Comparative Study of Early Cold-Regulated Proteins by Two-Dimensional Difference Gel Electrophoresis Reveals a Key Role for Phospholipase Dα1 in Mediating Cold Acclimation Signaling Pathway in Rice*

Chenmin Huo‡**, Baowen Zhang‡**, Hui Wang‡, Fawei Wang§, Meng Liu‡, Yingjie Gao‡, Wenhua Zhang§, Zhiping Deng¶, Daye Sun‡, and Wenqiang Tang‡ ‡‡

To understand the early signaling steps that regulate cold responses in rice, two-dimensional difference gel electrophoresis (2-D DIGE) was used to study early cold-regulated proteins in rice seedlings. Using mass spectrometry, 32 spots, which represent 26 unique proteins that showed an altered expression level within 5 min of cold treatment were identified. Among these proteins, Western blot analyses confirmed that the cellular phospholipase Dα1 (OsPLDα1) protein level was increased as early as 1 min after cold treatment. Genetic studies showed that reducing the expression of OsPLDα1 makes rice plants more sensitive to chilling stress as well as cold acclimation increased freezing tolerance. Correspondingly, cold-regulated proteome changes and the expression of the cold-responsive C-repeat/dehydration-responsive element binding 1 (OsDREB1) family of transcription factors were inhibited in the pldα1 mutant. We also found that the expression of OsPLDα1 is directly regulated by OsDREB1A. This transcriptional regulation of OsPLDα1 could provide positive feedback regulation of the cold signal transduction pathway in rice. OsPLDα1 hydrolyzes phosphatidylcholine to produce the signal molecule phosphatidic acid (PA). By lipid-overlay assay, we demonstrated that the rice cold signaling proteins, MAP kinase 6 (OsMPK6) and OsSIZ1, bind directly to PA. Taken together, our results suggest that OsPLDα1 plays a key role in transducing cold signaling in rice by producing PA and regulating OsDREB1s’ expression by OsMPK6, OsSIZ1, and possibly other PA-binding proteins. Molecular & Cellular Proteomics 15: 10.1074/mcp.M115.049759, 1397–1411, 2016.

As one of the most important food crops, rice (Oryza sativa) provides food for more than half of the world’s population. Compared with other major crops like maize and wheat, the growth and yield of rice is more susceptible to environmental low temperature stress. Therefore, revealing the cellular mechanisms that could lead to the generation of low temperature stress resistant rice cultivars is important for meeting the increasing food demand due to increasing world population.

When exposed to a period of non-lethal low temperature, plants can develop a number of cellular changes, including an increase in intracellular calcium concentration (1, 2), alteration of cold-regulated (COR) gene expression (3), changes in lipid composition (4, 5), scavenging of reactive oxygen species (6), accumulation of osmotic adjustment substances (7), and metabolic reprogramming (8) to enhance tolerance to lethal freezing temperature. This process is called cold acclimation. Using Arabidopsis thaliana as a model system, several cold acclimation regulating cold stress signal transduction pathways have been identified (9). Among these pathways, the C...
repeat/dehydration-responsive element binding protein1 (CBF/DREB1)-dependent pathway is the best characterized and is considered to be the major cold acclimation regulatory pathway. In this pathway, CBF regulates the expression of a number of COR genes (10, 11), and the expression of CBF is controlled by the inducer of CBF expression1 (ICE1) family of transcription factors (12) and/or calmodulin-binding transcription activator 3 (CAMTA3) (13). ICE1 is phosphorylated by open stomata 1 (OST1) kinase during cold acclimation (14). The phosphorylation of ICE1 activates and stabilizes ICE1 by E3 small ubiquitin-like modifier (SUMO)-protein ligase SIZ1 (SAP and Miz1)-mediated sumoylation (15), which prevents ICE1 ubiquitination mediated by an ubiquitin E3 ligase high expression of osmotically responsive gene1 (HOS1) (16).

In comparison with what is known in Arabidopsis, the mechanism for cold acclimation is not well studied in rice. Sequence homology searches have identified homologous OsHOS1, OsICE1, and OsDREB1s genes in the rice genome (17–19). Similar to what has been observed in Arabidopsis, OsHOS1 is able to regulate the protein abundance of OsICE1 (19). Also, the expression of OsDREB1A and OsDREB1B is induced by cold stress, and overexpression of these two genes increases the cold stress tolerance of transgenic rice (20), which suggests that the CBF/DREB1 cold signaling pathway is conserved in rice. In addition to OsDREB1s and OsICE1, a number of other proteins such as OsCOIN (21), OsMYB3 (22), OsPRP3 (23), and OsMAPK6 (24) have been shown to regulate cold stress responses in rice. Whether these proteins function as COR genes or as primary regulators of early cold responses in rice needs to be further explored.

Despite considerable progress in studying plant cold signal transduction mechanisms, our understanding of the cold stress signal perception and early signaling transduction steps leading to CBF/DREB1s activation is still very limited. With the development of mass spectrometry technologies, quantitative proteomic approaches have proven to be very powerful tools, complementing traditional genetic approaches, in finding new signaling components as well as revealing new signaling mechanisms (25, 26). There has been a number of reports studying cold-regulated proteins in rice using proteomic approaches (27). However, the rice plants in these studies were cold-stress-treated for hours or days. It was reported that cold induces an increase in cytoplasmic calcium levels as early as 40 s after cold treatment (1) and the expression of CBFs in Arabidopsis is dramatically increased within 15 min after cold treatment (10). These results suggest that early cold stress signal transduction occurs within 15 min or less after a plant is exposed to cold temperature. Therefore, it is likely that cold-regulated proteins previously identified in rice using proteomic approaches were mostly late cold-regulated proteins whose expression was regulated by DREB1s and other cold-regulated transcription factor-mediated transcriptional networks.

In order to help better understand early cold stress signaling, two-dimensional difference gel electrophoresis (2D DIGE) technology was applied to characterize rapid cold-regulated proteomic changes and identify novel early cold-regulated proteins in 1-week-old rice seedlings. A total of 32 spots, which represent 26 unique proteins whose abundance changed in response to a five-minute cold stress treatment, were identified by mass spectrometry. Additional genetic, biochemical, and physiological evidence suggest one of these early cold-regulated proteins, OsPLDα1, plays a key role in the early cold stress signal transduction pathway in rice.

**EXPERIMENTAL PROCEDURES**

**Protein Extraction and Two-Dimensional Difference Gel Electrophoresis**—Because we were interested in finding proteins whose abundance changed rapidly in response to cold stress, a water bath was used for the cold treatment to make sure all the rice seedlings were instantly exposed to a similar temperature at the same time. Wild-type (Oryza sativa japonica cv. Nipponbare) rice seedlings were first germinated in double distilled water in darkness for 3 days. Seedlings were then transferred to Hoagland’s liquid growth medium to grow 1 more week at 28 °C, 50% humidity and long-day conditions. After being submerged in double distilled water at 28 °C or 0 °C for 5 min, the seedlings were quickly tap-dried with tissue paper and snap frozen using liquid nitrogen. Cold-treated and untreated samples were handled in parallel for protein extraction and separation to reduce variations.

