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ORIGINAL PAPER

Overexpression of a *PLDα1* gene from *Setaria italica* enhances the sensitivity of *Arabidopsis* to abscisic acid and improves its drought tolerance

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Abstract Phospholipase D (PLD) plays an important role in various physiological processes in plants, including drought tolerance. Here, we report the cloning and characterization of the full-length cDNA of *PLDα1* from foxtail millet, which is a cereal crop with high water use efficiency. The expression pattern of the *SiPLDα1* gene in foxtail millet revealed that it is up-regulated under dehydration, ABA and NaCl treatments. Heterologous overexpression of *SiPLDα1* in *Arabidopsis* can significantly enhance their sensitivity to ABA, NaCl and mannitol during post-germination growth. Under water deprivation, overexpression of *SiPLDα1* in *Arabidopsis* resulted in significantly enhanced tolerance to drought stress, displaying higher biomass and RWC, lower ion leakage and higher survival percentages than the wild type. Further analysis indicated that transgenic plants showed increased transcription of the stress-related genes, *RD29A*, *RD29B*, *RAB18* and *RD22*, and the ABA-related genes, *ABI1* and *NCED3* under dehydration conditions. These results demonstrate that *SiPLDα1* is involved in plant stress signal transduction, especially in the ABA signaling pathway. Moreover, no obvious adverse effects on growth and development in the *35S::SiPLDα1* transgenic plants implied that *SiPLDα1* is a good candidate gene for improving crop drought tolerance.

Keywords *Setaria italica* · *PLDα1* · ABA sensitivity · Drought tolerance

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Introduction

Drought is one of the most devastating abiotic stresses limiting plant growth and crop productivity (Boyer 1982; Valliyodan and Nguyen 2006), and the plant response to drought is consequently complex. Because many genes are involved in plant responses to drought, it is important to study the functions of these stress-related genes for understanding the molecular mechanisms of stress tolerance (Zheng et al. 2010).

Phospholipase D (PLD) is a major family of lipid-hydrolyzing enzymes. Increasing evidence suggests that PLD is activated in response to various stresses, such as high salinity (Darwish et al. 2009), water deficits (Mane et al. 2007), freezing (Welti et al. 2002; Li et al. 2004), wounding (Lee et al. 1997), ethylene and abscisic acid (ABA) (Fan et al. 1997). Several PLD genes have been isolated from various plant species, such as castor bean, *Arabidopsis*, rice and maize, and their molecular characterization has highlighted their role in early signaling

events (Wang 2000). In *Arabidopsis*, there are 12 identified PLDs that are classified into six types, PLDα(3), -β(2), -γ(3), -δ(1), -ε(1) and ζ(2) (Qin and Wang 2002). Different AtPLDs have distinct functions, which explain in part the different effects of PLD in stress responses (Wang et al. 2006). For example, PLDδ promotes production under rapid dehydration and high salinity (Katagiri et al. 2001) and is also involved in freezing tolerance (Li et al. 2004), PLDζ is involved in the plant response to phosphate deficiency (Cruz-Ramirez et al. 2006), and PLDε plays a role in nitrogen signaling (Hong et al. 2009).

Previous studies have revealed that the *PLDα* genes in plants play important roles in various stresses. The *Arabidopsis* PLDα group has three members, PLDα1, -α2 and -α3. AtPLDα3 positively mediates plant responses to hyperosmotic stresses (Hong et al. 2008). Although AtPLDα2 appears to be a constitutive form of PLD, its functions in plant response to stresses have not yet been reported (Wang 2000). PLDα1 has been reported to promote ABA-mediated stomatal closure which decreases water loss in response to water deficit (Jacob et al. 1999; Sang et al. 2001; Zhang et al. 2004; Mishra et al. 2006; Hong et al. 2010). PLDα1 also plays a role in superoxide production in plants through the generation of PA as a lipid messenger (Sang et al. 2001; Welti et al. 2002). In addition, PLDα1 is involved in the regulation of the jasmonic acid (JA) signaling pathways (Wang 2000). Altogether, these results indicate that the *PLDα1* gene has important and active roles in various stresses and is a key step in the ABA signaling pathway, underscoring the significance in cloning and studying the *PLDα1*.

Foxtail millet is native to China and is regarded as an elite drought-tolerant crop (Cheng and Liu 2003) Therefore, cloning and characterization of *PLDα1* from foxtail millet may have potential benefits for genetic improvement of other cereal crops, such as rice, wheat and maize. In our previous work, we isolated a group of water stress up-regulated ESTs from foxtail millet seedlings using suppression subtractive hybridization and cDNA microarray (Zhang et al. 2007). Two of those ESTs are up-regulated by dehydration stress and aligned well with two regions of the same rice gene *OsPLDα1*. Here, we report the cloning, characteristics and biological functions of *SiPLDα1* from foxtail millet in order to provide the molecular basis for potentially exploiting this gene in genetic engineering.

Materials and methods

Plant materials, stress treatments

Foxtail millet cv. Mar51 was selected as a typical drought variety according to our previous evaluation (Zhang et al. 2005). Seeds were surface-sterilized in 3% sodium hypochloride for 20 min and rinsed 15 times (1 min/each) in distilled water. After 3 days of germination, the seeds were planted in pots with a mixture of peat/forest and vermiculite (1:1 v/v). Plants were grown in the greenhouse (28°C day/20°C night, 14 h photoperiod, natural lighting, 70% relative humidity). When the seedlings were 5 weeks old, the stress treatments were performed as described by EI Maarouf et al. (1999), with minor modifications. In dehydration experiments, leaves were cut off and dehydrated on filter paper as described by Yamaguchi-Shinozaki and Shinozaki (1994) at ambient temperature (26°C) and under light for 0, 0.5, 1, 2, 4 and 6 h. The effect of ABA on the detached leaves was tested by plunging them into a 100 μM ABA solution for 0, 0.5, 1, 2, 4 and 6 h. For NaCl stress, the detached leaves were submerged into a 250 mM NaCl solution for 0, 0.5, 1, 2, 4 and 6 h. The treated leaves were sampled at the indicated times and stored at −80°C for RNA extraction.

