INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0230/2018/20–2–323–332
DOI: 10.17957/IJAB/15.0494
http://www.fspublishers.org

Full Length Article

Cloning of a *dhn* Gene from *Pinus massoniana* and Characterization of the Responses to Drought Stress in *Arabidopsis thaliana*

Qiong Cai[1], Gui-jie Ding[1*], Bo Ding[1], Xiao-Peng Wen[2] and Ming-Feng Du[1]
[1]*Research Institute of Forest Resources and Environment, Guizhou University, Guiyang, Guizhou, China 550025*
[2]*Guizhou Key Laboratory of Agricultural Bioengineering, Guizhou University, Guiyang, Guizhou, China 550025*
[*]For correspondence: ding_gzu@sina.com; dukecq@sina.com

Abstract

Dehydrins (DHNs) play important roles as hydrophilic proteins in plant response to abiotic stress conditions such as salinity, cold, heavy metals, and drought. To determine the genes involved in drought stress in *Pinus massoniana*, we cloned a DHN gene using degenerate primers and a RACE strategy, and evaluated the gene function using transgenic *Arabidopsis thaliana* plants overexpressing the DHN gene of *P. massoniana* under drought stress. The designated full-length *Pmdhn* (1,232 bp) was found to include a 199 bp 5′- and a 316 bp 3'-terminal untranslated region (UTR), and a 717 bp open reading frame (ORF). *Pmdhn* was characterized as a mature 238 amino acid protein, containing a Ser-rich domain and three conserved replications of the characterized Lys-rich domain. The qRT-PCR analysis revealed that the *Pmdhn* expression levels in roots and stems were higher than in leaves, and the gene was involved in a broad spectrum of physiological processes under drought stress. No differences in the phenotypes and root lengths between the wild-type and transgenic *A. thaliana* were observed under normal conditions. Nevertheless, the transgenic *A. thaliana* lines had longer roots than in the wild-type lines under drought stress. RT-PCR and GFP analysis revealed that *Pmdhn* can be transcribed stably. Furthermore, the *Pmdhn*-overexpressing plants showed lower levels of malondialdehyde (MDA), higher water and proline contents under drought stress. These findings indicate that *Pmdhn* likely plays a role in the process of drought stress. © 2018 Friends Science Publishers

Keywords: Dehydrin; *Pinus massoniana*; Cloning; Transgenic; *Arabidopsis thaliana*

Introduction

Plants are often threatened by drought stress, resulting in physiological and biochemical alterations, slow growth, and even death (Mahajan and Tuteja, 2005). To address this important issue, many studies have been conducted to ameliorate the hereditary drought-related characteristics of plants and to develop genetic engineering techniques for producing drought-resistant plants. Among the drought-resistant gene candidates, the major proteins of the Late Embryogenesis Abundant (LEA) are of particular interest. Recently, LEA proteins have been isolated and identified in a number of plants, such as *Stipa purpurea* (Yang *et al*., 2014), *Malus domestica* (Liang *et al*., 2012), *Saussurea involucrate* (Qiu *et al*., 2014), *Ammopiptanthus mongolicus* (Sun *et al*., 2013), *Prunus mume* (Du *et al*., 2013), and *Quercus robur* (Sunderlíková *et al*., 2009). Based on sequence similarities, LEA proteins have been divided into seven categories (Dure *et al*., 1989; Hundertmark and Hincha, 2008; Hunault and Jaspard, 2010).

