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Cloning and stress response analysis of the *PeDREB2A* and *PeDREB1A* genes in moso bamboo (*Phyllostachys edulis*)

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ABSTRACT. Moso bamboo is a large woody bamboo with the highest ecological, economic, and cultural value among all bamboos in Asia. However, environmental stress influences its growth and development and limits its geographic distribution. Therefore, improving its resistance to environmental stress is extremely important. Dehydration responsive element binding (DREB) transcription factors perform an important role in the regulation of stress-related genes, enhancing the resistance of plants to abiotic stress. In the current study, two novel *DREB* genes, *PeDREB2A* and *PeDREB1A* (Gene ID No. PH01000046G1730 and PH01000668G0350), were isolated from moso bamboo and the sequences were identified and characterized (coding sequence lengths were 795 and 825 bp, respectively). The *PeDREB2A* and *PeDREB1A* proteins were estimated to have typical AP2/ERF domains, molecular weights of 28.96 and 28.84 kDa, and isoelectric points of 9.47 and 5.34, respectively. RT-PCR analysis revealed that *PeDREB2A* and *PeDREB1A* were tissue-specific genes, expressed in leaves, young stems, and roots, with similar expression levels in leaves and young stems. qRT-PCR analysis of leaves

demonstrated that *PeDREB2A* transcription levels rapidly accumulate following exposure to drought and salt stress, peaking at 12 and 0.5 h, respectively, but only low expression levels were observed under cold stress. *PeDREB1A* exhibited a strong response to cold stress, reaching a peak in expression 3 h after exposure, but demonstrated only a slight response to drought and salt stress. In roots, *PeDREB2A* was down-regulated, and *PeDREB1A* was initially upregulated but then declined, under stress conditions. Two plant expression vectors, pCAMBIA2300-CaMV35S-PeDREB2A and pCAMBIA2300-CaMV35S-PeDREB1A were also successfully constructed.

Key words: Moso bamboo; DREB transcription factor; Drought stress; Cold stress; Salt treatment

INTRODUCTION

Environmental stress includes both abiotic stress, such as drought, low temperature and high salinity, and biotic stress, such as pathogens and disease attacks, all of which adversely affect the growth and development of plants (Liu et al., 2013). Under stressful conditions, plants have evolved a complex signaling network at the molecular, cellular, and system levels to maintain normal metabolism in order to survive (Sharoni et al., 2011). Various functional and regulatory proteins have rapidly accumulated to improve environmental stress tolerance in plants. Transcription factors (TFs) have crucial functions in regulating stress signal transduction and activating target gene expression. In a previous study, 1968 genes that encoded TFs were identified in *Arabidopsis*, with 7.4% of all *Arabidopsis* genes and the major TFs (such as MYB, AP2/EREBP, bHLH, NAC, MADS, bZIP, and WRKY) being determined (Iida et al., 2005).

The AP2/ERF family is one of the most important TF families in plants. The AP2/ERF proteins possess one or two highly conserved AP2/ERF DNA-binding domains, consisting of 60-70 amino acid residues and their structure consists of a three-stranded β-sheet and an α-helix sheet running almost parallel to the β-sheet. The conserved DNA-binding domain was first observed in the homeotic gene AP2 (Jofuku et al., 1994), and then the presence of EREBP was reported in tobacco (Ohme-Takagi and Shinshi, 1995), and two years later, it was also reported to exist in the Pti-4, Pti-5, and Pti-6 proteins in tomato (Zhou et al., 1997). Based on the number of AP2/ERF domains and the sequence structure, the 144 *Arabidopsis* AP2/ERF family genes have been divided into four subfamilies: AP2 (containing two AP2/ERF domains); RAV (possessing a single AP2/ERF domain and a B3 domain); and dehydration responsive element binding (DREB) and ERF (both containing a single AP2/ERF domain) (Sakuma et al., 2002).

The DREB subfamily of TFs activates abundant abiotic stress-related genes, regulating stress tolerance in plants (Lata and Prasad, 2011). In general, DREB TFs induce multiple dehydration/cold-regulated (RD/COR) genes by recognizing their dehydration-/C repeated-responsive element (A/GCCGAC) present in the promoters of RD/COR genes (Liu et al., 2012), which are responsive to drought and cold stress, such as *COR15A* and *COR15B*, *RD29A*, and *RD29B* (Thalhammer et al., 2010; Bihmidine et al., 2013). DREB proteins have been divided into two categories, DREB1 and DREB2, which are involved in two separate signal transduction pathways under low temperature and dehydration, respectively (Liu et al., 1998;

Lata and Prasad, 2011). For example, expression of *CiDREB1A* and *CiDREB1B* was found to be induced during low temperature adverse conditions but not in response to other abiotic stresses (Liang et al., 2014), and the *BdDREB2* gene was significantly expressed in response to drought and salt stress (Zhang et al., 2014). In previous studies, a number of DREB TFs have been isolated from many plant species, including *Physcomitrella patens* (*PpDBF1*) (Liu et al., 2007), *Hordeum vulgare* (*HvDREB1-a*) (Xu et al., 2009), *Medicago falcate* (*MfDREB1* and *MfDREB1s*) (Niu et al., 2010), *Glycine soja* (*GsTIFY11b*) (Zhu et al., 2012), oil palm (*EABF* and *EABF1*) (Omidvar et al., 2012), *Avicennia marina* (*AmCBF1*, *2*, and *3*) (Peng et al., 2013a), and *Malus sieversii* Roem (*MsDREB2C*) (Zhao et al., 2013). Interestingly, it has been found that over-expression of stress inducible DREB TFs may regulate the expression of all target genes that contain a DRE element within their promoters, resulting in transgenic plants with improved stress tolerance. For example, over-expression of the *BdDREB2* or *PpDBF1* genes in tobacco, the *MsDREB2C* or *HvDREB1* genes in *Arabidopsis*, and the *AtDREB2A* gene in soybean have been demonstrated under drought, cold or salt stress, suggesting that the transgenic plants may have improved tolerance of abiotic stress (Liu et al., 2007; Xu et al., 2009; Zhao et al., 2013; Engels et al., 2013; Zhang et al., 2014). Thus, *DREB* genes are promising candidates in terms of molecular breeding for stress resistance in plants.

The moso bamboo, *Phyllostachys edulis* (Carrière) J. Houzeau (synonym *Phyllostachys heterocycla* Carrière), is a large woody bamboo with the highest ecological, economic, and cultural value among all bamboos in Asia (Peng et al., 2013b). However, adverse environmental factors, especially drought, cold and salt stress, not only reduce the economic value of moso bamboo, but also limit its range. For these reasons, improvements in the stress-resistance ability of moso bamboo are extremely important. At present, the bamboo draft genome sequence has been completed (Peng et al., 2013c), providing a vast valuable resource for bamboo molecular research. In the current study, we isolated and characterized two *DREB* homologs (*PeDREB2A* and *PeDREB1A*) from moso bamboo in order to study the mechanism of abiotic stress tolerance in moso bamboo. We found that *PeDREB2A* and *PeDREB1A* responded variably to several abiotic stresses. Our novel findings offer a valuable insight into the role of *PeDREB2A* and *PeDREB1A* during abiotic stress.