Cloning of the *Setaria italica PLDα1* full-length cDNA

In a previously established PEG-induced subtracted cDNA library, two ESTs sequences, which encode the same PLDα1 protein, RCRST0_005783 (near 5′ end, Accession No. EC612614) and CL185Contig1 (near 3′ end, Accession No. EC612075), were shown to be induced by PEG treatment (Zhang et al. 2007). Here, the RCRST0_005783 and CL185Contig1 sequences were used as templates for designing RACE amplification primers. Amplification of 5′ RACE and 3′ RACE was performed using the First-Choice RLM-RACE Kit (Ambion). The 5′- and 3′-nested PCR primers are listed in Supplemental Table S1. The amplified RACE products were purified and cloned into the pGEM-T Easy Vector (Promega) and sequenced. After assembling the 5′ RACE and 3′ RACE fragments of *SiPLDα1* cDNA, the ORF sequence was amplified with the specific primers, which are listed in Supplemental Table S1. The PCR products were cloned into the pGEM-T Easy Vector and sequenced. The complete sequence was deposited in GenBank under the accession number (GU480026).

Vector construction and *Arabidopsis* transformation

The full-length cDNA of the *SiPLDα1* gene was amplified using the *SiPLDα1* 5′ forward primer 5′-GGATCCTCTA-GACTCCCGATCCTCAACAGCCTCT-3′ with a *Bam*HI restriction site and the *SiPLDα1* 3′ reverse primer 5′-GT GAGCTCCAGTCTGCCCTCTATGTGGTGA-3′ with a *Sac*I restriction site. The *SiPLDα1* gene and the 35S promoter were subcloned into the plant expression vector pBI121 using the *Bam*HI and *Sac*I restriction sites. The

resultant construct, named pBISiPLDα1, contains *nptII* as the selectable marker, which can be used for screening transgenic plants by exploiting its resistance to kanamycin. These constructs were mobilized into *Agrobacterium tumefaciens* GV3101 and then introduced into wild-type *Arabidopsis* ecotype (Col-0) by floral dip (Clough and Bent 1998). Homozygous transgenic lines were selected in the T3 generation and used for further assays.

Germination study and stress treatment

Arabidopsis thaliana, ecotype Col-0, was used in this study as the transformation receptor. *Arabidopsis* seeds were sterilized and kept for 3 days at 4°C in the dark to break dormancy. After 1 week, the seedlings were transferred to a pot filled with a mixture of peat/forest soil and vermiculite (1:1 v/v) in a greenhouse at 22°C, with a light intensity of 50 μmol m^{-2} s^{-1} and 70% RH under a 16 h-light photoperiod.

For germination analysis, approximately 30 seeds each from the wild-type and transgenic lines were surface-sterilized and plated on MS medium supplemented with different concentrations of ABA, NaCl, or mannitol, incubated at 4°C for 3 days and then transferred to 22°C under long-day light conditions. Germination (emergence of radicles) and green cotyledons were scored at the third and seventh day, respectively. All experiments were performed in triplicate, and the SD is shown as an error bar. For dehydration stress, the water solution was supplemented with mannitol to a final concentration of 350 mM, and the plants were placed in a container with a mannitol solution for 0, 2 and 4 h to simulate dehydration stress. For drought-stress treatment, 3-week-old plants were transferred to a growth chamber at 22°C, with no further addition of water for 12 days. Samples were collected and stored at −80°C for RNA extraction. The biomass of transgenic and wild-type plants was estimated by measuring fresh weights of plants.

Drought resistance evaluation

For drought resistance evaluation, 30 seedlings each of transgenic and wild-type plants were grown in pots (10-cm diameter) with normal watering every 3 days and a commercial fertilizer every 2 weeks before water was withheld. After 2 weeks, the plants were divided into two groups for stress treatments. One group was subjected to drought stress by withholding water, and a control group was watered normally. After 2 weeks of drought treatment, all of the pots were rewatered simultaneously, and the survival rate was scored 7 days later. Plants were considered dead if there was no growth 7 days after watering.

Relative water content measurements

The relative water content (RWC) was essentially determined as reported by Gaxiola et al. (2001). The RWC values of transgenic and WT plants were determined during a 10-day drought-stress experiment. Leaves of drought- and non-stressed transgenic and WT seedlings were carefully excised, and their fresh weight was immediately scored. After being floated on deionized water at 4°C for overnight, their rehydrated weight was determined. Finally, they were dried in an oven at 80°C overnight and weighed. The RWC was calculated as RWC = (fresh weight − dry weight)/(rehydrated weight − dry weight).

Measurement of ion leakage

To measure the ion leakage, leaves were detached and rinsed with distilled water and then immersed in 15 ml of distilled water in glass tubes. After degassing under vacuum for 30 min to remove air bubbles on the leaf surface, samples were incubated with gentle agitation for 3 h (Fan et al. 1997). The initial conductivity was measured with a conductivity meter, and then the samples were boiled in a water bath for 20 min. The total conductivity was measured again after cooling to room temperature. The ion leakage is expressed as a percentage of the initial conductivity over the total conductivity.

RNA isolation, semiquantitative RT-PCR and quantitative real-time PCR

Total RNA was extracted by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 100 mg of young leaves of foxtail millet and *Arabidopsis* seedlings, and then treated with RNase-free DNase to remove contaminating DNA. For the RT-PCR reaction, 1 μg of DNase-treated RNA samples from each sample was used for the reverse transcription reaction. Subsequently, the first-strand cDNA (1 μl) was used as templates for PCR amplification. The gene-specific primers for semiquantitative RT-PCR were 5783 3′ RACE primer 5′-CATGATGACTTCCACC AGCCA-3′ and CL185 5′ RACE primer 5′-CAGG-GAAGCCAAAAGCAGCACCA-3′. Real-time PCR analysis was performed and statistically analyzed as described by Livak and Schmittgen (2001). Triplicate quantitative assays were performed on 1 μl of each cDNA dilution with SYBR Green Master Mix and an ABI 7300 sequence detection system. The gene-specific primers for *SiPLDα1* in the foxtail millet seedlings were 5′-CATGATG ACTTCCACCAGCCA-3′ and 5′-CAGGGAAGCCAAAA GCAGCACCA-3′. The gene-specific primers for the real-time RT-PCR in *Arabidopsis* plants are listed in Supplemental Table S2. Actin from foxtail millet and *His2A*

from *Arabidopsis* were used as internal controls to quantify the relative transcript levels of each target gene in our assays.