Dehydrins (DHNs), known as Group 2 LEA proteins, attract the most attention in higher plants at present (Hara, 2010). DHNs are characteristically rich in glycine, but lack of cysteine and tryptophan. The Y, K, S segments are located near the N-terminus, the C-terminus and midway between the Y and the K fragments, respectively (Close, 1996; Shih *et al*., 2008). Based on the permutations of the conserved motifs, DHNs are classed into five sub-classes (YnSKn, SKn, Kn, YnKn and KnS), (Campbell and Close, 1997; Rorat *et al*., 2006). To date, the expression levels and functions of DHNs have been extensively studied. Shekhawat *et al*. (2011) found that *MusaDHN-1*, a SK_3-type dehydrin gene, enhanced drought-stress tolerance of the transgenic banana plants. Moreover, DHNs from *Capsicum annuum* and *Lycopersicon chilense* were constitutively expressed and up-regulated by drought stress (Szabala *et al*., 2014). In addition, the overexpression of the *tas14* gene also contributed to drought resistance in transgenic tomato plants (Muñoz-Mayor *et al*., 2012). Perdiguero *et al*. (2012; 2013; 2015) found that dehydrins have been induced by drought stress in *Pinus pinaster*, and conducted the analysis on Ulmus transcriptome under the constant threat of Dutch elm disease. Lorenz *et al*. (2006) found that many genes in *P. taeda* were essential in drought tolerance, such as dehydrins.

Pinus massoniana (21º41′~33º56′ N; 102º10′~123º14′ E) is widely distributed in 17 provinces of southern China. It has excellent characteristics, including a high growth capability, fast growth, high yield, wide range of uses, and a

To cite this paper: Cai, Q., G.J. Ding, B. Ding, X.P. Wen and M.F. Du, 2018. Cloning of a *dhn* gene from *Pinus massoniana* and characterization of the responses to drought stress in *Arabidopsis thaliana*. *Int. J. Agric. Biol*. 20: 323–332

high level of comprehensive uses (Ding *et al*., 2006). Previous studies mainly focused on the investigations of the physiological and biochemical variations in *P. massoniana* under drought stress (Chen and Ding, 2004; Shi *et al*., 2004; Xu and Ding, 2006; Chen *et al*., 2011; He *et al*., 2011; Han and Ding, 2012; Wang and Ding, 2013). Presently, there have been only a limited number of studies on the molecular events such as on the lignin formation (Zhang *et al*., 2014) and plant defenses against insects and pathogens (Wang *et al*., 2008).

The scarcity of water resources resulting from global climate changes necessitate the development of drought-tolerant plants to adapt to adverse environmental conditions. However, conventional breeding methods are limited by time and geography. This study aims to demonstrate the feasibility of cloning drought-tolerance genes from *P. massoniana* using genetic engineering technology, and to characterize the functions of the *Pmdhn* gene in transgenic *A. thaliana*. This study also describes the cloning of *Pmdhn* and detailed assays for improving drought tolerance in transgenic *A. thaliana* plants.

Materials and Methods

Plant Material

P. massoniana (No. 83 family), an elite genotype with a significantly higher tolerance to drought (Shi *et al*., 2004; Yuan *et al*., 2008), was used as the research material in this study. One year old seedlings collected from Guangxi Province China were cultivated in a greenhouse using a mixture of yellow soil derived from quaternary red clay and clean river sand (volume ratio 9:2) under normal conditions (relative water content of soil: 70%±5% for control) and drought stress conditions (relative water content of soil: 45%±5%) for1, 3, 5, 10, 15, 20 and 25 days (Han *et al*., 2012; Wang and Ding, 2013) separately. *P. massoniana* used in this study had similar height with an average height of 17.2 cm. Plants were washed thoroughly, frozen immediately, and then stored at -80°C. Leaves, stems and roots of *P. massoniana* were collected for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses. Wild-type (Columbia ecotype) and *Pmdhn*-overexpressing *A. thaliana* seeds were washed multiple times with sterile water and then disinfected with alcohol for growth in Murashige and Skoog medium (Solarbio, USA). Plastic petri dishesused for plant culture were kept at 25°C in a growth chamber.