To prepare different cellular fractions for proteomic studies, −6 g liquid nitrogen ground tissue powder were mixed with extraction buffer (25 mM HEPES, pH 7.5, with NaOH, 0.33 M sucrose, 10% glycerol, 0.6% polyvinylpyrrolidone, 5 mM ascorbic acid, 5 mM Na2-EDTA, 50 mM NaF, 2 mM imidazole, 1 mM sodium molybdate, 1 mM PMSF, 5 mM DTT, 1 mM sodium orthovanadate, 1 μM E-64, 1 μM bestatin, 1 μM pepstatin, 2 μM leupeptin) on ice, filtered through two layers of miracloth to remove cell debris, and centrifuged at 10,000 × g for 15 min to collect the pellet fraction. The supernatant was further centrifuged at 60,000 × g for 45 min to separate soluble and microsomal fractions. The soluble, pellet, and microsomal fractions were mixed with SDS extraction buffer (100 mM Tris-HCl, pH 8.0, 2% SDS, 1% β-mercaptoethanol, 5 mM EGTA, 10 mM EDTA, 1 μM E-64, 1 μM bestatin, 1 μM pepstatin, 2 μM leupeptin) and incubated at 65 °C for 10 min, followed by phenol extraction and methanol precipitation as described previously (28). The proteins were resuspended in DIGE buffer (7 M urea, 2 M thiourea, 4% CHAPS) at a concentration of 5–10 μg/μl.

Cy3 and Cy5 labeling of the proteins and two-dimensional electrophoresis were performed as described previously (29, 30). 2D DIGE images were acquired using a Typhoon Trio scanner (GE Healthcare, Piscataway, NJ). The images were analyzed using DeCyder 6.5 software (GE Healthcare). Spots that showed consistent cold stress regulated changes in at least four biological repeat samples were picked using a robotic spot picker (GE healthcare).

**Mass Spectrometry and Protein Identification**—Selected protein spots were in-gel digested by trypsin as previously described (28, 29). The extracted peptides were dissolved in 10 μl 0.1% formic acid. The peptides were loaded onto a C18 reverse phase column (100 μm × 150 mm, Thermo Fisher Scientific, Waltham, MA) coupled online to a LTQ- XL linear ion trap mass spectrometer (Thermo Fisher Scientific) at a flow rate of 350 nL/min. Peptides were separated using a gradient from 100% of A (0.1% formic acid) to 45% of B (0.1% formic acid, 95% acetonitrile) for 60 min. MS1 spectra were acquired in a positive mode using the data dependent automatic survey MS scan in the m/z range using the data dependent automatic survey MS scan.
range between 400 and 2000. The 10 most intense ions that over 500 counts were selected for MS2 acquisition using normalized collision induced dissociation (CID) with 35% collision energy, and activation Q value was set to 0.25. CID product ions were analyzed on the linear ion trap in centroid mode. The dynamic exclusion activation time was set to 30 s to prevent same m/z ions from being selected after its acquisition.

LC-MS/MS peak lists were searched against database generated from Oryza sativa subset of the NCBI database (date 20/8/2010, 84,086 entries searched), using the SEQUEST search algorithm. Peptides were searched in the mass range between 400–5000 amu. The following search parameters were applied: mass tolerance for precursor ions and fragment ions were set to 2.00 amu and 1.00 amu, respectively; two incomplete cleavages were allowed; alkylation of cysteine by carbamidomethylation and oxidation of methionine were considered as possible modification. Search result option filter was set as the following: Delta CN ≥ 0.1; Xcorr (≤1, 2, 3) = 1.5, 2.0, 2.5; peptide probability ≤0.01.

Isolation of OsPLDα1 T-DNA Mutant—T-DNA insertional mutant for OsPLDα1 (PGF-IA-21508) was obtained from the Rice T-DNA Insertion Sequence Database (http://cbi.khu.ac.kr/RISD_DB.html), and was generated in Oryza sativa japonica cv Dongjin background. The gene-specific primers used for genotyping the mutant were: 5′-ccctcctgcagccccgcatag-3′ and 5′-taatcactgatcctctgctt-3′. Primers used for semi-quantitative RT-PCR analysis of OsPLDα1 expression level were: 5′-ggtgacctgacagagccatgctag-3′ and 5′-ggcatgcttcagcagctggt-3′ for OsPLDα1; 5′-tcattgacctgcagcagaga-3′ and 5′-ttgggaagtcgagcaagttgaggcagcat-3′ for OsTubulin (Os03g51600).

Molecular Cloning and Generation of Transgenic Rice—Oryza sativa japonica cv Dongjin was used for all the rice transgenic studies. The genome sequence and coding sequence of OsPLDα1 were PCR amplified, cloned into pENTRY/S.D./D-TOPO vector (Thermo Fisher Scientific), and subcloned into pMDMC107, pGWB3, or pMDCB3 designation vectors by LR clonase (Thermo Fisher Scientific) to generate pOsPLDα1:OsPLDα1-GFP, pOsPLDα1:OsPLDα1-GUS, or pSSS: OsPLDα1-GFP expressing binary vectors. To generate the RNAi vectors for OsPLDα1 and OsDREB1A, a conserved 420-bp (2042–2461) coding sequence of OsPLDα1 and a conserved 234-bp (117–350) coding sequence of OsDREB1A was amplified by RT-PCR using the following primer sets: 5′-ggagctagatagacagagcatcagatccttagctggtg-3′ and 5′-cctgatgcttcagcagctggtg-3′ for cloning of OsPLDα1; 5′-ggttgaaccgcagacagcagccatgctgctag-3′ and 5′-ccagatcctgacccgagagcttggagaggtg-3′ for BamH1 + Kpn1 cloning of OsDREB1A; 5′-ggagctagatagacagagcatcagatccttagctggtg-3′ and 5′-ccagatcctgacccgagagcttggagaggtg-3′ for SpeI + SacI cloning of OsDREB1A. The PCR products were sequentially subcloned into pCTK303 RNAi binary vector by BamH1 + Kpn1 followed by SpeI + SacI-mediated double digestion and ligation as described (31). The binary vectors were introduced into EHA105 strain of Agrobacterium tumefaciens and transformed into rice plants using Agrobacterium-mediated callus transformation. T2 homozygous transgenic rice seedlings were used for the cold stress phenotype analysis.