Results

Isolation and characterization of full-length cDNA of *SiPLDα1*

From the subtracted foxtail millet cDNA libraries, as described above, two ESTs (RCRST0_005783 and CL185Contig1) were isolated as differentially expressed sequences in response to PEG stress (Zhang et al. 2007). According to the BLAST results, RCRST0_005783 and CL185Contig1 align to two regions of the same rice gene *OsPLDα1*, leading us to speculate that the two fragments may belong to the same cDNA of foxtail millet. The two regions were separated by a 4-bp nucleotide sequence. Using the specific 5783 3′ RACE primer 5′-CATGATGACTT CCACCAGCCA-3′ and CL185 5′ RACE primer 5′-CAG GGAAGCCAAAAGCAGCACCA-3′, a 292-bp fragment was amplified by RT-PCR, which covered partial sequences from the two ESTs and the gap sequence between them. Then, 5′ RACE and 3′ RACE were carried out to obtain the 5′ and 3′ end sequences of the *PLDα1* cDNA. Finally, the open reading frame of this gene was amplified by RT-PCR. Since this gene was the first member of *PLDα* family from *Setaria italica*, we named it as *SiPLDα1*. The *SiPLDα1* gene (Accession No: GU480026) had a 2,938-bp full-length cDNA with an open reading frame of 2,436 nucleotides, which encodes 811 amino acids with a predicted molecular mass of 92.0 kDa and a pI of 5.41 (Fig. 1). A putative conserved domain detection tool (http://www.ebi.ac.uk/InterProScan/) indicated that SiPLDα1 includes a C2 domain (Calcium/lipid-binding domain) and two conserved HXKXXXXD domain (X represents any amino acid). The SiPLDα1 has 92, 90, 89 and 78% identities with ZmPLDα, OsPLDα1, TaPLDα and AtPLDα1, respectively (Fig. 1).

Phylogenetic analysis of *SiPLDα1* gene

To analyze the closeness of *SiPLDα1* gene with related *PLD* genes, a phylogenetic tree was constructed based on their amino acid sequences. Based on this phylogram, the *SiPLDα1* gene is most similar to the *ZmPLDα1* gene among all of the plant PLDs. In the 12 *Arabidopsis* PLD genes, *SiPLDα1* is more similar to *AtPLDα1* than the others (Fig. 2).

Expression of the *SiPLDα1* in response to various abiotic stresses

Real-time quantitative RT-PCR was used to analyze the expression pattern of *SiPLDα1* under various stress treatments. The results showed that *SiPLDα1* expression in shoots is strongly up-regulated by ABA, NaCl and dehydration stresses. As shown in Fig. 3a, the transcript of *SiPLDα1* began to increase after 0.5 h of drought treatment and gradually accumulated up to the highest level after 6 h of treatment. In the case of ABA treatment, expression of the *SiPLDα1* gene was induced at 1 h of ABA treatment. The accumulation of the *SiPLDα1* transcripts increased up to 6 h, but a lower expression level at 4 h was observed in comparison to that after 2 h of treatment (Fig. 3b). Under NaCl treatment, the *SiPLDα1* expression level also continuously increased with the stress time, except there was less induction after the 2 h treatment compared to that after 1 h (Fig. 3c). Furthermore, expression of the *SiPLDα1* gene under the dehydration treatment was induced more sharply than under the ABA and NaCl treatments (Fig. 3). The *SiPLDα1* transcription level was very low in normal growth conditions, suggesting that the *SiPLDα1* gene plays an active role in response to drought and osmotic stress in the shoots of foxtail millet.

Molecular characterization of the *SiPLDα1*-transgenic *Arabidopsis* lines

The full-length ORF of the foxtail millet *SiPLDα1* was cloned into the pBI121 vector under control of the 35S promoter (Fig. 4a). The *SiPLDα1* gene was transformed into wild-type *Arabidopsis thaliana* by the floral dip method. More than ten *35S::SiPLDα1* transgenic plantlets were selected on culture medium with 50 μg/ml of kanamycin. Six independent T3 homozygous lines showed high expression of *SiPLDα1* by semi-quantitative RT-PCR of young leaves (Fig. 4b). A quantitative real-time PCR analysis was carried out to determine the expression level of the *SiPLDα1*-transgenic lines. The transgenic events L2 and L32, respectively, representing low and high levels of *SiPLDα1* gene expression, were selected for further stress-tolerance tests (Fig. 4c). Genetic analyses of both L2 and L32 also showed that *SiPLDα1* was co-segregated with the kanamycin resistance in a 3:1 ratio, indicating T-DNA insertion in the two lines at a single locus.

Overexpression of *SiPLDα1* enhances the sensitivity of *Arabidopsis* to ABA and osmotic stress

To investigate whether *SiPLDα1* overexpression affects the ABA response, we conducted analyses of germination and early seedling growth (i.e., radicle emergence) of *35S::SiPLDα1* and wild-type *Arabidopsis* plants. There was no difference in seed germination between the wild-type and transgenic plants under ABA treatment (data not shown). However, altered ABA sensitivity was observed during post-germination growth. In the presence of exogenous ABA, the