Identification of the *Pmdhn* Gene

Total RNA was extracted from *P. massoniana* seedlings using Trizol Reagent (Invitrogen, USA) and PureLink® Plant RNA Reagent (Invitrogen, USA). First, based on the conserved sequences of DHNs registered in NCBI, a forward primer (5′-GCGG(T/C)ATGTT(T/C)GG(A/T/C/G)TTAT-3′) and reverse primer (5′-T(T/C)TTGTCCTT(A/T/C/G)GA(A/T/C/G)CCTTT-3′) were designed. Secondly, the *Pmdhn* gene fragment was obtained using PCR performed in a 25.0 μL reaction volume containing ~1.0 μL template, 1.0 μL forward primer, 1.0 μL reverse primer, 12.5 μL 2×*Taq* PCR MasterMix (Tiangen, China) and 9.5 μL double distillation H_2O. The amplification procedure is standard, including a denaturation at 94°C for 5 min, followed by 35 cycles with 1 min at 94°C, 30 s at 54.0°C and 1 min at 72°C, and finally a 10 min extension at 72°C. Thirdly, the 5′ end of the cDNA was amplified using the 5′-RACE system for rapid amplification of cDNA ends (Invitrogen, USA). Three gene-specific primers, GSP1 (5′-GAGGAGCAGCTGGATA-3′), GSP2 (5′-CTGAGCAGGAGTTTGCACAA-3′) and GSP3 (5′-TTGCTTTCCCTCCTCTTCCT-3′), were designed and the amplification procedures are detailed in the manufacturer's instructions. The 3′ end of the cDNA was amplified using the 3′-Full RACE Core Set with PrimeScript™RTase (Takara, China). The 3′-RACE products were obtained by outer and inner PCR. Gene specific outer (5′-CGAACACG GGCATGGTCACGAGGGG-3′) and inner (5′-AGCTGAG CAACAGAAACACACGGGT-3′) primers were constructed. Finally, the full length cDNA of *Pmdhn* was confirmed through PCR amplification using specific primers (forward: 5′-ATGGCGGAAGAAGCAGCAGAGC -3′; reverse: 5′-TTAATGATGGTGCTTCTTCTCT-3′).

qRT-PCR Analysis

The gene-specific primers (F: 5′-CGGTATGTTCGGCTTA TTC-3′ and R: 5′-GACCCGTGTGTTTCTGTTG-3′) were used in qRT-PCR. The control PCR primers amplified 18S (F: 5′-TTAGGCCATGGAGGTTTGAG-3′ and R: 5′-GAGTTGATGACACGCGCTTA-3′) (Fan *et al*., 2014). All of the qRT-PCR reactions were carried out using an ABI 7500 (ABI, USA) with SuperReal PreMix Plus (SYBR Rreen) (Tiangen, China). Quantifications of the relative changes in transcriptional levels were performed using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Construction of Plant Expression Vector

The cloning vector pMD18-T containing the in-frame coding cDNA of *Pmdhn* and the plant binary expression vector pBI121 were first digested with the restriction enzyme *Xba*I and *Bam*HI, respectively and then directly connected to the vector usingT4DNA ligase. The product was named pBI121-*Pmdhn*.

A. thaliana Transformation

The pBI121-*Pmdhn* was transformed into *A. thaliana* plants using *Agrobacterium* LBA4404. *Pmdhn*-overexpressing *A. thaliana* plants were confirmed by PCR using the primers5′-GGGTCTAGAATGGCGGAAGAAG-3′ and 5′-CTTGGATCCATGATGGTGCTTC-3′. The T_2 generation plants were selected and used for further studies.

Investigation on the *Pmdhn* transcript in *A. thaliana* with RT-PCR

The WT and transgenic *A. thaliana* were cultured in flasks in MS medium containing 0% or 3.5% PEG-6000 at 20 days. Total RNA was extracted. The *Actin2* gene of *A. thaliana* was used as a housekeeping gene (Zhang *et al*., 2013), and RT-PCR was carried out using the gene-specific primers (F: 5′-CGGTATGTTCGGCTTATTC-3′ and R: 5′-GACCCGTGTGTTTCTGTTG-3′). The 10.0 μL reaction contained ~0.8 μL template, 0.2 μL forward primer, 0.2 μL reverse primer, 5.0 μL 2×*Taq* PCR MasterMix (Tiangen, China), and 3.8 μL ddH_2O. Standard amplification procedures were used with a denaturation at 94°C for 5 min, followed by 29 cycles of 1 min at 94°C, 40 s at 57.0°C and 1 min at 72°C, and a final 10 min extension at 72°C.