MATERIAL AND METHODS

Plant material and growth conditions

Moso bamboo seeds were picked from Guilin, Guangxi Province, China, and germinated on sterile filter paper in culture dishes. After approximately two weeks, seedlings were transferred to plastic pots with vermiculite and grown in an illumination incubator with a 16:8 h light/dark regime, a day/night temperature of 25°/18°C, 80% humidity, and watered Hoagland nutrient solution. Plants were cultivated for a total of three months.

For tissue-specific expression analysis of *PeDREB2A* and *PeDREB1A* genes, leaves, young stems, and roots were collected from plants grown without exposure to stress, frozen immediately in liquid nitrogen, and stored at -80°C prior to RNA extraction. For drought stress treatments, a solution containing 20% PEG-6000 was poured over the culture medium, and for salinity treatments, a solution containing 250 mM NaCl was poured over the culture medium. For low temperature stress treatments, plants were transferred to an illumination incubator at 4°C, with the other environmental conditions remaining unchanged. Plant leaves and roots

were collected for analysis from plants exposed to stress at 0-, 0.5-, 3-, 6-, 12-, 24-, 48-, and 72-h time points following exposure, frozen immediately in liquid nitrogen, and stored at -80°C prior to RNA extraction.

Isolation and analysis of *PeDREB2A* and *PeDREB1A*

Total RNA was extracted from moso bamboo seedlings using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocol and extensively pre-treated using RNase-free DNase I (Promega, Madison, WI, USA) to digest any genomic DNA. RNA quality was characterized initially on a 1% agarose gel with Tris-acetate-EDTA buffer and using a NanoDrop 8000 spectrophotometer (NanoDrop, Thermo Scientific), and then the integrity of RNA samples was further evaluated using an Agilent 2100 Bioanalyzer (Agilent, USA). For first-strand cDNA synthesis, 2 μg total RNA in a 20-μL reaction volume treated with DNase was transcribed using M-MLV reverse transcriptase in accordance with the manufacturer protocol (Promega). The cDNA quality was analyzed on a 1% agarose gel and using a NanoDrop 8000 spectrophotometer.

Based on the moso bamboo genome database, two coding sequences (CDS) of *DREB* genes were identified and characterized. The *PeDREB2A* sequence was amplified using PCR using primers incorporating *Bam*HI (PeDREB2A-*Bam*HI-F, 5'-ATGGATCCATGGACGACA GCCTCCGCACA-3') and *Hin*dIII (PeDREB2A-HindIII-R, 5'-GCAAGCTTCTAGAAGTTA AGCATCTCCCA-3') sites, and the primers used to isolate *PeDREB1A* were constructed using *Bam*HI (PeDREB1A-*Bam*HI-F, 5'-GCGGATCCATGGAAGCAGACGCAAGCCAT-3') and *Hin*dIII (PeDREB1A-*Hin*dIII-R, 5'-TAAAGCTTCTACTCGGCCCACAAGAGT-3') sites. Primers were selected using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0).

PCR conditions were as follows: initial denaturation at 94°C for 5 min; then 32 cycles of 94°C for 30 s, 60°C for 40 s, 72°C for 1 min; and a final extension step at 72°C for 10 min. After PCR amplification and gel-purification, PCR products were inserted into the pMD18-T vector (Takara Dalian), named pMD18T-PeDREB2A and pMD18T-PeDREB1A, and transferred into a DH5α bacterial strain (*Escherichia coli*). Positive clones were selected from a blue-white selection experiment, verified using double enzyme-digestion, and then sequenced.

Bioinformatic analysis

The ProtParam software (http://web.expasy.org/protparam/) was used to predict the molecular weight (MW) and isoelectric point (pI) of the genes; protein subcellular localization was predicted using Softberry ProtComp 9.0 (http://linux1.softberry.com/); the AP2 domain position was queried using the SMART software; the DNAMAN software was used to analyze the gene sequences of *PeDREB2A* and *PeDREB1A* and encode the protein sequences; and the gene structure display server software was used to analyze introns and exons within the *PeDREB2A* and *PeDREB1A* genes. DREB family TFs in *Arabidopsis thaliana* were obtained from the *Arabidopsis* genome database TRIA (http://www.arabidopsis.org/); alignment of the AP2/ERF domain region within *DREBs* was deduced from amino acid sequences of *Arabidopsis*, *PeDREB2A*, and *PeDREB1A* using the ClustalX software 1.83, and an unrooted tree was constructed using MEGA 6.0, and evolutionary relationships were analyzed.

Tissue-specific gene expression analysis

Total RNA was extracted from the leaves, young stems, and roots of 3-month-old seedlings not exposed to stress treatment using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was obtained using the procedure described above. Tonoplast intrinsic protein 41 (*TIP41*) (Fan et al., 2013) was used as a reference gene with the following primer pair: TIP41-F (5'-AAAATCATTGTAGGCCATTGTCG-3') and TIP41-R (5'-ACTAAATTAAGCCAGCGGGAGTG-3'). For the *PeDREB2A* and *PeDREB1A* genes, the same primers used for the cloning described above were used (PeDREB2A-*Bam*HI-F and PeDREB2A-*Hin*dIII-R, PeDREB1A-*Bam*HI-F and PeDREB1A-*Hin*dIII-R, respectively). Conditions for RT-PCR were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 40 s, 72°C for 1 min; then a 10 min extension step at 72°C.

Quantitative real-time PCR (qRT-PCR) analysis

Moso bamboo seedlings were exposed to 20% PEG6000, 4°C, or 250 mM NaCl for 0, 0.5, 3, 6, 12, 24, 48, and 72 h. Total RNA was extracted from leaves or root tissue samples as described above and quantified using a NanoDrop 8000 spectrophotometer. *TIP41* was used as the internal housekeeping gene, the primers for *PeDREB2A* were PeDREB2A-F (5'-CGAGG AGAACAAGGAGAACG-3') and PeDREB2A-R (5'-ATCAACTCGGGATCAAACGA-3'), and the primers for *PeDREB1A* were PeDREB1A-F (5'-CCTCCTCGTTGTTGTCCACT-3') and PeDREB1A-R (5'-GCTCTCCTCTCCCTGTCCAT-3'). The qRT-PCR reactions were carried out using the LightCycler480 System (Roche, USA) and the SYBR Premix EX Taq™ kit (Roche). The 20-µL reaction volume contained 0.4 µL 10 mM of each primer, 2 µL 20 ng cDNA, 10 µL SYBR Green I Master, and ddH_2O to the final volume, following the manufacturer protocol. Amplification reactions were performed as follows: 95°C for 10 s, 60°C for 10 s, and 72°C for 20 s. Other reaction parameters were set to the system default settings. All reactions were performed in triplicate, both technical and biological, and data were analyzed using the Roche manager software.