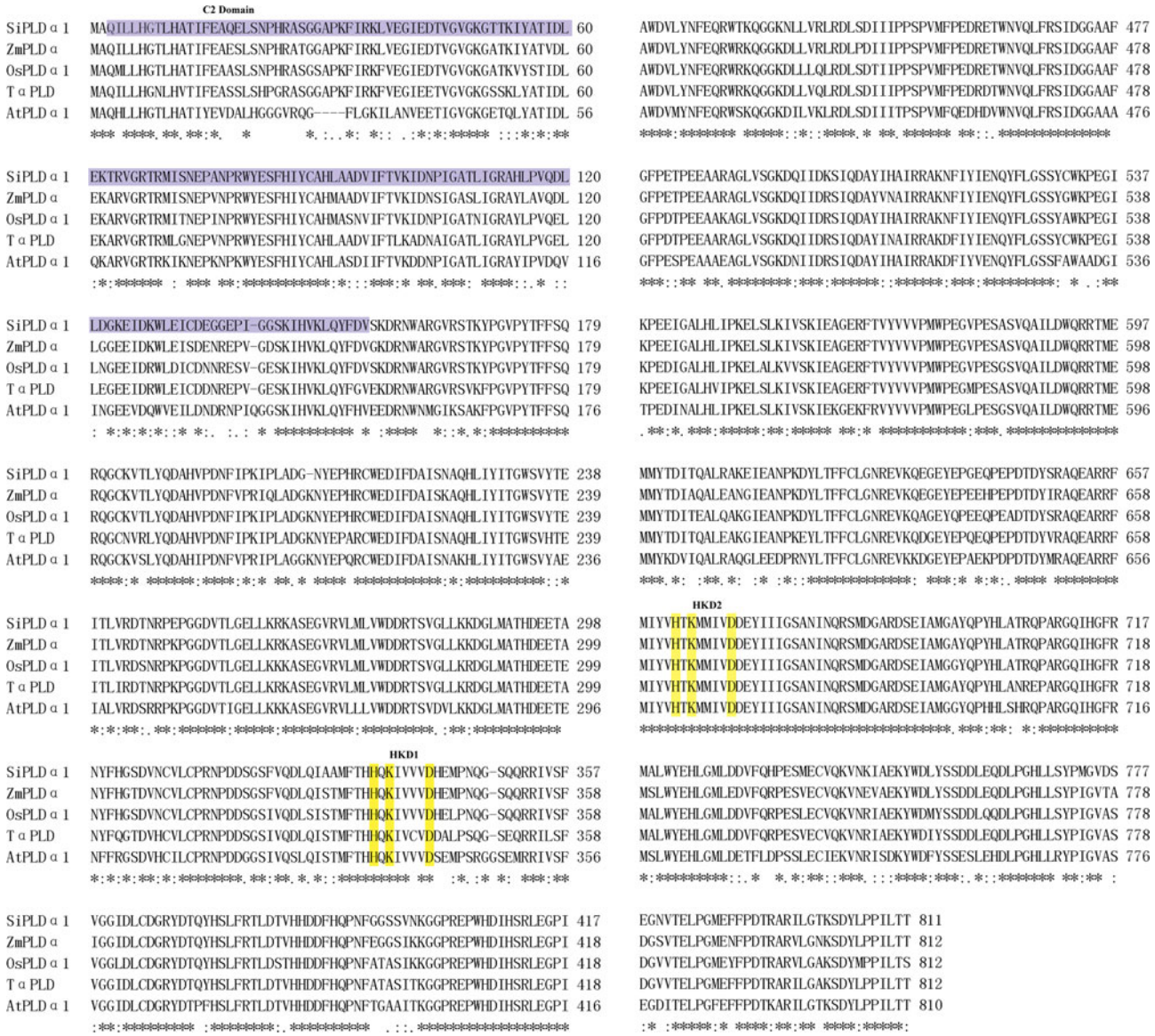

Fig. 1 Alignment of amino acid sequences of SiPLDα1 with PLDs from other plant species. The predicted SiPLDα1 protein sequence was compared with PLD sequences from rice (*OsPLD*), maize (*ZmPLD*), wheat (*TaPLD*) and *Arabidopsis* (*AtPLD*). Alignment of the amino acid sequences was performed with the CLUSTALW(1.83) program on the EBI server. Species abbreviations are: Si, *Setaria italica*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Zea mays*; Ta, *Triticum aestivum*

seedling growth of both wild-type and *35S::SiPLDα1* were inhibited significantly, but transgenic seeds were inhibited to a greater extent (Fig. 5). For example, on 0.5 μM ABA MS medium, the *35S::SiPLDα1* seeds revealed only 13% green cotyledon rates, whereas the wild-type seeds displayed approximately 30% with green cotyledons, demonstrating that the growth of the aerial parts of *35S::SiPLDα1* seedlings was more sensitive to ABA.

To investigate the response to osmotic stress, germination and early growth in media supplemented with 100 mM NaCl or 300 mM mannitol were performed for *35S::SiPLDα1* transgenic and wild-type lines. No significant differences in germination rates were observed between the wild-type and *35S::SiPLDα1* seeds grown on MS medium with 100 mM NaCl and 300 mM mannitol. In contrast, the post-germination seedling growth of wild-type and transgenic lines was affected significantly by 100 mM NaCl and 300 mM mannitol (Fig. 5). Transgenic lines showed severe inhibition of early growth by osmotic stress than the wild-type seeds. As shown in Fig. 5, the numbers of green cotyledon *35S::SiPLDα1* seeds were significantly lower than those of wild type (Fig. 5).

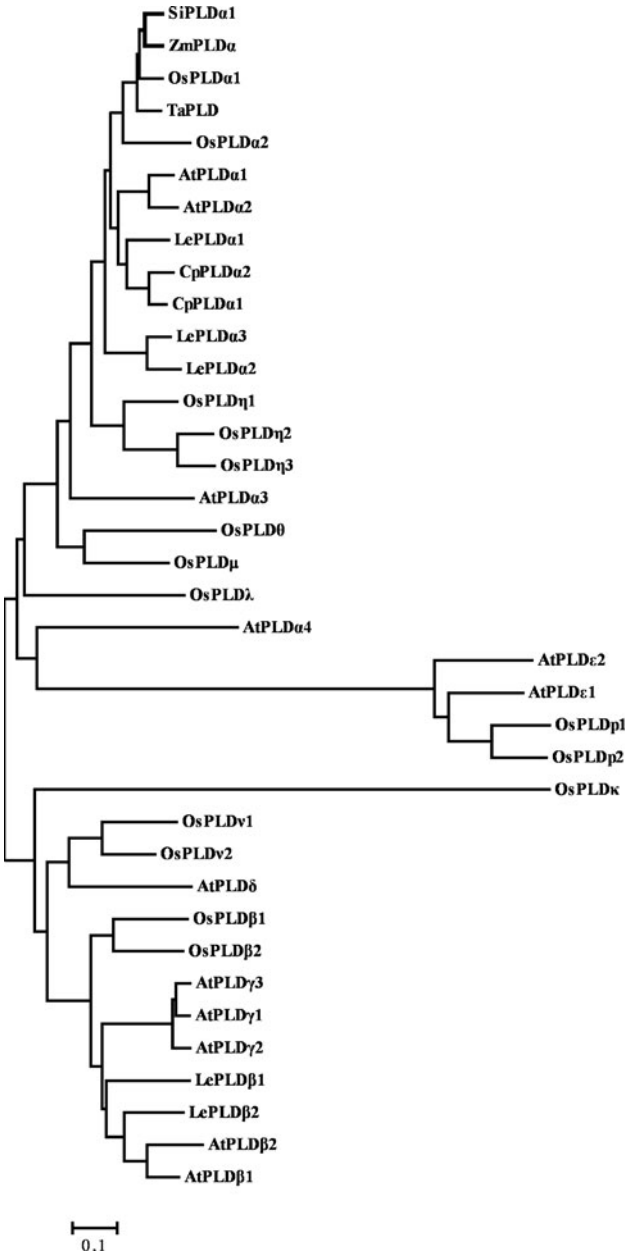

Fig. 2 Phylogenetic tree analysis of the predicted relationships between the SiPLDα1 protein of foxtail millet and those of other plant species. The full-length amino acid sequences of each protein were aligned using ClustalW. Fifteen rice *PLD* genes (*OsPLD*), five tomato genes (*LePLD*), one wheat PLD (*TaPLD*), one maize PLD (*ZmPLD*) gene and two resurrection plant PLD (*CpPLD*) genes were compared with *SiPLDα1*. Si, *Setaria italica*; At, *Arabidopsis thaliana*; Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Zm, *Zea mays*; Ta, *Triticum aestivum*; Cp, *Craterostigma plantagineum*