Cold Tolerance Assays—Cold tolerance assays of rice seedlings were performed as described with modifications (21, 32, 33). For germination assays, a minimum of 40 wild-type or plda1 mutant seeds were submersed in 25 ml double distilled water in a glass Petri dish in darkness at 28 °C or 16 °C for up to 14 days. The seeds were checked daily for their germination rate. Seed germination was determined by emergence of coleoptile tips. For cold sensitivity assays, seedlings were first germinated in water in darkness for 3 days at 28 °C. A minimum of 30 seedlings with similar coleoptile length were then transferred to Hoagland’s liquid growth medium at 28 °C or 12 °C, with a daily photoperiodic cycle of 16 h light and 8 h dark (long day) with 50% humidity for 30 days before coleoptile length and seeding weight measurements were made. Alternatively, the seedlings were allowed to grow in Hoagland’s liquid growth medium at 28 °C, long day with 50% humidity for 7 days before being subjected to 4 °C, long day with 50% humidity and allowed to continue growing for up to 7 days. The cold-treated seedlings were allowed to recover at 28 °C, long day with 50% humidity conditions for 7 more days before taking pictures and calculating the survival rates. All the experiments shown in this study have been performed at least three times with similar results. Representative data from one repetition are shown.

Freezing Tolerance Assays—Freezing tolerance assays of rice seedlings were performed according to Li et al. (34). Seedlings were grown in soil at 28 °C, long-day conditions with 50% humidity for 6 weeks. Freezing stress was performed using a programmable freezing chamber (model AR33L, Percival Scientific, Perry, IA). The plants were first kept at -1 °C for 3 h. Temperature was decreased at -1 °C per hour to -2 °C, -4 °C, or -6 °C. After holding at the designated freezing temperature for 3 h, the temperature was increased at 1 °C per hour to -1 °C then incubated at 12 °C for 12 h before transferring to a greenhouse to recover for 7 days at 28 °C, long-day conditions with 50% humidity. Ion leakage was determined immediately after overnight incubation at 12 °C. Approximately 1 g of leaves was harvested and shaken in a falcon tube with 20 ml double distilled water at room temperature overnight. Leaves were then transferred to a new falcon tube with 20 ml double distilled water and boiled for 15 min. Conductivity of the solution was determined using a Leici conductivity meter (DDS-IIA, Shanghai, China). Relative ion leakage was calculated by dividing the conductivity collected from room temperature samples by the sum of conductivities collected from room temperature samples and boiled samples.

Quantitative Real-Time RT-PCR—Seven-day-old wild-type rice seedlings (Dongjin) grown in Hoagland’s liquid growth medium at 28 °C, long-day conditions with 50% humidity were subjected to 12 °C, long-day conditions with 50% humidity for different time intervals. Whole seedlings were collected and snap frozen using liquid nitrogen. Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse-transcribed in a 20 μl reaction using PrimeScript™ RT reagent Kit (TakaRa, Kyoto, Japan) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed by standard protocol using a PRISM 7500 system (ThermoFisher Scientific). Expression of OsPLDα1 and OsDREB1s was normalized by OsACTIN1 (Os03g50885). Relative expression of OsPLDα1 and OsDREB1s is presented as the ratio to the expression level in nontreated wild-type control plants. The primers used are: 5′-atggcatgatattcactcacggct-3′ and 5′-atggcatgatattcactcacggct-3′ for OsPLDα1; 5′-ggggtaccgcggaccaagttcagggagacgag-3′ and 5′-ccagatcctgacccgagagcttggagaggtg-3′ for OsDREB1A; 5′-tcggtgatctcggcaacaga-3′ and 5′-ggcatgcttcagcagctggt-3′ for OsTubulin (Os03g51600).
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cloned into pENTRY/S.D./D-TOPO vector (Thermo Fisher Scientific), and subcloned into Gateway-compatible vectors pGEX-4T-1 by LR clonase (Thermo Fisher Scientific). The GST-tagged proteins were expressed and purified from Escherichia coli using glutathione Sepharose 4B beads by standard protocols. For in vitro lipid overlay assay, phospholipids (Avanti polar lipids, Alabaster, AL) dissolved in chloroform were spotted on a nitrocellulose membrane and dried at room temperature. The membrane was incubated with blocking solution containing 3% (w/v) fatty-acid-free BSA (Sigma-Aldrich, St. Louis, MO), 10 mM Tris-HCl, pH 8.0, 140 mM NaCl, and 0.1% Tween-20 for 1 h. The membranes were incubated with 2 μg/ml GST-tagged proteins in blocking buffer for 12 h at 4 °C with gentle shaking. After washing three times with blocking buffer, the membrane was incubated with a rabbit HRP conjugated anti-GST antibody (Sigma-Aldrich 1:5000 in blocking buffer) for 1 h. Binding of proteins to phospholipids was visualized with FluorChemQ Multimaging III (Alpha Innotech, San Leandro, CA) using the SuperSignal West Dura chemiluminescent reagent (Thermo Fisher Scientific).

Electrophoretic Mobility Shift Assay—Double-stranded oligonucleotide sequence for electrophoretic mobility shift assay (EMSA) was: 5’-ccgacctggacgcctgggagcacg-3’. EMSA assay was performed using a modified 10 μl mixture, which contained 1.5 μl (0.5 μg/μl) purified protein, 1 μl biotin-labeled oligonucleotides, 1 μl 10 × binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT, pH 7.5), 0.5 μl 50% glycerol, 0.5 μl 1% Nonidet P-40, 0.5 μl 1 mM KCl, and 5 μl ultrapure water. The reactions were incubated at 4 °C for 60 min and loaded onto a 6% native polyacrylamide gel in TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.3). The gel was transferred to a nylon membrane (Millipore, Billerica, MA) using the 0.5 × TBE buffer at 100 V for 60 min in cold room. Nylon membrane was cross-linked at 120 mJ/cm² for 45–60 s using a UV cross-linker. Biotin-labeled DNA was detected by using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to manufacturer’s instruction.

Transient Luciferase Expression Assay—To generate OsPLDα1: firefly luciferase, a 2.5 kb promoter upstream of translation start codon ATG of OsPLDα1 was PCR amplified, cloned into pENTRY/S.D./D-TOPO vector (Thermo Fisher Scientific), and subcloned into pHGWLT7.0 vector by LR clonase (Thermo Fisher Scientific). A full-length coding sequence of OsDREB1A without stop codon was PCR amplified and inserted into a p35S:EGFP-HA expression vector by EcoRI + SalI to replace the enhanced green fluorescent protein (EGFP) sequence to generate a p35S:OsDREB1A-HA expression vector. Transient luciferase expression assay was performed according to Yoo et al. (35). In brief, rice protoplasts were prepared from leaves of 7-day-old wild-type rice seedlings (Dongjin) plants grown at 28 °C under long-day conditions with 50% humidity. The expression vectors were transformed into rice protoplasts by polyethylene glycol (PEG) 4000 (Sigma-Aldrich) mediated transformation. An p35S-driven renilla luciferase expression vector was cotransformed to calibrate the transfection efficiency, and 16 h after transformation, luciferase luminescence was recorded for 10 s using a microplate luminometer (Centro LB 960, Berthold technologies, Bad Wildbad, Germany). Quantitation of luminescent signal from each of the luciferase reporter enzymes was performed using a dual-luciferase® reporter assay kit (Promega, Fitchburg, WI) according to manufacturer’s instruction.