Plant expression vector construction

Using the restriction enzymes *Bam*HI and *Hin*dIII, the double enzyme-digested recombinant plasmids pMD18T-PeDREB2A and pMD18T-PeDREB1A, and the double enzyme-digested plant expression vector pCAMBIA2300-CaMV35S, products were purified using agarose gel electrophoresis, and purpose fragments were connected to expression vector fragments in order to construct the plant expression vector. Detected using double enzyme-digestion and sequencing, the right positive plasmids were named pCAMBIA2300-CaMV35S-PeDREB2A and pCAMBIA2300-CaMV35S-PeDREB1A.

RESULTS

Isolation and analysis of *PeDREB2A* and *PeDREB1A*

Based on the moso bamboo genome database, two novel genes, *PeDREB2A* and *PeDREB1A* (Gene ID No. PH01000046G1730and PH01000668G0350), were isolated from

moso bamboo. The results of the PCR and double enzyme-digested products verified using agarose gel are shown in Figures 1 and 2, respectively. Sequence analysis determined that the full-length CDSs of *PeDREB2A* and *PeDREB1A* were 795 and 825 bp, respectively, encoding 264- and a 274-amino acid proteins, with predicted MWs of 28.96 and 28.84 kDa, and predicted pIs of 9.47 and 5.34, respectively. Subcellular localization prediction results showed that the two genes were located in the nucleus. The two genes did not contain introns (Figure 3). The two deduced proteins both contained very conserved AP2/ERF domains, consisting of 64 amino acid residues, and located between site 92 leucine (L) to site 155 arginine (R) for *PeDREB2A*, and between site 106 serine (S) to 169 L for *PeDREB1A*. The AP2 domain of *DREB* genes commonly has a conserved valine (V) at position 14 and a glutamate (E) at position 19, which play important roles in the determination of the DNA-binding specific AP2/ERF domain, but conservation of amino acids at position 19 sites is lower than that at position 14 V sites (Liu et al., 1998; Sakuma et al., 2002; Dubouzet et al., 2003; Agarwal et al., 2007). The position 14 sites of *PeDREB2A* and *PeDREB1A* were V and the position 19 site of *PeDREB1A* was E, whereas the position 19 site of *PeDREB2A* was L (Figure 4).

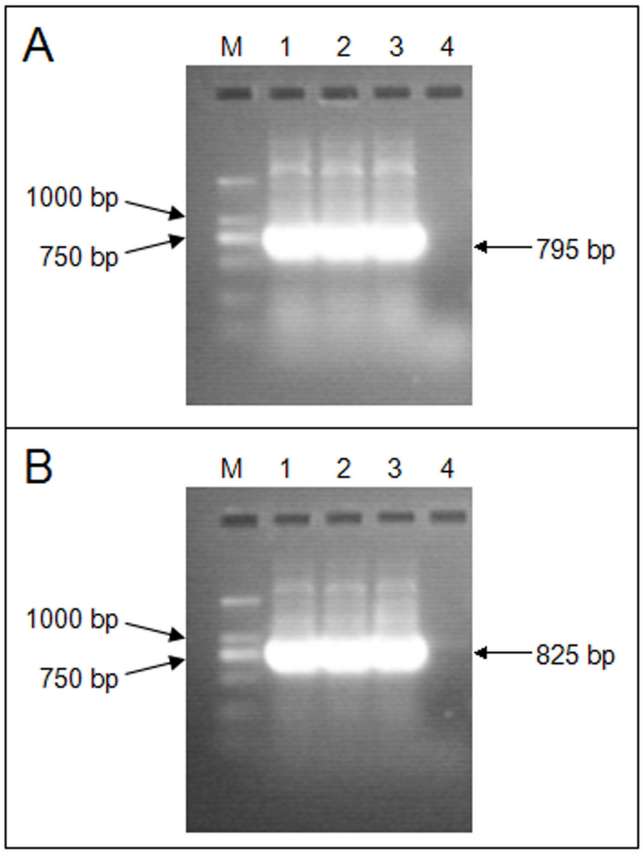

Figure 1. Electrophoresis of PCR products for *PeDREB2A* (**A**) and *PeDREB1A* (**B**) genes. *Lane M* = marker molecule DL2000; *lanes 1*, *2*, *3* = PCR products based on cDNA template; *lane 4* = PCR product based on ddH_2O.

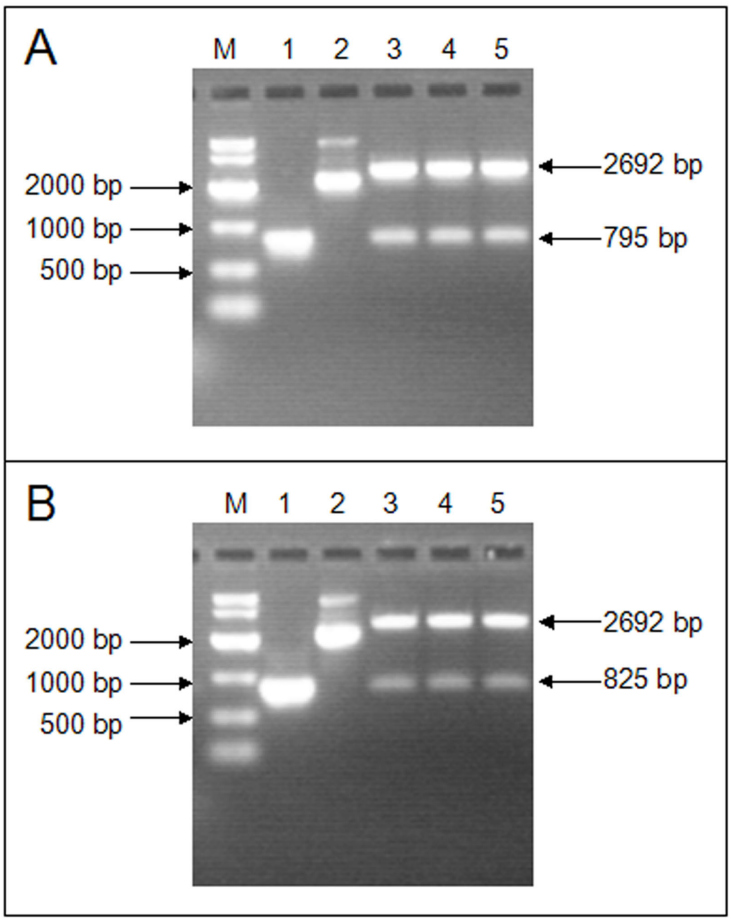

Figure 2. Electrophoresis of the double enzyme-digested pMD18T-PeDREB2A (**A**) and pMD18T-PeDREB1A (**B**). *Lane M* = marker molecule DL10000; *lane 1* = recycled product of PCR; *lane 2* = plasmid; *lanes 3*, *4*, and *5* = double enzyme-digested products of plasmid.

