N. Transformation of *Arabidopsis* with the *codA* gene for choline oxidase enhances freezing tolerance of plants. *Plant J.* **22**, 449–453 (2000).
25. McKersie, B.D., Bowley, S.R. & Jones, K.S. Winter survival of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* **119**, 839–848 (1999).
26. Dubouzet, J.G. *et al. OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J.* **33**, 751–763 (2003).
27. Hardy, S., Langelier, Y. & Prentki, M. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Res.* **60**, 6353–6358 (2000).
28. Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J. & Klessig, D.F. A fatty acid desaturase modulates the activation of defense signaling pathways in plants. *Proc. Natl. Acad. Sci. USA* **98**, 9448–9453 (2001).
29. Fan, L., Zheng, S. & Wang, X. Antisense suppression of phospholipase Dα retards abscisic acid- and ethylene- promoted senescence in postharvest *Arabidopsis* leaves. *Plant Cell* **9**, 2183–2196 (1997).
30. Pappan, K., Zheng, S. & Wang, X. Identification and characterization of a novel phospholipase D that requires polyphosphoinositides and submicromolar calcium for activity in *Arabidopsis*. *J. Biol. Chem.* **272**, 7048–7054 (1997).
31. Magne, C. & Larher, F. High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Anal. Biochem.* **200**, 115–118 (1992).
32. Bravo, L.A., Zúñiga, G.E., Alberdi, M. & Corcuera, L.J. The role of ABA in freezing tolerance and cold acclimation in barley. *Physiol. Plant.* **103**, 17–23 (1998).