RESULTS

OsPLDα1 Is an Early Cold-Regulated Protein—To investigate the early proteomic response of rice to cold stress, 1-week-old rice seedlings were treated with 0 °C water bath for 5 min. Cellular proteins were then separated into soluble, microsomal, and pellet (which include most of the organelles) fractions to reduce the complexity of the protein samples, and analyzed independently by two-dimensional difference in gel electrophoresis (2D DIGE). Samples from six independent preparations were analyzed. On average, 2683 ± 120 spots were detected in our 2D DIGE gels, proteins that showed consistent cold-regulated changes (over 1.5 fold) in abundance in at least four independent experiments were chosen for protein identification.

By LC-MS/MS, 32 spots, which represent 26 unique proteins, were identified. Interestingly most of these spots were found in the soluble and pellet fractions of our samples (Figs. 1A-1C and Table I). Only six spots whose abundance was consistently regulated by cold treatment were identified from the microsomal fraction (Fig. 1B), suggesting a relative delay in the expression changes of membrane proteins to cold stress. Functional classification showed these proteins are involved in processes such as cellular metabolism, stress responses, protein folding, cytoskeleton rearrangement, and lipid metabolism. A comparison of our results with previous cold stress proteomic studies in rice showed that 20 (76.9%) of our identified proteins had not been previously reported. For the proteins we identified that had also been identified by previous cold stress proteomic studies, our results indicate that these proteins are actually early cold-regulated proteins and thus likely contribute to early cellular responses to cold stress treatment.

Of all the early cold-regulated proteins identified, OsPLDα1 was chosen for further studies (Fig. 1D) because it was previously reported that suppressing the expression of PLDα1 in Arabidopsis made plants more resistant to freezing temperature (5). We first validated the proteomic results by Western blot analysis, using a polyclonal antibody raised specifically against the conserved C-terminal 12-amino acid sequence found in both AtPLDα1 and OsPLDα1 (36). Surprisingly, the increase of the OsPLDα1 protein level inside the cell can be seen as early as 1 min after cold stress treatment (Figs. 1E and 1F). This increase in OsPLDα1 protein level was sustained for 5 min and then decreased to return to the basal level 10 min after cold treatment. We also tested whether the abundance of OsPLDα1 transcript is rapidly regulated by cold stress. Semi-quantitative RT-PCR results showed that the expression of OsPLDα1 is decreased by cold treatment for 1 min, possibly due to sudden increase in protein translation, and then continues to increase from 5 min to 30 min after cold treatment (Figs. 1G and 1H).

Expression Pattern and Subcellular Localization of OsPLDα1—To examine the tissue-specific expression pattern of OsPLDα1, genomic sequence, without the stop codon and 3′ untranslated region (UTR), of OsPLDα1, which includes 2537-bp promoter and 5′ UTR sequence before ATG and two introns, was cloned, fused with a β-glucuronidase (GUS) reporter enzyme at the C terminus, and introduced into wild-type rice plants. T2 homozygous pOsPLDα1:OsPLDα1-GUS transgenic plants were used for histochemical staining for GUS activity. As shown in Fig. 2, GUS expression was mostly
detected at the tips of roots and coleoptiles of rice seedlings that had just germinated (Fig. 2A). When plants were 7 days old, strong GUS activity was detected in whole roots. Except for weak OsPLD1/H9251-GUS expression at the tips of the leaves, no detectable GUS activity was found in coleoptiles and young leaves of 7-day-old rice seedlings grown under normal conditions. A 5 min treatment with 0 °C water bath induced a significant increase in the expression of OsPLD1/H9251-GUS in the coleoptile and leaf, which further confirmed that the level of OsPLD1 protein is increased by cold treatment (Fig. 2B). During tillering stages, OsPLD1-GUS is also expressed in stem. A close examination of hand cross-dissected stem showed that the expression of OsPLD1 in rice stems is very strong in vascular tissues (Fig. 2C). When plants enter the flowering stage, OsPLD1 promoter activities can be detected in hulls but not in stamens and pistils (Fig. 2D).

We also generated pOsPLD1:OsPLD1-GFP-expressing transgenic rice and observed the subcellular localization pat-
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Early cold-regulated proteins identified by mass spectrometry. The cold-regulated abundance changes were the averages of at least four biological repeats. For MS/MS identification, number of unique peptides, sequence coverage of the identified proteins and the best matched scores are listed. Refs indicate same protein was identified from previous cold stress proteomic studies in rice

| Spot | Gene locus | Protein name | Unique peptide | Coverage (%) | Score | Fold change | p value | Ref. |
|------|------------|--------------|----------------|--------------|-------|-------------|---------|------|
| 1    | Os05g33570 | Pyruvate, phosphate dikinase | 14 | 19.20 | 130.28 | –1.61 ± 0.03 | 3.0E-03 |
| 2    | Os05g33570 | Pyruvate, phosphate dikinase | 20 | 24.50 | 180.27 | –1.59 ± 0.01 | 1.0E-03 |
| 6    | Os03g04410 | Aconitate hydratase protein | 3 | 3.65 | 30.24 | –2.14 ± 0.31 | 4.2E-02 |
| 7    | Os02g53860 | Putative subtilase 22 | 2 | 3.70 | 20.20 | 3.08 ± 0.28 | 3.7E-02 |
| 11   | Os01g25484 | Ferredoxin–nitrite reductase | 12 | 17.34 | 120.22 | 2.57 ± 0.30 | 4.7E-02 |
| 12   | Os01g25484 | Ferredoxin–nitrite reductase | 11 | 15.66 | 100.21 | 2.31 ± 0.40 | 4.4E-02 |
| 15   | Os02g38210 | Elongation factor Tu | 16 | 40.90 | 150.24 | 1.66 ± 0.13 | 1.1E-02 |
| 17   | Os08g41990 | Aminotransferase | 2 | 4.60 | 20.18 | 4.32 ± 0.51 | 4.7E-02 |
| 19   | Os08g27840 | Phosphoenolpyruvate carboxylase | 3 | 3.32 | 30.19 | 1.62 ± 0.06 | 2.7E-02 |
| 21   | Os08g27840 | Phosphoenolpyruvate carboxylase | 3 | 3.32 | 30.18 | 1.57 ± 0.01 | 9.6E-03 |
| 22   | Os06g40940 | Glycin dehydrogenase | 4 | 3.48 | 40.17 | –1.91 ± 0.36 | 3.2E-02 |
| 23   | Os09g32650 | Leucyl-tRNA synthetase | 7 | 7.30 | 80.1 | 2.31 ± 0.07 | 2.2E-02 |
| 24   | Os02g32350 | TUDOR protein | 2 | 5.98 | 50.21 | –1.91 ± 0.31 | 4.3E-02 |
| 25   | Os03g28330 | Sucrose synthase | 17 | 20.80 | 170.21 | –1.63 ± 0.21 | 3.1E-02 |
| 26   | Os03g28330 | Sucrose synthase | 6 | 8.95 | 60.22 | 1.64 ± 0.13 | 1.4E-02 |
| 30   | Os02g41630 | Phenylalanine ammonia-lyase | 5 | 5.56 | 60.19 | –1.67 ± 0.13 | 1.3E-02 |
| 31   | Os08g60100 | O-methyltransferase | 4 | 8.42 | 30.16 | –2.27 ± 0.25 | 1.6E-02 |
| 32   | Os10g21266 | ATP synthase subunit beta | 3 | 4.20 | 30.19 | –3.74 ± 0.19 | 1.3E-02 |