Overexpression of *SiPLDα1* improves the drought tolerance of *Arabidopsis*

Under normal growth conditions, the transgenic plants did not show any obvious morphological or developmental abnormalities (Fig. 6a). However, when subjected to drought stress for 2 weeks, as shown in Fig. 6, the wild-type plants showed severe wilting and chlorosis on the rosette leaves, whereas the transgenic plants were turgid and their leaves remained green; at the same time, the transgenic lines had higher biomasses than the wild type under drought stress (Fig. 6b). The leaves of transgenic lines also had higher RWC and lower relative electrolyte leakage under drought stress (Fig. 6c, d). These results indicated that overexpression of *SiPLDα1* in *Arabidopsis* improved their drought tolerance. Moreover, 14-day-old plants grown on soil were deprived of water for 2 weeks. After re-watered, the transgenic plants showed a stronger growth recovery and higher survival rate than the wild-type plants (Table 1).

Overexpression of *SiPLDα1* affects the expression of drought stress-related genes

To elucidate the molecular mechanism of drought tolerance, quantitative RT-PCR experiments were conducted to compare the transcript levels of nine drought stress-related genes between WT and *35S::SiPLDα1* lines using gene-specific primers (Supplemental Table S2). The results revealed that the ABA and dehydration stress-inducible genes (i.e., *RD29A*, *RD29B*, *RD22* and *RAB18*) showed higher expression levels in both the wild type and transgenic plants under dehydration, but the expression levels of these genes in transgenic seedlings were significantly higher than in WT (Table 2). For example, under dehydration, the relative expression level of *RD29B* drastically increased, reaching 237.22 folds in transgenic seedlings, but only 124.82 folds in wild type after 2 h of dehydration. However, the expression levels of *P5CS1*, which is the rate-limiting enzyme in the biosynthesis of Proline, showed no significant difference between WT and transgenic plants. Except for *RD29B*, we observed a consistent trend that the expression levels of transgenic plants were significantly lower than WT under normal conditions.

Discussion

Overexpression of *SiPLDα1* in *Arabidopsis* increased the drought tolerance

Previous studies have revealed that the *PLDα* genes in plants play an important role in various stresses. Here we reported the cloning of the *SiPLDα1* full-length cDNA from foxtail millet and analysis of its expression pattern in various stresses. The results showed that the expression of *SiPLDα1* is significantly induced by dehydration, NaCl and ABA treatments, which is consistent with previous reports in other species (Ryu and Wang 1995; EI Maarouf et al.

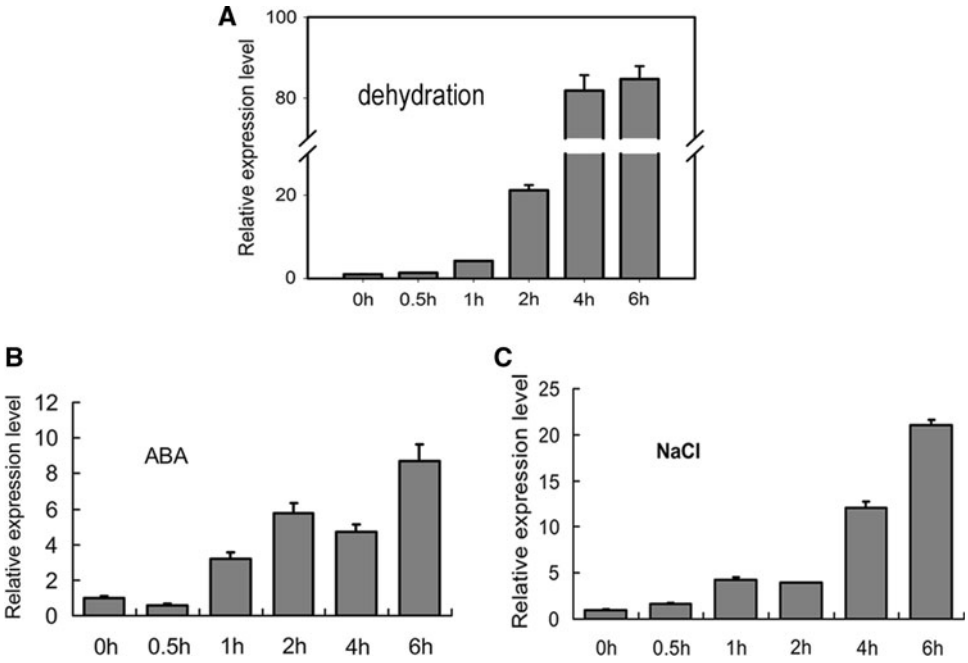

Fig. 3 Real-time RT-PCR analysis of *SiPLDα1* gene expression in foxtail millet shoots treated with ABA, NaCl and dehydration stress. **a** *SiPLDα1* expression profiles in foxtail millet shoots treated with dehydration stress. **b** *SiPLDα1* expression profiles of foxtail millet shoots treated by ABA. **c** *SiPLDα1* expression profiles of foxtail millet shoots treated by NaCl. *Actin* was used as an internal control. Data represent mean ± SD ($n = 3$ experiments)