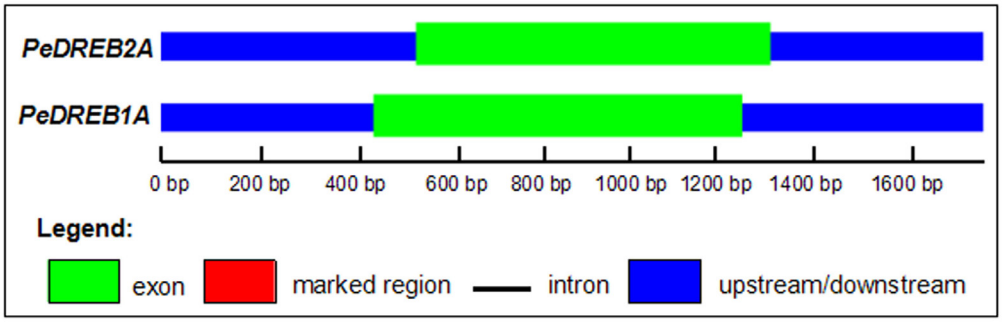

Figure 3. Exon/intron structures of the *PeDREB2A* and *PeDREB1A* genes in moso bamboo.

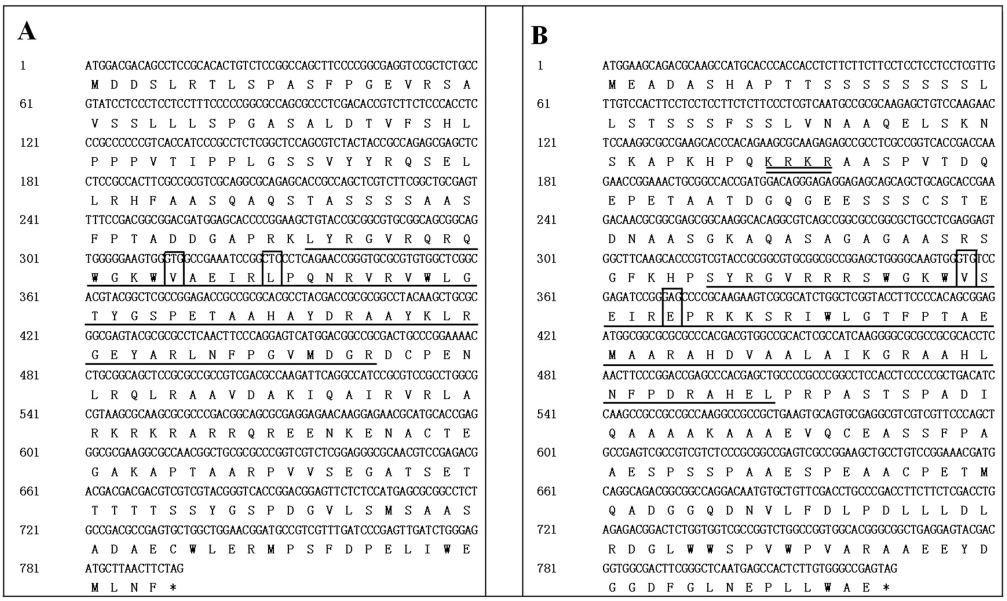

Figure 4. Nucleotide sequences and deduced amino acid sequences of *PeDREB2A* (**A**) and *PeDREB1A* (**B**). The AP2/ERF domain is indicated by a single underline; a predicted nuclear localization signal of *PeDREB1A* is indicated by a double underline. The valine residue at position 14 (V14) and the glutamic acid at position 19 (L/E19) of the AP2/ERF domain are boxed.

An unrooted tree was constructed for *PeDREB2A, PeDREB1A*, and 53 DREB proteins of *Arabidopsis* from a previous study using MEGA 6.0, and the results are shown in Figure 5. *PeDREB2A* was clustered with group A6 of the AtDREB subfamily genes and *PeDREB1A* was clustered with group A4 of the AtDREB subfamily members, indicating *PeDREB2A* and *PeDREB1A* belong to groups A6 and A4 of the moso bamboo DREB subfamily, respectively. According to evolutionary conservation of transcription factors, *PeDREB2A* and *PeDREB1A* may also have significant functions relating to abiotic stress response. Comparative sequence analysis of *PeDREB2A* with group A6 and *PeDREB1A* with group A4 of the AtDREB subfamily, respectively, is shown in Figure 6. For *PeDREB2A*, 37 conserved residues were found, including 1 L, 2 Y, 3 R, 4 G, 5 V, 6 R, 7 Q, 8 R, 10 W, 11 G, 12 K, 13 W, 14 V, 15 A, 16 E, 17 I, 18 R, 20 P, 23 R, 25 R, 27 W, 28 L, 29 G, 30 T, 37 A, 38 A, 40 A, 41 Y, 42 D, 45 A, 49 R, 50 G, 53 A, 55 L, 56 N, 57 F, and 58 P; for *PeDREB1A*, 26 conserved residues were detected, including 3 R, 5 R, 7 R, 12 K, 13 W, 14 V, 15 S, 16 E, 17 I, 18 R, 20 P, 25 R, 27 W, 28 L, 29 G, 36 M, 37 A, 40 A, 42 D, 44 A, 45 A, 50 G, 55 L, 56 N, 57 F, and 58 P. The results imply that highly conserved amino acid residues have important roles in determining the functions of DREB homologous genes. Two extremely conserved motifs were also observed in the AP2/ERF domain, namely the YRG and RAYD motifs.

In previous studies, *PeDREB2* (Liu et al., 2011) and *PeDREB1* (Liu et al., 2012) have been characterized and their functions identified in moso bamboo. In order to verify that the *PeDREB2A* and *PeDREB1A* genes observed in our study were novel genes, the alignment of *PeDREB2*, *PeDREB1*, *PeDREB2A*, and *PeDREB1A* amino acid sequences were aligned using

the ClustalX 1.83 software, and the results are shown in Figure 7. As expected, only the AP2/ERF domain region has high homology, and outside the AP2/ERF domain region homology is relatively low. These results indicate that two novel genes were isolated from moso bamboo in the current study.