| Metabolism | Protein name | Coverage (%) | Score | Fold change | p value |
|-------------|--------------|--------------|-------|-------------|---------|
| 1           | HSP99        | 7.90         | 70.20 | 1.67 ± 0.24 | 3.9E-02 |
| 10          | Peptidyl-prolyl isomerase | 8.79 | 50.18 | –1.80 ± 0.38 | 3.6E-02 |
| 9           | ATP-dependent Clp protease | 12.82 | 120.23 | –2.11 ± 0.18 | 1.5E-02 |
| 13          | ATP-dependent Clp protease | 13.60 | 120.23 | 2.66 ± 0.20 | 3.5E-02 |
| 14          | ATP-dependent Clp protease | 3.38 | 20.23 | 2.44 ± 0.39 | 4.3E-02 |
| 27          | HSP60 BETA   | 2.46         | 20.21 | –1.88 ± 0.24 | 2.2E-02 |
| 29          | CHAPERONIN 60 | 3.62         | 30.23 | 1.60 ± 0.01 | 1.6E-02 |

| Cytoskeleton | Protein name | Coverage (%) | Score | Fold change | p value |
|--------------|--------------|--------------|-------|-------------|---------|
| 5            | Villin protein | 7.30         | 80.1  | 2.31 ± 0.07 | 2.2E-02 |
| 16           | Actin 5       | 9.28         | 40.15 | 5.74 ± 0.14 | 4.3E-02 |
| 28           | Actin 7       | 6.47         | 30.15 | –1.73 ± 0.08 | 7.5E-03 |

| Lipid metabolism | Protein name | Coverage (%) | Score | Fold change | p value |
|------------------|--------------|--------------|-------|-------------|---------|
| 3                | OsPLDα1      | 25.49        | 170.31| 1.96 ± 0.14 | 2.6E-02 |
| 4                | OsPLDα1      | 54.40        | 250.2 | 4.09 ± 0.11 | 5.2E-04 |
| 20               | Lipoxygenase-2| 5.31         | 50.22 | –1.97 ± 0.05 | 4.3E-03 |

| Other | Protein name | Coverage (%) | Score | Fold change | p value |
|-------|--------------|--------------|-------|-------------|---------|
| 18    | Ribosomal L1P family proteins | 10.31 | 30.21 | 1.70 ± 0.05 | 5.0E-02 |

The isolated T-DNA mutant was backcrossed with wild-type (Dongjin cultivar) plants twice to clean up the background. Segregated homozygous plda1 mutant plants, which contained only one T-DNA insertion site (based on the segregation ratio of F2 hygromycin resistant seedlings) and showed no obvious growth phenotype, were used for chilling tolerance studies. One-week-old wild-type and plda1 mutant rice seedlings were exposed to chilling stress (4 °C) for various durations. After recovering at 28 °C for 7 days, the seedlings were photographed and their survival rate was calculated. Compared with slightly reduced survival rate of wild-type plants, about half of the plda1 mutant seedlings died after growing at 4 °C for 5 days (Figs. 3D and 3E). Similar results were also observed for seeds germinated at 16 °C or seedlings grown at 12 °C for 30 days. On average, the germ-
Suppressing the expression of OsPLDα1 reduces the cold acclimation increased freezing tolerance of rice plants. We also tested the freezing tolerance of the pldα1 mutant under acclimation and nonacclimation conditions. Six-week-old rice seedlings were subjected to various freezing temperatures for 3 h and allowed to recover at 28 °C for 7 days. Because of the space limitation in the growth chamber used for freezing treatment, a maximum of eight plants (four mutants and four wild type) could be treated at the same time. Thus, the experiment was independently repeated five times, and the average number of green leaves per plant from all of the experiments after recovery was calculated and used to evaluate the freezing tolerance of the mutant.

Suppressing the expression of OsPLDα1 in rice seedlings had no significant effect to the development of leaves. Six-week-old wild-type and pldα1 mutant rice plants developed 8.25 ± 1.21 and 8.08 ± 1.83 leaves on average, respectively. There was no obvious difference in the green leaves of freeze-treated wild-type and pldα1 mutant rice plants under nonacclimation conditions at all freezing temperatures tested (Figs. 4A and 4B), suggesting the expression level of OsPLDα1 does not regulate the basal freezing tolerance of rice. Cold acclimation at 12 °C under long-day conditions for 7 days significantly increased the freezing tolerance of wild-type rice plants. However, compared with the wild-type control, the increased freezing tolerance in pldα1 mutant was greatly reduced (Figs. 4A and 4B). Correspondingly, freeze-induced electrolyte leakage, an indicator of plasma membrane damage, in the pldα1 mutant was dramatically increased under cold acclimation condition compared with wild-type plants. Based on the chilling and freezing tolerance assays of the pldα1 mutant, we conclude that OsPLDα1 is a positive regulator of the rice cold acclimation processes.

Seedlings overexpressing OsPLDα1-GFP are hypersensitive to cold and freezing stresses. To further assess the role of OsPLDα1 in cold and freezing tolerance of rice plant, the full-length coding sequence of OsPLDα1 was expressed under the control of p35S cauliflower mosaic virus promoter. The expression of OsPLDα1-GFP was detected by GFP antibody (Fig. 5A). Rice seedlings were subjected to cold (4 °C) treatment or cold acclimation at 12 °C for 7 days followed by freezing (−6 °C) treatment. Unexpectedly, after recovery at 28 °C for 7 days, the survival rate of OsPLDα1-GFP overexpressing rice seedlings was lower than wild-type control seedlings but higher than the pldα1 mutant seedlings (Figs. 5B and 5C). It appears that the protein level of PLDα1 has to be carefully controlled and maintained at an appropriate level. Overexpressing OsPLDα1-GFP may cause overaccumulation of PA inside the cell and the corresponding decrease in phosphatidylcholine (PC) concentration in the membrane, which might stimulate the formation of hexagonal phases of membrane and increase membrane damage when plant cells encounter low temperature (37).