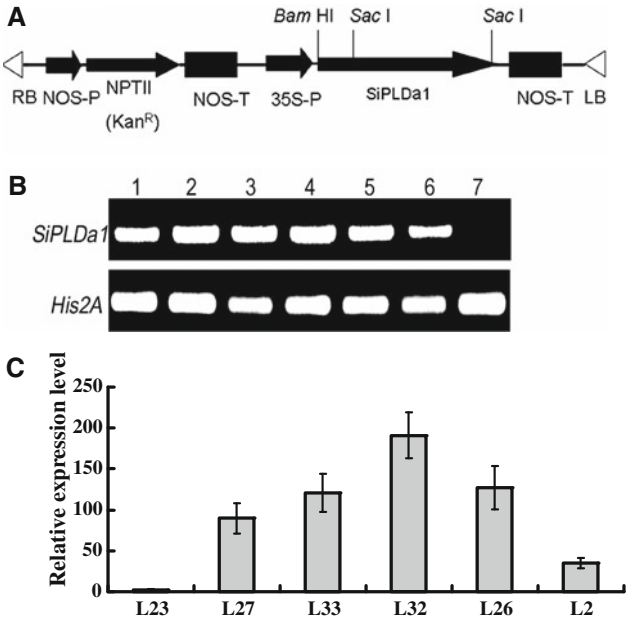

Fig. 4 Expression analysis of *SiPLDα1* in the transgenic and WT plants. **a** Schematic diagrams of the pBISiPLDα1 construct. The *SiPLDα1* gene was inserted between the 35S CaMV promoter and the nopaline synthase promoter terminator (NOS-T), and the *nptII* gene was flanked by the nopaline synthase promoter (NOS-P) and terminator (NOS-T). **b** Analysis by semi-quantitative RT-PCR of transgenic *SiPLDα1* lines. Specific 292-bp PCR products were detected in six transgenic lines, 1, L23; 2, L27; 3, L33; 4, L32; 5,L26; 6, L2; 7,WT. *His2A* was amplified by RT-PCR as an internal control. **c** Analysis by real-time PCR of transgenic *SiPLDα1* lines. Relative expression levels of *SiPLDa1* in six transgenic lines were calculated according to $2^{-\Delta CT}$ methods based on *Arabidopsis His2A* as internal controls

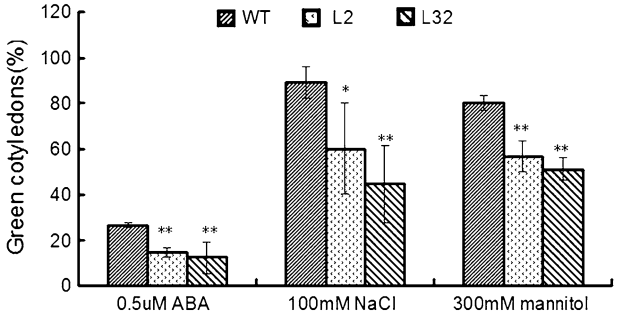

Fig. 5 Early seedling growth of transgenic and WT seeds in response to ABA, NaCl and mannitol. Seeds were germinated on MS agar plates supplemented with 0.5 μM ABA, 100 mM NaCl and 300 mM mannitol, and the green cotyledons were scored 7 days after germination. *Bars* indicate SD ($n = 3$). * and ** indicate significant and highly significant differences from the wild type at $P < 0.05$ and $P < 0.01$, respectively, by Student *t* tests

1999; Darwish et al. 2009). The results also showed that the overexpression of *SiPLDα1* in *Arabidopsis* significantly increased the drought tolerance of transgenic plants, which was revealed by less chlorosis and growth inhibition, higher biomass and RWC in transgenic plants than in the wild type.

Generally, increased drought tolerance accompanies hypersensitivity to ABA during seed germination and early seedling growth (Hu et al. 2006; Ko et al. 2006; Dai et al. 2007). The plant hormone ABA is a principal stress hormone and is involved in many aspects of plant responses to abiotic stresses, such as drought and salt stress (Yamaguchi-Shinozaki and Shinozaki 1994). Several reports have

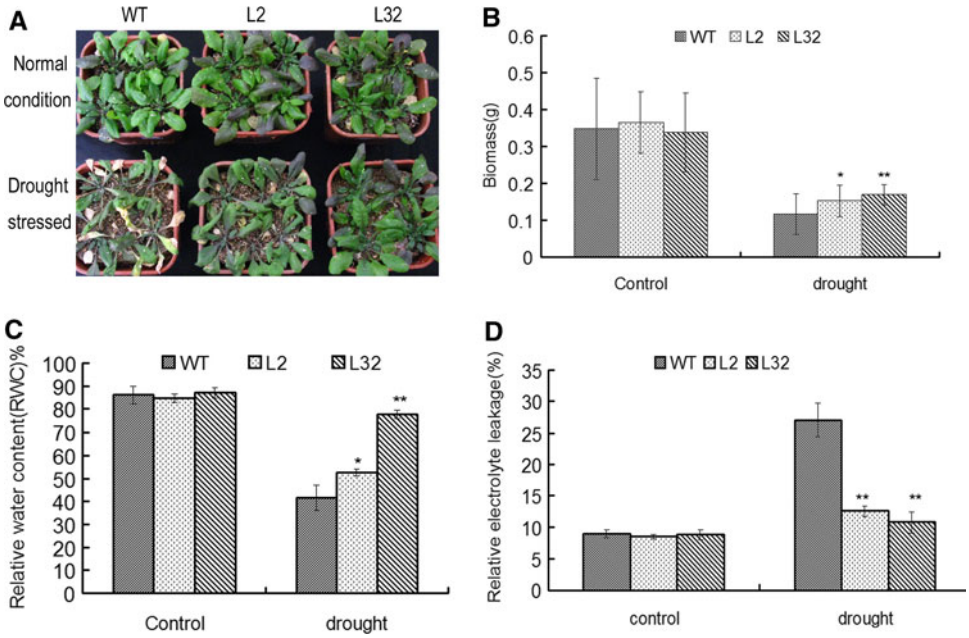

Fig. 6 Drought stress tolerance of *35S::SiPLDα1* transgenic plants. **a** Drought-tolerant test after 3-week-old plants was stopped watering for 12 days. **b** Biomass comparison of wild-type and transgenic plants under drought stress. **c** Relative water content of wild-type and transgenic plants under water-deficit stress. Each of the ten wild-type and transgenic plants were stressed for 12 days. One leaf per plant (three plants of each group) was removed from the seedlings for analysis. **d** Relative electrolyte leakage of *SiPLDα1* transgenic plants and wild type in response to drought stress. The leaves were harvested for measurement. *Bars* indicate SD ($n = 3$); * and ** indicate significant and highly significant differences from the wild type at $P < 0.05$ and $P < 0.01$, respectively, by Student *t* tests

Table 1 Survival rates of transgenic and WT plants under severe drought stress

Lines	Total number of plants	Number of survival plants[a]	Percentage of survival plants (%)[b]
WT	30	6	20.0
L02	30	29	96.7
L32	30	30	100

[a] 2-week-old soil-grown *Arabidopsis* plants were withheld from water for 2 weeks, then rewatered. Plants were considered dead if all of the leaves were brown and there was no growth 7 days after rewatering

[b] Survival rates (%) were calculated from the number of survival plants over the total number of plants

shown that osmotic stress delays seed germination and arrests early seedling development, primarily through ABA action. Usually, the ABA level is increased under osmotic stress, and transgenic lines are generally more sensitive to ABA than the wild type (Saez et al. 2004, 2006). In this study, we observed the hypersensitive phenotypes of *SiPLDα*-transgenic plants conferred under ABA, NaCl and mannitol treatment (Fig. 4). Therefore, we suggest that the increased drought tolerance in *35S::SiPLDα1* transgenic plants is carried out by their high sensitivity to the ABA signal.