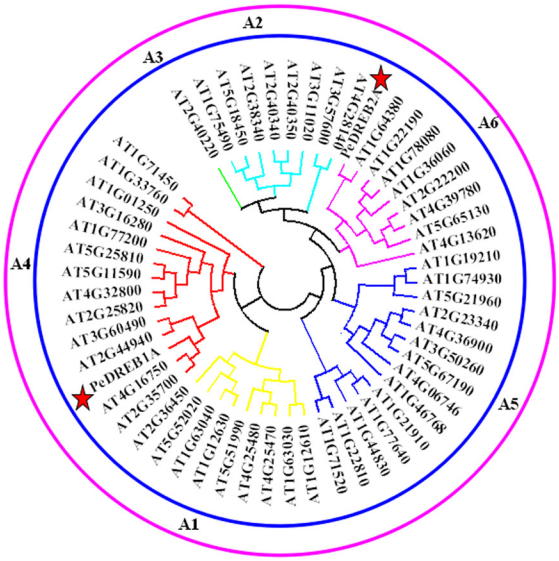

Figure 5. Phylogenetic tree of the *PeDREB2A*, *PeDREB1A*, and *Arabidopsis thaliana DREB* genes. An unrooted tree was constructed using MEGA 5.0 and the neighbor-joining method. The red and pink lines indicate clusters of group A4 and A6, respectively; the red stars indicate *PeDREB2A* and *PeDREB1A*.

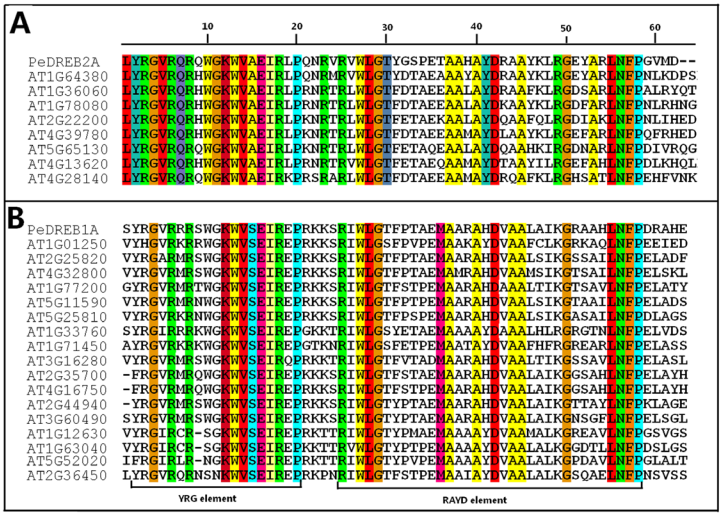

Figure 6. Comparison of the deduced amino acid sequences of the AP2/ERF domains of the DREB subfamily proteins from groups A6 and A4 in *Arabidopsis thaliana* and *PeDREB2A* (**A**) and *PeDREB1A* (**B**) in moso bamboo. The colored background represents highly conserved amino acid residues. The location of the conserved YRG and RAYD elements is indicated by brackets.

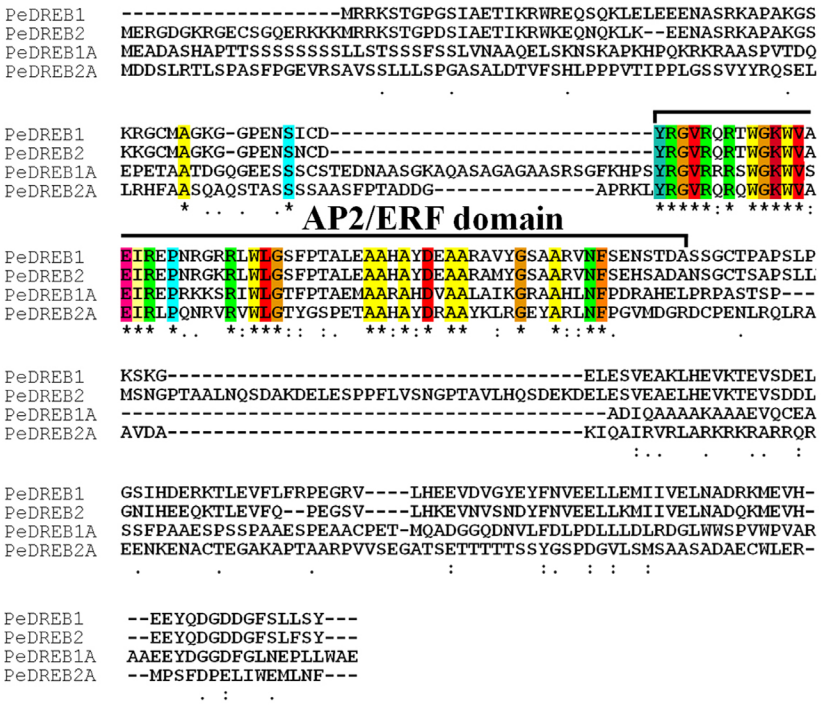

Figure 7. Comparison of the deduced amino acid sequences of *PeDREB1*, *PeDREB1A*, *PeDREB2*, and *PeDREB2A* in moso bamboo. The colored background represents highly conserved amino acid residues. The location of the conserved AP2/ERF domain is indicated by brackets.

Tissue-specific gene expression

The results of the tissue-specific gene expression analysis revealed that the *PeDREB2A* and *PeDREB1A* genes were expressed in leaves, young stems, and roots (Figure 8), thus, the two genes are tissue-specific genes. Expression of *PeDREB2A* in roots was significantly higher than that in leaves and stems, and expression levels in leaves and stems were low with no obvious difference in expression patterns between these two tissues. Transcripts of *PeDREB1A* in the leaves and stems of moso bamboo were very high with similar patterns, whereas low expression was observed in root tissue. The expression patterns of the two genes in different tissues illustrate the tissue specific expression characteristics of the two genes. Expression of *PeDREB1A* in leaves and stems was clearly higher than that of *PeDREB2A*, but expression levels of the two genes was similar in the roots. The difference in expression levels between tissues also suggests that these two genes are different genes.

qRT-PCR analysis

The expression of *PeDREB2A* and *PeDREB1A* genes was induced by drought, and the results are shown in Figures 9 and 10. In leaves, *PeDREB2A* was up-regulated and transcript levels initially increased, reaching a peak at 98.3-fold after 12 h, and then decreased. In

roots, *PeDREB2A* was down-regulated, with only a 0.05-fold change in expression after 6 h, returning to basal levels after 24 h. The results illustrate that *PeDREB2A* plays a very important role in terms of enhancing moso bamboo drought resistance in leaves. *PeDREB1A* was induced and up-regulated at each time point, and levels of transcripts in leaves were slightly elevated, whereas in roots the primary increase occurred after 12 h, peaking at 17.13-fold after 48 h, and then decreased. This implies that *PeDREB1A* has an obvious function in roots in response to drought stress. Expression of *PeDREB2A* was higher in leaves than in roots and this gene was up-regulated under drought stress, thus, it may be associated with tissue-specific expression of genes.