Cold-stress-regulated proteomic changes in pldα1 mutant. Using 2D-DIGE, we compared the cold-stress-regulated proteomic changes in wild-type (Dongjin) and pldα1 mutant seedlings. Because we are interested in finding cold-regulated proteins downstream of PLDα1, 1h cold treatment in 0 °C water bath was chosen for the proteomics analyses. We prepared and analyzed six independent biological repeats of soluble protein sample sets. Spots that showed consistent cold-regulated abundance changes in at least four independent experiments were chosen for future protein identification. LC-MS/MS identified 25 spots, which represents 18 proteins,
whose abundance was regulated by 1 h cold treatment from wild-type seedlings (Fig. S1 and Table II). Compared with Table I, only eight spots, which represents seven proteins (38.9%) including a villin (Os03g24220), an actin 3 (Os03g61970), an actin 7 (Os05g01600), an aminotransferase (Os08g41990), an ATP-dependent Clp protease (Os04g32560), a putative subtilisin (Os02g53860), and a sucrose synthase (Os03g28330), showed similar up- or down-regulated patterns. The differences in cold-stress-regulated proteomic changes we observed might be due to using a different cultivar of rice or the difference in the treatment duration. Of the 1 h cold-treatment-regulated proteins identified, the abundance changes of nine spots, which represents eight proteins (44.4%) showed significant differences between wild-type control and plda1 mutant seedlings (Table II and Fig. S2). These results further support that cold regulates the abundance change of a group of proteins through PLDα1 in rice.

**Chilling-Induced Expression of OsDREB1s Is Impaired in OsPLDα1 Mutant**—Cold acclimation has been shown to increase plant chilling/freezing tolerance by up-regulating the expression of CBF/DREB family transcription factors. There are 10 CBF/DREB homologs (OsDREB1A to OsDREB1J) in the rice genome. It has been shown that the expression of OsDREB1A, OsDREB1B, OsDREB1E, OsDREB1F, and OsDREB1G is regulated by cold acclimation (17, 38). To investigate whether the hypersensitive phenotype of the plda1
mutant in response to chilling/freezing stress is related to the endogenous level of OsDREBs, the chilling-induced expression of OsDREBs was examined in the pldα1 mutant. Quantitative real-time PCR analysis showed 12 °C chilling stress induced a dramatic increase of OsDREBs’ expression in wild-type rice seedlings. In comparison, the increase in expression of OsDREBs was greatly reduced in pldα1 mutant (Fig. 6), which suggests OsPLDα1 is required for the chilling-induced increase in OsDREBs expression during the cold acclimation process. We also found that the expression of OsPLDα1 in wild-type plants increased by chilling treatment but remained at a basal level in the pldα1 mutant (Fig. 6). As the cold acclimation process is sustained for 6 h, the expression of OsPLDα1 decreases, which could help prevent the overhydrolysis of PC in the membrane.

PA Interacts with OsMPK6 and OsSIZ1 In Vitro—PLDα1 is a phospholipase that hydrolyzes phospholipids, such as phosphatidylcholine to produce the signaling molecule phosphatidic acid (PA). PA is able to bind to a variety of signaling proteins, regulating their subcellular localization or their activity to transduce signals to downstream components (39). To investigate whether a similar mechanism exists in the rice cold-stress-signaling pathway, recombinant OsMPK6, Arabidopsis RCN1, was used as a positive control. As shown, RCN1, OsMPK6, AtSIZ1, and OsSIZ1 all bound strongly to PA in a dose-dependent manner but not to other lipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG). In contrast, OsOST1 does not bind to any of the lipids we tested, similar to the GST protein control (Fig. 7).
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Fig. 5. OsPLDα1 overexpressing transgenic rice seedlings are hypersensitive to cold and freezing stress. (A) Western blot analysis of the transgenic rice seedlings overexpressing OsPLDα1-GFP fusion protein. OX8 and OX12 are two independent transgenic lines. (B) One-week-old transgenic rice seedlings overexpressing OsPLDα1-GFP were transferred to 4 °C, long-day condition and allowed to continue to grow for 5 days. Survival of the seedlings was examined after recovery at 28 °C for 7 more days. (C) Six-week-old transgenic rice seedlings overexpressing OsPLDα1-GFP were cold acclimated and treated with freezing temperature (–6 °C) for 3 h. Seedlings were allowed to recover at 28 °C for 7 days, and the green leaves per plants were calculated. Shown are average results from three independent experiment with at least 24 plants per treatment. One-way ANOVA test was performed. Statistically significant differences are indicated by different lowercase letters (p < 0.05). Error bars represent ±S.D.

**Table II**

Early cold-regulated proteins in wild-type and pldα1 mutant seedlings identified by mass spectrometry. The cold-regulated abundance changes were observed in at least four biological repeats. For MS/MS identification, number of unique peptides, sequence coverage of the identified proteins, and the best matched scores from wild type are listed. Refs indicate same protein was identified from previous cold stress proteomic studies in rice. Proteins with abundance changes that are significantly different between wild type and the pldα1 mutant are underlined.