To elucidate the molecular mechanism of *SiPLDα1* in the drought response, we analyzed the expression levels of nine drought stress-related genes involved either in stress tolerance or in ABA biosynthesis and signaling processes. It is well known that *RD29A*, *RD29B*, *RD22* and *RAB18* are induced by dehydration- and ABA-responsive genes, and that the higher expression of stress-responsive genes improves plant tolerance under stress (Shinozaki and Yamaguchi-Shinozaki 1997; Zhu 2002). In our study, these genes showed up-regulation in both wild-type and transgenic plants under dehydration, but their expression levels in *35S::SiPLDα1* seedlings are significantly higher than those in wild type (Table 2). The transcript levels of ABA biosynthesis and signaling genes (*NCED3* and *ABI1*) were higher in *35S::SiPLDα1* seedlings than in wild type after drought treatment, which may suggest that *SiPLDα1* is an upstream gene regulating the ABA biosynthesis and signaling transduction. Stress-responsive genes, including the alcohol dehydrogenase gene *ADH1* and *KIN2*, were found up-regulated by ABA and drought stress, and their transcript levels were also higher in the *35S::SiPLDα1* transgenic seedlings than in the wild-type plants under drought stress. *P5CS1* is the rate-limiting enzyme in the

Table 2 Relative expression levels of stress-responsive genes in response to dehydration treatment

Gene	Plant lines	Dehydration stress[a]		
		0 h	2 h	4 h
RD22	WT	1.00 ± 0.08	1.87 ± 0.13	1.83 ± 0.15
	35S::SiPLDa1	0.83 ± 0.03	2.70 ± 0.11	2.35 ± 0.10
RD29A	WT	1.00 ± 0.13	45.33 ± 3.14	15.00 ± 1.73
	35S::SiPLDa1	0.36 ± 0.04	70.34 ± 5.49	16.77 ± 0.66
RD29B	WT	1.00 ± 0.05	124.82 ± 3.52	71.10 ± 4.19
	35S::SiPLDa1	1.94 ± 0.07	237.22 ± 42.64	60.76 ± 17.06
RAB18	WT	1.00 ± 0.10	5.42 ± 0.58	12.71 ± 0.50
	35S::SiPLDa1	0.29 ± 0.01	17.23 ± 1.99	13.08 ± 1.81
ABI1	WT	1.00 ± 0.07	15.29 ± 0.62	4.39 ± 0.18
	35S::SiPLDa1	0.47 ± 0.1	21.65 ± 1.69	9.00 ± 0.74
NCED3	WT	1.00 ± 0.28	20.56 ± 5.05	8.05 ± 1.11
	35S::SiPLDa1	0.46 ± 0.02	38.00 ± 6.71	19.80 ± 2.45
ADH1	WT	1.00 ± 0.04	3.36 ± 0.11	4.23 ± 0.21
	35S::SiPLDa1	0.84 ± 0.03	11.59 ± 0.36	7.50 ± 0.11
KIN2	WT	1.00 ± 0.07	5.47 ± 0.40	3.49 ± 0.07
	35S::SiPLDa1	0.98 ± 0.03	4.95 ± 0.14	5.61 ± 1.30
P5CS1	WT	1.00 ± 0.11	3.52 ± 0.61	2.96 ± 0.11
	35S::SiPLDa1	0.28 ± 0.05	3.74 ± 0.26	2.58 ± 0.10

[a] 0, 2 and 4 h represent samples at 0, 2 and 4 h after dehydration treatment, respectively

biosynthesis of proline and it is up-regulated by drought stress (Hu et al. 1992; Yoshiba et al. 1999), but the expression of *P5CS1* in our transgenic seedlings did not display obvious change. Interestingly, the nine drought stress-related genes except for *RD29B* were down-regulated in transgenic lines to various degrees under normal conditions, suggesting that the expression levels of these genes are partially repressed by *SiPLDα1*, similar to previously reported results for *ABF2* (Uno et al. 2000; Kim et al. 2004). However, the mechanism of this is still unknown.

The application of SiPLDα1 in crop improvement for drought tolerance

In this study, we transferred the *SiPLDα1* from foxtail millet into *Arabidopsis* and certified that the *SiPLDα1* confer drought tolerance in *Arabidopsis* plants, a feature that can be attributed to the faster/stronger activation of signaling pathways downstream of *SiPLDα1*. Unlike other transcription factors, such as DREB factors (Kasuga et al. 1999) and ABFs (Kim et al. 2004), constitutive overexpression of *SiPLDα1* had no obvious adverse effects on the growth and development of transgenic plants. These results indicate that *SiPLDα1* has great potential in the genetic improvement of plants for drought tolerance. It will be important to investigate the overexpression of the *PLDα1* gene in other plants, such as maize, wheat and rice, with the aim of improving crop tolerance to multiple environmental stresses. A stress-inducible promoter may be necessary to drive *SiPLDα1* overexpression, because constitutive expression of *SiPLDα1* is likely to compromise the tolerance of crop plants (Agarwal et al. 2006).