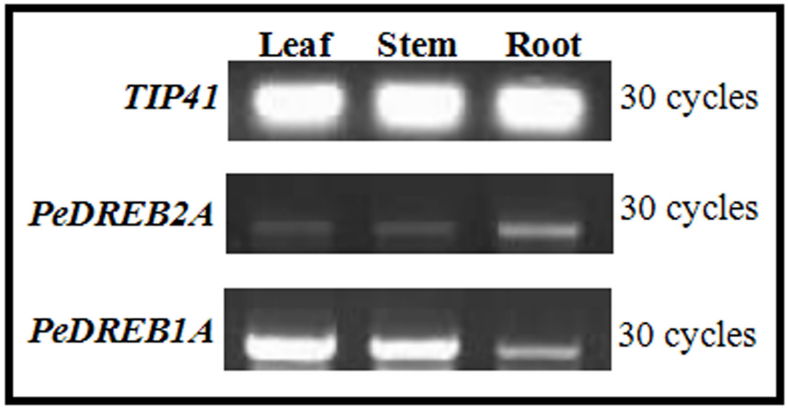

Figure 8. RT-PCR analyses of the *PeDREB2A* and *PeDREB1A* genes in different tissues from moso bamboo. TIP41 denotes the reference gene Tonoplast intrinsic protein 41.

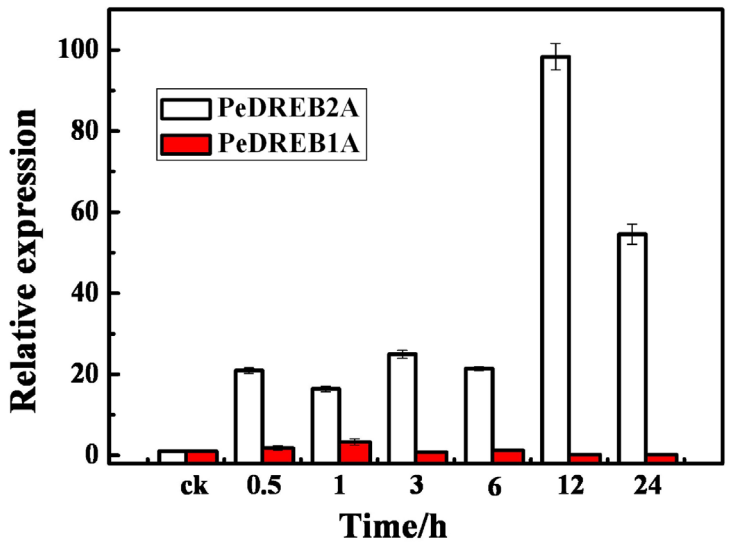

Figure 9. Relative expression of the *PeDREB2A* and *PeDREB1A* genes in leaves of moso bamboo over time following treatment with 20% PEG6000. The CK represented the control check group.

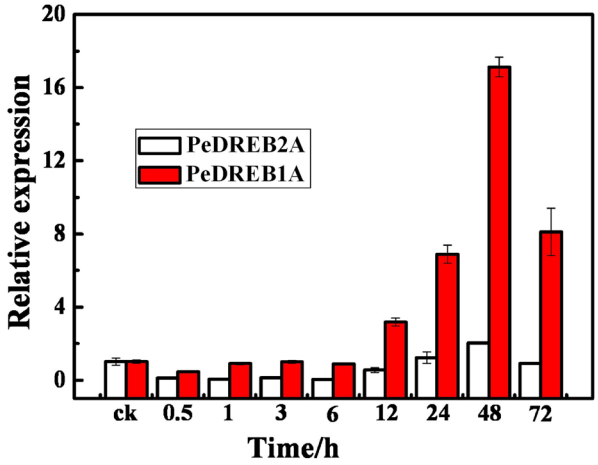

Figure 10. Relative expression of the *PeDREB2A* and *PeDREB1A* genes in roots of moso bamboo over time following treatment with 20% PEG6000. The CK represented the control check group.

The expression of *PeDREB2A* and *PeDREB1A* was induced under cold stress, and the results are presented in Figures 11 and 12. *PeDREB2A* was primarily induced and up-regulated in leaves, but the number of transcripts decreased after 1 h, returning to basal levels, and total expression levels did not change significantly. However, in roots, *PeDREB2A* was down-regulated and even dropped to undetectable levels. Through analysis of expression patterns for *PeDREB2A* under low temperature, the very low relative expression values indicate that *PeDREB2A* provides little function under cold conditions. Transcripts of *PeDREB1A* in leaves and roots were induced and up-regulated with an initial increase followed by a decrease. Expression levels peaked at 13.71- and 4.49-fold after 3 h in leaves and roots, respectively. The high expression level of *PeDREB1A* under cold conditions implies that the *PeDREB1A* gene plays a significant role in cold resistance, especially in leaves.

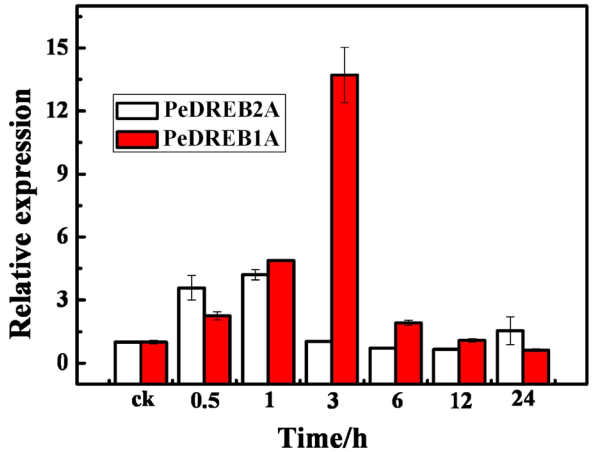

Figure 11. Relative expression of the *PeDREB2A* and *PeDREB1A* genes in leaves of moso bamboo over time following treatment at 4°C. The CK represented the control check group.

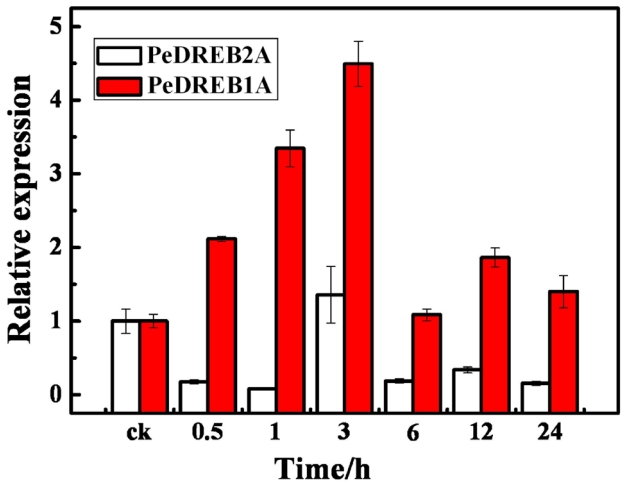

Figure 12. Relative expression of the *PeDREB2A* and *PeDREB1A* genes in roots of moso bamboo over time following treatment at 4°C. The CK represented the control check group.