| Spot | Gene locus | Protein name | Unique peptides | Coverage (%) | Score | Fold changes | p value | Ref. |
|------|------------|--------------|----------------|--------------|-------|--------------|---------|------|
| U3   | Os02g53860 | Putative subtilisin 22 | 8 | 16.50 | 80.25 | 3.23 ± 0.31 | 1.51 ± 0.33 | 1.2E-02 |
| U4   | Os02g07870 | V-type ATPase alpha subunit | 12 | 20.61 | 130.27 | 1.91 ± 0.29 | 1.87 ± 0.23 | 1.1E-01 |
| U5   | Os02g07870 | V-type ATPase alpha subunit | 5 | 9.50 | 40.23 | 1.53 ± 0.24 | 1.44 ± 0.11 | 3.2E-01 |
| U6   | Os02g07870 | V-type ATPase alpha subunit | 22.22 | 110.28 | 1.63 ± 0.14 | 1.24 ± 0.19 | 4.2E-02 |
| U8   | Os02g52700 | Alpha-amyrase precursor | 4 | 9.81 | 40.21 | 2.73 ± 0.22 | 1.10 ± 0.04 | 4.3E-02 |
| U10  | Os08g41990 | Aminotransferase | 5 | 17.36 | 50.23 | 1.82 ± 0.43 | 1.11 ± 0.10 | 1.1E-01 |
| U11  | Os03g28330 | Sucrose synthase | 26 | 33.44 | 270.28 | 1.52 ± 0.08 | 1.35 ± 0.07 | 2.4E-01 |
| U5   | Os02g46520 | Hydrolase | 3 | 9.93 | 30.19 | 1.19 ± 0.07 | 1.07 ± 0.10 | 3.5E-02 |
| U7   | Os03g28330 | Sucrose synthase | 16 | 19.61 | 150.22 | 2.74 ± 0.63 | 1.11 ± 0.16 | 1.7E-02 |
| D7   | Os03g28330 | Sucrose synthase | 20 | 27.08 | 230.26 | 1.69 ± 0.33 | 1.14 ± 0.23 | 3.0E-02 |
| D8   | Os03g28330 | Sucrose synthase | 32 | 48.32 | 420.22 | 1.98 ± 0.63 | 1.14 ± 0.23 | 3.0E-02 |
| D9   | Os03g28330 | Sucrose synthase | 26 | 33.44 | 270.28 | 1.52 ± 0.08 | 1.35 ± 0.07 | 2.4E-01 |
| D10  | Os01g71830 | Glycosyl hydrolases | 5 | 21.56 | 60.28 | 1.59 ± 0.10 | 1.03 ± 0.07 | 7.6E-02 |
| U10  | Os03g04970 | HSP60 BETA | 30 | 46.27 | 290.31 | 1.59 ± 0.25 | 1.40 ± 0.31 | 3.7E-01 |
| U11  | Os04g35570 | Sucrose synthase | 24 | 31.25 | 240.23 | 2.12 ± 0.14 | 2.33 ± 0.02 | 3.0E-02 |
| D11  | Os01g71830 | Glycosyl hydrolases | 17 | 25.16 | 60.28 | 1.59 ± 0.10 | 1.03 ± 0.07 | 7.6E-02 |
| U12  | Os03g04970 | HSP60 BETA | 30 | 46.27 | 290.31 | 1.59 ± 0.25 | 1.40 ± 0.31 | 3.7E-01 |
| U13  | Os04g35570 | Sucrose synthase | 24 | 31.25 | 240.23 | 2.12 ± 0.14 | 2.33 ± 0.02 | 3.0E-02 |
| D1   | Os03g60620 | DnaK family protein | 21 | 31.90 | 220.27 | 2.00 ± 0.11 | 1.86 ± 0.04 | 1.8E-01 |
| D2   | Os03g64210 | HSP60 ALPHA | 14 | 26.54 | 130.28 | 1.62 ± 0.11 | 1.60 ± 0.07 | 4.8E-01 |
| D4   | Os05g01310 | Ankyrin repeat family protein | 10 | 32.39 | 90.26 | 2.27 ± 0.03 | 1.45 ± 0.02 | 5.1E-03 |
| U7   | Os03g24220 | Villin protein | 22 | 33.96 | 180.29 | 1.63 ± 0.21 | 1.06 ± 0.14 | 1.7E-02 |
| U9   | Os03g61970 | Actin-3 | 3 | 9.81 | 30.21 | 1.69 ± 0.28 | 1.14 ± 0.32 | 5.5E-02 |
| D6   | Os05g01600 | Actin-7 | 7 | 19.10 | 70.23 | 1.57 ± 0.18 | 1.16 ± 0.11 | 8.7E-03 |
| Other | Os07g46370 | WD domain family protein | 21 | 18.19 | 100.21 | 2.16 ± 0.09 | 1.33 ± 0.12 | 3.1E-02 |
| U2   | Os07g46370 | WD domain family protein | 17 | 19.50 | 170.27 | 2.46 ± 0.06 | 1.25 ± 0.15 | 4.4E-04 |
| U14  | Os04g01540 | Uncharacterized protein | 5 | 21.51 | 50.22 | 1.53 ± 0.69 | 1.23 ± 0.12 | 6.6E-02 |
| D5   | Os04g35570 | Regulator of chromosome condensation family protein | 16 | 44.37 | 160.25 | 1.95 ± 0.12 | 1.76 ± 0.04 | 2.2E-01 |

**Expression of OsPLDα1 is Directly Regulated by OsDREB1A—**

CBF/DREB family transcription factors are able to bind to a CCGAC core sequence of C-repeat (CRT)/dehydration responsive elements (DRE) and regulates COR gene expression (40). The finding that the expression of OsPLDα1 is up-regulated by chilling treatment motivated us to search the promoter of
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To investigate whether the expression of OsPLDα1 can be directly regulated by OsDREB1s, RNAi technology was used to knock down the expression of OsDREB1A in rice plants. Quantitative real-time PCR analysis showed that when endogenous OsDREB1A transcript was knocked down by RNAi, chilling (12 °C)-induced expression of OsPLDα1 is inhibited (Fig. 8B). We also tested whether OsDREB1A activates the expression of OsPLDα1 using a transient expression assay. A 2537-bp promoter sequence upstream ATG of OsPLDα1 was fused upstream of the firefly luciferase gene. The generated pOsPLDα1:firefly luciferase reporter construct was cotransformed into protoplast generated from 1-week-old rice seedlings with a p35S:OsDREB1A-HA or p35S:EGFP-HA expression vector. As shown in Fig. 8C, expression of OsDREB1A-HA led to a fivefold increase of firefly luciferase activity compared with the EGFP-HA control, indicating that OsDREB1A protein activates OsPLDα1 promoter in vivo. We also showed using gel mobility shift assay (EMSA) that recombinant OsDREB1A protein purified from E. coli was able to bind specifically with a CRT/DRE containing 30-bp promoter sequence (-618 to -589 bp) of OsPLDα1 (Fig. 8D). Together, these results suggest that OsDREB1A activates the expression of OsPLDα1 by binding directly to OsPLDα1 promoter.

DISCUSSION

The CBF/DREB1-dependent cold signaling pathway has been shown to play an important role in regulating cold acclimation in both Arabidopsis and rice. Many studies have investigated the regulation of CBF/DREB1 by ICE1 and their upstream regulators HOS1, SIZ1, and OST1, but the understanding about the early cold signal perception and signaling mechanisms leading to activation of HOS1, SIZ1, and OST1 is
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still very limited. Using 2D-DIGE, we identified 26 unique proteins from rice seedlings, whose abundance is regulated rapidly by a 5 min chilling treatment. Of these proteins, genetic studies showed reducing the expression of OsPLDα1 by RNAi or T-DNA insertion impaired chilling tolerance and cold acclimation increased freezing tolerance of rice plants. In addition, chilling-induced expression of OsDREB1s is greatly reduced in pldα1 mutant, suggesting OsPLDα1 plays an important role in regulating the cold acclimation signaling pathway leading to activation of OsDREB1s in rice.