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References

Agarwal M, Hao Y, Kapoor A, Dong C, Fujii H, Zheng X, Zhu J (2006) A R2R3-type MYB transcription factor is involved in the cold-regulation of CBF genes and in acquired freezing tolerance. J Biol Chem 281:37636–37645

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Cheng R, Liu Z (2003) Evolution of breeding objectives of foxtail millet and its developing tendency in China. J Hebei Agricult Sci 7:95–98

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Cruz-Ramirez A, Oropeza-Aburto A, Razo-Hernandez F, Ramirez-Chavez E, Herrera-Estrella L (2006) Phospholipase Dζ2 plays an important role in extraplastidic galactolipid biosynthesis and phosphate recycling in Arabidopsis roots. Proc Natl Acad Sci USA 103:6765–6770

Dai X, Xu Y, Ma Q, Xu W, Wang T, Xue Y, Chong K (2007) Overexpression of an R1R2R3 MYB gene, OsMYB3R–2, increases tolerance to freezing, drought, and salt stress in transgenic Arabidopsis. Plant Physiol 143:1739–1751

Darwish E, Testerink C, Khalil M, El-Shihy O, Munnik T (2009) Phospholipid signaling responses in salt-stressed rice leaves. Plant Cell Physiol 50(5):986–997

EI Maarouf H, Zuily-Fodil Y, Gareil M, d'Arcy-Lameta A, Pham-Thi AT (1999) Enzymatic activity and gene expression under water stress of phospholipase D in two cultivars of *Vigna unguiculata* L. Walp. differing in drought tolerance. Plant Mol Biol 39(6):1257–1265

Fan L, Zheng S, Wang X (1997) Antisense suppression of phospholipase D alpha retards abscisic acid- and ethylene-promoted senescence of postharvest Arabidopsis leaves. Plant Cell 9:2183–2196

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc Natl Acad Sci USA 98:11444–11449

Hong Y, Pan X, Welti R, Wang X (2008) Phospholipase Dα3 is involved in the hyperosmotic response in *Arabidopsis*. Plant Cell 20(3):803–816

Hong Y, Devaiah SP, Bahn SC, Thamasandra BN, Li M, Welti R, Wang X (2009) Phospholipase D epsilon and phosphatidic acid enhance Arabidopsis nitrogen signaling and growth. Plant J 58(3):376–387

Hong Y, Zhang W, Wang X (2010) Phospholipase D and phosphatidic acid signalling in plant response to drought and salinity. Plant Cell Environ. doi:10.1111/j.1365-3040.2009.02087.x

Hu CA, Delauney AJ, Verma DP (1992) A bifunctional enzyme (delta 1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA 89:9354–9358

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103:12987–12992

Jacob T, Ritchie S, Assmann SM, Gilroy S (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. Proc Natl Acad Sci USA 96:12192–12197

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17:287–291

Katagiri T, Takahashi S, Shinozaki K (2001) Involvement of a novel *Arabidopsis* phospholipase D, AtPLDδ, in dehydration inducible accumulation of phosphatidic acid in stress signaling. Plant J 26(6):595–605

Kim S, Kang JY, Cho DI, Park JH, Kim SY (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. Plant J 40(1):75–87

Ko JH, Yang S, Han K (2006) Upregulation of an Arabidopsis RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis. Plant J 47:343–355

Lee S, Suh S, Kim S, Crain R, Kwak JM, Nam HG, Lee Y (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. Plant J 12(3):547–556

Li W, Li M, Zhang W, Wang X (2004) The plasma membrane bound phospholipase Dδ enhances freezing tolerance in *Arabidopsis thaliana*. Nat Biotechnol 22:427–433

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25:402–408

Mane SP, Vasquez-Robinet C, Sioson AA, Heath LS, Grene R (2007) Early PLDa-mediated events in response to progressive drought stress in *Arabidopsis*: a transcriptome analysis. J Exp Bot 58(2):241–252

Mishra G, Zhang W, Deng F, Zhao J, Wang X (2006) A bifurcating pathway directs abscisic acid effects on stomatal closure and opening in Arabidopsis. Science 312:264–266

Qin C, Wang X (2002) The Arabidopsis phospholipase D family. Characterization of a calcium-independent and phosphatidylcholine selective PLDζ-1 with distinct regulatory domains. Plant Physiol 128:1057–1068

Ryu BS, Wang X (1995) Expression of phospholipase D during castor bean leaf senescence. Plant Physiol 108:713–719

Saez A, Apostolova N, Gonzalez-Guzman M, Gonzalez-Garcia MP, Nicolas C, Lorenzo O, Rodriguez PL (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signaling. Plant J 37:354–369

Saez A, Robert N, Maktabi MH, Schroeder JI, Serrano R, Rodriguez PL (2006) Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant Physiol 141:1389–1399

Sang Y, Zheng S, Li W, Huang B, Wang X (2001) Regulation of plant water loss by manipulating the expression of phospholipase D alpha. Plant J 28:135–144

Shinozaki K, Yamaguchi-Shinozaki K (1997) Gene expression and signal transduction in water-stress response. Plant Physiol 115:327–334

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity. Proc Natl Acad Sci USA 97:11632–11637

Valliyodan B, Nguyen H (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin Plant Biol 9:1–7

Wang X (2000) Multiple forms of phospholipase D in plants: the gene family, catalytic and regulatory properties, and cellular functions. Prog Lipid Res 39(2):109–149

Wang X, Devaiah SP, Zhang W, Welti R (2006) Signaling functions of phosphatidic acid. Prog Lipid Res 45:250–278

Welti R, Li W, Li M, Sang Y, Biesiada H, Zhou H, Rajashekar CB, Williams TD, Wang X (2002) Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. J Biol Chem 277:31994–32002

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salinity stress. Plant Cell 6:251–264

Yoshiba Y, Nanjo T, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Stress-responsive and developmental regulation of Delta(1)- pyrroline-5-carboxylate synthetase 1 (P5CS1) gene expression in *Arabidopsis thaliana*. Biochem Biophys Res Commun 261:766–772

Zhang W, Qin C, Zhao J, Wang X (2004) Phospholipase D alpha1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc Natl Acad Sci USA 101:9508–9513

Zhang J, Wang M, Bai Y, Jia J, Wang G (2005) Rapid evaluation on the drought tolerance of foxtail millet at seedling stage. J Plant Genet Resour 6:59–62 (in Chinese)

Zhang J, Liu T, Fu J, Zhu Y, Jia J, Zheng J, Zhao Y, Zhang Y, Wang G (2007) Construction and application of EST library from *Setaria italica* in response to dehydration stress. Genomics 90:121–131

Zheng J, Fu J, Gou M, Huai J, Liu Y, Jian M, Huang Q, Guo X, Dong Z, Wang H, Wang G (2010) Genome-wide transcriptome analysis of two maize inbred lines under drought stress. Plant Mol Biol 72:407–421

Zhu J (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273