Under salt treatment, different expression patterns of *PeDREB2A* and *PeDREB1A* were tested and these are displayed in Figures 13 and 14. In leaves, the expression of *PeDREB2A* rapidly increased after 0.5 h, peaking at 15.86-fold, and then decreased to basal levels. However, there was very low expression, almost down-regulation, of *PeDREB2A* in roots, signifying that *PeDREB2A* in leaves has a strong, rapid reaction in response to salt exposure. Transcripts of *PeDREB1A* in leaves remained unchanged and in roots, the expression level was slightly altered, verifying that the *PeDREB1A* gene was not induced by high salinity stress.

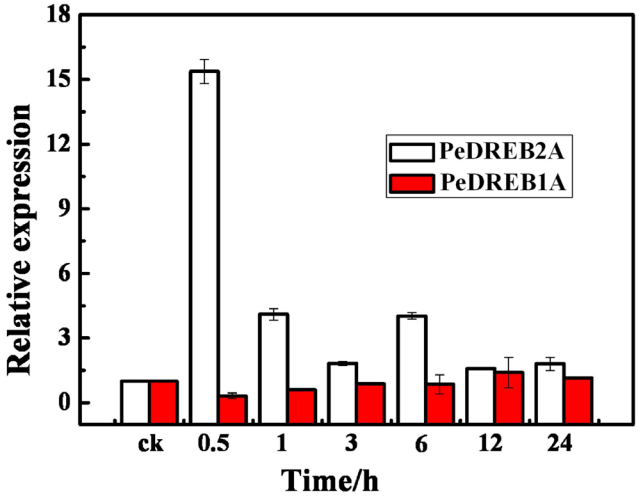

Figure 13. Relative expression of the *PeDREB2A* and the *PeDREB1A* genes in leaves of moso bamboo over time following treatment with 250 mM NaCl. The CK represented the control check group.

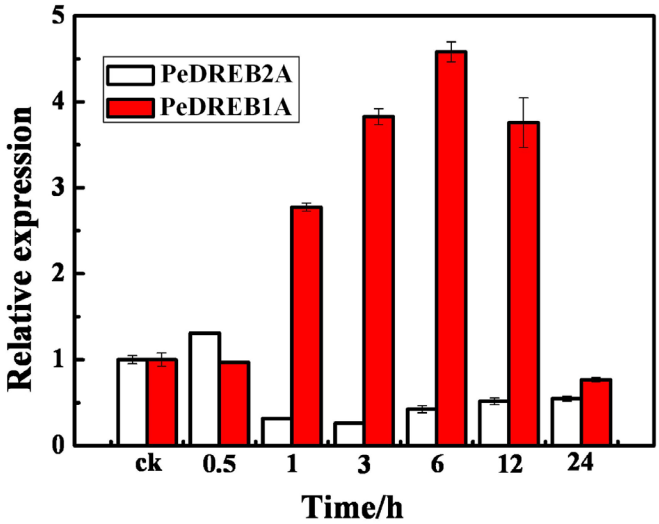

Figure 14. Relative expression of the *PeDREB2A* and *PeDREB1A* genes in roots of moso bamboo over time following treatment with 250 mM NaCl. The CK represented the control check group.

Plant expression vector construction

Purified plasmids pCAMBIA2300-CaMV35S-PeDREB2A and pCAMBIA2300-CaMV35S-PeDREB1A were double enzyme-digested using the restriction enzymes *Bam*HI and *Hin*dIII, and analysis of these products using agarose gel found that the small-sized fragments were completely matched with *PeDREB2A* and *PeDREB1A*, respectively, and the large-sized fragments were similar to the plant expression vector pCAMBIA2300-CaMV35S (Figure 15). Sequencing of purified recombinant plasmids for further analysis showed that inserted fragments were completely aligned with the corresponding purpose genes, indicating that interesting recombinant plasmids were successfully constructed (Figure 16).

DISCUSSION

DREB TFs involved in abiotic stress signal transduction pathways induce the expression of numerous stress related genes, resulting in improved abiotic stress resistance in plants, especially in terms of resistance to drought, low temperature, and high salinity stress. In our study, two *DREB* genes were isolated, *PeDREB2A* and *PeDREB1A*, both containing an AP2/ERF conserved domain that has very high homology and similar structural characteristics with *Arabidopsis DREB* genes, e.g., conservative amino acid residues and motifs. The conservative sequence structure may perform specific roles on a gene's function within a TF family. Highly conserved amino acid residues were observed within the AP2/ERF domain region: a V at position 14 sites and an E at position 19 sites. This features distinguishes between DREB and ERF family members, with ERF family genes having alanine (A) at position 14 sites and aspartate (D) at position 19 sites (Agarwal et al., 2007). Previous research has reported that conservation of amino acid residues at position 19 sites was lower than that at position 14 sites (Sakuma et al., 2002). For example, *At1G64380* and *At4G39780* have L at position 19 sites,

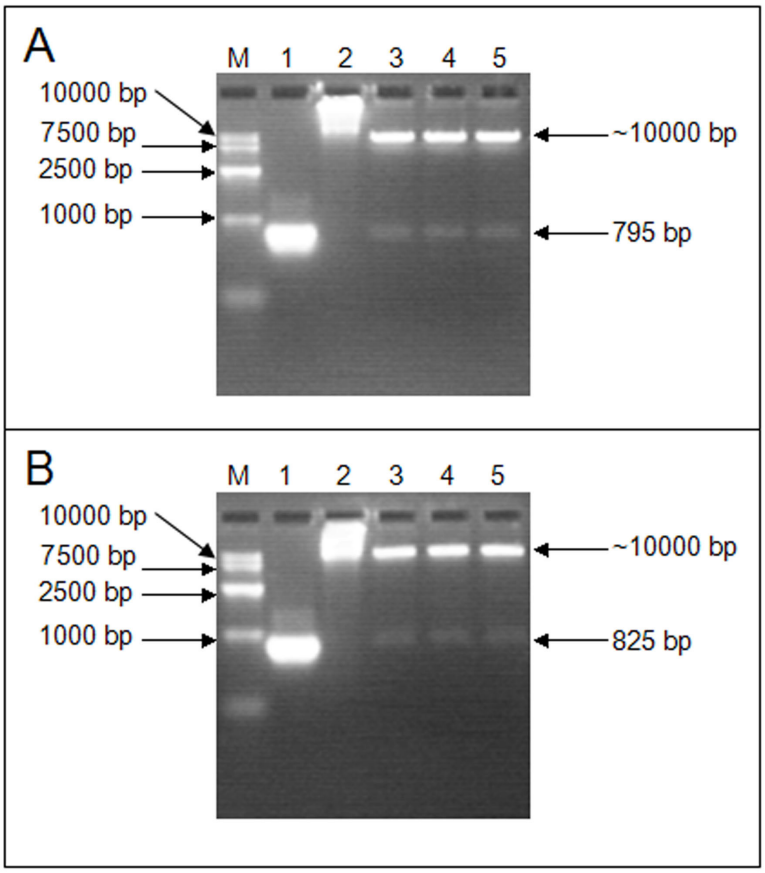

Figure 15. Electrophoresis of the double enzyme-digested pCAMBIA2300-PeDREB2A (**A**) and pCAMBIA2300-PeDREB1A (**B**). *Lane M* = marker molecule DL10000; *lane 1* = recycled product of PCR; *lane 2* = plasmid; *lanes 3*, *4*, and *5* = double enzyme-digested products of the plasmid.