The activity of PLD inside a cell is regulated by intrinsic and extrinsic signals (42). Once activated, PLD hydrolyzes phospholipids such as phosphatidylcholine to produce a lipid messenger phosphatidic acid (PA), which has been

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### Table 1

| Protein | Gene | Regulation |
|---------|------|------------|
| OsDREB1s | OsDREB1 | Increased |
| OsPLDα1 | OsPLDα | Decreased |

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**Fig. 8. OsPLDα1 expression is directly regulated by OsDREB1A.** (A) Diagram shows various stress-related regulatory elements on 1.5 kb promoter regions of OsPLDα1 and AtPLDα1. Numbers indicate the starting nuclear acid of the cis-element. (B) Quantitative real-time RT-PCR analysis of chilling-induced expression of OsDREB1A and OsPLDα1 in wild-type and DREB1A RNAi transgenic plants. One-week-old seedlings were treated with or without chilling stress (12 °C) for 1 h. (C) Relative luciferase activities in rice protoplasts transformed with a pPLDα1: luciferase reporter vector together with a p35S-OsDREB1A-HA or p35S-EGFP-HA vector. Data shown in B and C represent the mean value from three independent experiments. Asterisks indicate values that differ significantly from control samples (*p* > 0.05). (D) OsDREB1A protein binds directly to the CRT/DRE motif from OsPLDα1 promoter. Upper panel shows the sequence of probe and mutant probe (mProbe) for EMSA assay. Core OsDREB1A binding sequences are underlined, and the mutated bases are marked in red. Lower panel shows mobility shift of recombinant OsDREB1A-GST protein incubated with biotin-labeled probe in the absence or presence of unlabeled wild-type or mutated probes. This experiment has been repeated four times; representative result from one experiment is shown. (E) A model showing a proposed OsPLDα1-mediated cold signaling transduction pathway.
shown to regulate cellular processes by promoting membrane fusion, altering protein-membrane interactions and modulating protein activities (39). By lipid overlay assay, we demonstrated that OsMPK6 and OsSIZ1, which are cold-stress-signaling components upstream of OsDREB1s, are able to bind specifically to PA in a dose-dependent manner, thus providing insight into the mechanism by which OsPLDα1 regulates cold signaling pathway in rice. It will be interesting to investigate whether the cellular localization or protein activity of additional components in the rice cold signaling pathway are also regulated by PA.

There are 12 and 17 PLDs encoded in Arabidopsis and rice genome, respectively. Based on differences in their biochemical and structural properties, these PLDs can be further grouped into two subfamilies: C2-PLDs (PLDα, β, γ, δ and ε) and PX/PH-PLDs (PLDζ) (39). Recent discoveries indicate that PLDs play an important role in regulating growth, development, and abiotic stress responses in plants (43). There are also studies showing the involvement of PLDs in plant cold stress responses. For example, the expression or protein activities of PLD in Arabidopsis (5), rice (44), cotton (45), Chorispora bungeana (46), and Jatropha curcas (47) have been shown to be regulated by cold stress. Suppressing the expression of AtPLDδ in Arabidopsis makes the mutant less tolerant to freezing, whereas overexpression of AtPLDδ increases freezing tolerance of the transgenic plants (34). In contrast, suppressing the expression of AtPLDα in Arabidopsis makes the plant more resistant to freezing stress (5). It remains to be determined whether the substrate selection or tissue-specific localization of AtPLDα and AtPLDδ is responsible for the distinct roles of these two PLDs in regulating freezing tolerance in plants. In this study, we found that cellular OsPLDα1 protein abundance is increased as early as 1 min after chilling treatment, suggesting a role of OsPLDα1 in early cold-regulated cellular responses. We also found that chilling treatment increases the expression of OsPLDα1. Suppressing the expression of OsDREB1A in rice plants diminished the chilling-induced increase of OsPLDα1 expression. Motif search of the 1500-bp promoter region upstream ATG of OsPLDα1 identified three potential cold-responsive CRT/DRE elements. By EMSA assay, we demonstrated OsDREB1A protein binds directly with the CRT/DRE element (-609 to -601) that is closest to the ATG translation start site. Together these results suggest that OsPLDα1 is also a COR gene, and the chilling-induced expression of OsPLDα1 could provide positive feedback regulation during the cold acclimation process.

It is interesting that OsPLDα1 and AtPLDα1 play opposite roles in regulating cold stress responses in Arabidopsis and rice plants. An alignment indicates that OsPLDα1 and AtPLDα1 are 79% identical at the amino acid level. Amino acid sequences in domains that are responsible for enzymatic activity of OsPLDα1 and AtPLDα1 are even more conservative (Fig. S3). Thus, it is unlikely that differences in enzyme activity or substrate selection contribute to this functional discrepancy between OsPLDα1 and AtPLDα1. The difference in responses to cold and freezing stresses of transgenic plants with knocking down expression level of OsPLDα1 or AtPLDα1 suggested that the dynamics of phospholipids in responding to cold treatment is differently regulated in monocots and dicots. Meanwhile, examination of the 1500-bp promoter and the 5’UTR sequence upstream of the translation start codon of the OsPLDα1 gene revealed the presence of various stress-responsive cis-elements, including three copies of cold-responsive CRT/DRE elements. This suggests OsPLDα1 might play important roles in regulating responses of rice plant to various environmental stresses. In contrast, no CRT/DRE elements and only a few stress-responsive elements were found in AtPLDα1. The difference in transcription regulation suggests that tissue-specific expression and/or cellular accumulation levels of OsPLDα1 and AtPLDα1 protein are regulated differently, which might also contribute to the different roles of OsPLDα1 and AtPLDα1 in regulating cold responses in rice and Arabidopsis.

Given the important role of OsPLDα1 in regulating cold acclimation in rice plants, it is surprising to find that the expression of OsPLDα1 is not ubiquitously distributed throughout the whole plant. Histochemical staining of GUS activity in pOsPLDα1:OsPLDα1-GUS transgenic rice plants showed the expression of OsPLDα1-GUS protein was mostly in root, tip of young leaf, and stem and hulls. Very weak GUS activity can be detected in young and old leaves grown under normal conditions. This raises the question, where is the cold signal sensed and how does locally expressed OsPLDα1 regulate cold acclimation for the whole rice plant? Interestingly, a high level of GUS staining is detected in the vascular tissue of the stem. Recent studies have discovered the existence of phospholipids, such as PC and PA, and lipid-binding proteins in phloem exudates of Arabidopsis (48). Although the presence of long distance transport of lipid signal molecules still needs to be tested, the accumulation of OsPLDα1 in vascular tissue suggests that it is possible that cold stress increases the local PA concentration in vascular tissue, which could then regulate the cold resistance of surrounding cells and tissues in rice.

In conclusion, we found that the abundance of OsPLDα1 protein is rapidly increased by cold stress treatment and that OsPLDα1 is important for cold acclimation of rice plants. It has been demonstrated that the phospholipase activity of PLDα is regulated by the intracellular calcium level and that cold stress induces a rapid increase in the intracellular calcium level (49). Based on these observations and our current results, we propose a model for an OsPLDα1-mediated cold stress signal transduction pathway in rice (Fig. 7E). In this pathway, environmental chilling stress is sensed by a still unknown mechanism, which activates the phospholipase activity of OsPLDα1, possibly by increasing the intracellular calcium level. Activated OsPLDα1 uses PC as a substrate to produce PA. Then PA binds to cold signaling components, such as OsMPK6 and OsSIZ1, altering their cellular localization and/or
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protein activity and increasing the expression of OsDREB1s. The increased level of cellular OsDREB1s then can regulate the expression of OsPLDα1, which could provide a positive feedback enhancement of cold acclimation processes.

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To whom correspondence should be addressed: Tel.: 86 311 80787594; E-mail: tangq0@mail.hebu.edu.cn.

** These authors contributed equally to this work.

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