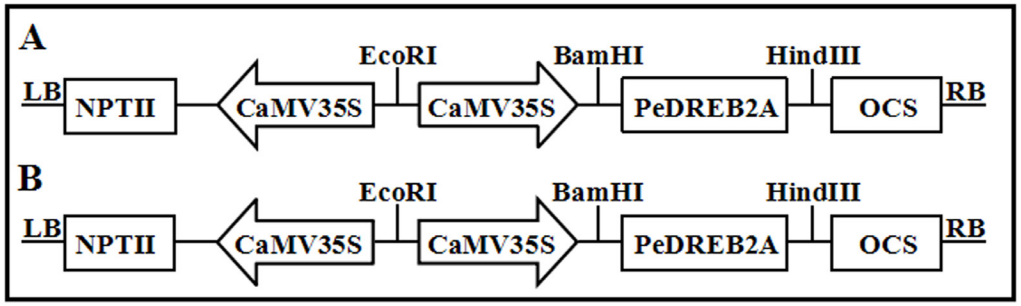

Figure 16. Structure maps of the pCAMBIA2300-PeDREB2A (**A**) and pCAMBIA2300-PeDREB1A (**B**) expressing framework.

indicating that the position 14 sites play a more important function in specific DNA-binding of target genes. Similar to *At1G64380* and *At4G39780*, L was observed at position 19 sites of the AP2/ERF domain region within *PeDREB2A*, as shown in Figure 6A.

Proteins within a family that share conserved motifs are more likely to share similar functions (Nakano et al., 2006). Two elements, YRG and RAYD, within the AP2/ERF domain have been documented as being significant in activating DNA-binding to modulate the expression of target genes in *Arabidopsis* (Okamuro et al., 1997). The YRG element aids in the binding of TFs to target genes and consists of 19-22 amino acid residues, almost all of which are basic amino acids. The RAYD element is 42 or 43 amino acid residues in length and contains an α-helix with 18 amino acid residues, and can change the YRG element conformation or interact with other proteins to modulate specific binding to target genes (Allen et al., 1997; Okamuro et al., 1997). The YRG and RAYD elements were conserved and identified in *PeDREB2A* and *PeDREB1A* genes, indicating their essential functions. Liu et al. (2011, 2012) isolated and identified two *DREB* genes, one was the drought induced gene *PeDREB2* and the other was the cold induced gene *PeDREB1*. Comparisons of the amino acid sequences of the two genes in the current study showed that they were significantly different genes with different ORF lengths and different amino acid residues, indicating our study obtained two novel *DREB* genes.

PeDREB2A and *PeDREB1A* were expressed in moso bamboo seedling roots, young stems, and leaves, demonstrating that the two genes were tissue-specific. In moso bamboo seedlings cultivated without any abiotic stress, the expression of *PeDREB2A* in roots was significantly higher than that in leaves and stems. On the contrary, levels of transcripts of *PeDREB1A* in leaves and stems were prominently higher than those in roots. This phenomenon may be explained by different amino acid sequences resulting in different features and different tissue expression patterns, and these results also provided preliminary information for further gene function verification in the next step of the analysis.

The DREB proteins namely, DREB1 and DREB2, involved in two separate signal transduction pathways under low temperature and dehydration, respectively, are important APETALA2 (AP2)/ethylene responsive factor (ERF) plant TFs that induce a set of abiotic stress-related genes (Lata and Prasad, 2011). The results of the analysis of *PeDREB2A* and *PeDREB1A* expression patterns under drought, low temperature, and salt treatment stress showed that *PeDREB2A* responded mainly to drought and high salinity stress, with very limited expression under low temperature stress, and the *PeDREB1A* gene was expressed under low temperature stress but had relatively low expression under drought and high salinity stress. These results vividly imply that the *PeDREB2A* gene is mainly involved in drought stress signal transduction pathways and the *PeDREB1A* gene plays an important role in low temperature stress signal transduction pathways. The expression levels of the *PeDREB2* gene in leaves under different stress conditions was also studied and the highest expression was observed under drought stress after 12 h and high salinity stress after 6 h, but no response was observed under low temperature stress (Liu et al., 2011). The expression patterns of the *PeDREB2A* gene in leaves were similar to those of *PeDREB2*; expression was observed under drought and high salinity stress, peaking at 12 and 0.5 h, respectively, and low expression levels were observed under cold stress conditions. Interestingly, peak expression of the *PeDREB2A* gene occurred earlier than *PeDREB2* under salt treatment, illustrating that different genes play various roles under the same stress conditions and also the timing of gene functions also differs. Another study has reported that transcripts of the *PeDREB1* gene

in moso bamboo accumulated under low temperature conditions, peaking at 3 h; however, little change in expression was observed under drought or salt treatments (Liu et al., 2012). *PeDREB1A* and *PeDREB1* also have similar expression patterns in leaves, being strongly expressed under low temperature treatments and with relative expression peaking at 3 h, while under drought and salinity conditions, expression levels remained close to basal levels. Most plant resistance research has only focused on the analysis of gene expression patterns in leaves (Niu et al., 2010; Zhao et al., 2013; Liang et al., 2014), and less research has focused on gene expression in roots. The current study also examined *PeDREB2A* and *PeDREB1A* gene expression patterns in moso bamboo roots under drought, low temperature, and salinity stress, providing further understanding of gene expression patterns in different plant tissues. In root tissue, the *PeDREB2A* gene was almost down-regulated under drought, low temperature, and salinity stress. However, the expression of *PeDREB1A* increased initially and subsequently decreased, and expression was especially high under drought stress. These results indicate that the *PeDREB1A* gene in roots has a more important function than the *PeDREB2A* gene under abiotic stress conditions. Comprehensive analysis of *PeDREB2A* and *PeDREB1A* gene expression patterns implies that the two genes perform several critical roles in abiotic stress responses in different tissues.

In conclusion, two vital *DREB* genes with functions of enhanced stress resistance in moso bamboo were isolated in our study. Furthermore, their expression patterns under drought, low temperature, and salt stress were clarified. Finally, the corresponding plant expression vectors of these two *DREB* genes were obtained. Nevertheless, these genes may have more complex functions and, thus, further research into the functions of the two genes may have important significance for bamboo research.

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