Southern Blot Analysis

Genomic DNA of both wild-type and transgenic *A. thaliana* plants were respectively extracted by the CTAB method (Porebski *et al*., 1997). Aliquots of genomic DNA (10 μg) was digested with EcoR I. The digested samples were electrophoresed on a 0.7% (w/v) agarose gel and blotted on Hybond N^+ nylon membranes (Amersham, Sweden). Full-length cDNA of *Pmdhn* amplified by PCR using the specific primers (F: 5′-ATGGCGGAAGAAGCAGCAGAG CACC-3′ and R: 5′-ATGATGGTGCTTCTTCTCTTCCT-3′) were used as the probe DNA in the analysis. The procedure was performed according to the instructions for the PCR DIG Probe Synthesis Kit (Roche, USA). The copy numbers in D1and D3 lines were determined.

GFP Fluorescence Detection on Roots of Transgenic *A. thaliana* by Fluorescence Microscope

The activity of green fluorescence protein on root in D3 line was detected by inverted fluorescence microscopy CFM-500 (Changfang, China). The photographs were taken with A3300 digital camera (Canon, China).

Growth Assays

T_2 seeds were selected for the drought stress analysis. The wild-type *A. thaliana* was labeled as WT and the overexpressing *Pmdhn* plant was labeled as OP. Six lines of *Pmdhn*-overexpressing *A. thaliana*—D1, D3, D5, D7, D8, D10—were used in effect analysis on phenotypes, root developments under well-watered (in MS medium containing 0% PEG-6000 at 15 days) and drought stress (in MS medium containing 3.5% PEG-6000 at 15 days) conditions, and the D3 line was used in fluorescent observation and physiological analysis. Water loss of leaves detached from transgenic and wild-type *A. thaliana* plants were measured by weighing the leaves harvested from 5-week-old plants at 1 h intervals for 4 h. Proline and MDA contents were determined using an established method described by Li (2006).

Data Analysis

All experiments in our study were repeated 3 times. Analysis of variance (ANOVA) was performed by SPSS (v16.0). Duncan's multiple range tests were applied to compare means at 0.05 level of significance. ORIGIN (v8.0) was used for cartography.

Results

Isolation of *Pmdhn* Full-Length cDNA

Several fragments sequenced by RT-PCR used as query in a BLAST analysis were generated (Fig. 1Sa). The results showed that the 420 bp fragment was highly similar to DHN (AFN44791). Subsequently, the 5′-upstream and 3′-downstream regions of the transcript were amplified (Fig. 1Sb and 1Sc), respectively. Then, the full-length cDNA was obtained using a nucleotide sequence alignment and the assembly of the middle fragment with the 5′- and 3′-RACE PCR products. The obtained *Pmdhn* full-length cDNA (1,232 bp) includes a 199 bp 5′-and a 316 bp 3′-terminal untranslated region, and a 717 bp open reading frame, which encodes a protein of 238 amino acids (Fig. 1Sd and 2S). The GenBank accession number is KF910087. The molecular mass and isoelectric point of *Pmdhn* were 25.85 kDa and 7.35 by ProtParam prediction (http://web.expasy.org/protparam/), respectively.

The *Pmdhn* is a high hydrophilic protein (hydrophilicity index 3.489) (http://web.expasy.org/protscale/), as described for other LEA2 protein family members. Furthermore, *Pmdhn* contains a S- motif located at residues 84-91 and three K-motifs (residues 134–149, 170–185, and 206–221) (Fig. 1). The sequence structure indicated that *Pmdhn* belongs to the SK_3-type DHNs. The cloned *Pmdhn*, containing the structural characteristics of DHNs, provided useful information for the further understanding and use of *P. massoniana* under drought stress.

Compared to other plant DHNs, such as *P. mugo*, *P. resinosa* and *P. sylvestris* proteins, *Pmdhn* showed a high sequence identity (92 to 98%), but a low sequence identity of 31 to 71% with *Picea abies*, *Corylus mandshurica*, *Jatropha curcas*, *Vitis riparia*, *A. thaliana*, and *Populus trichocarpa* (Table 1), which shared mainly the S- and K-motifs (Fig. 1). We selected 21 additional plants and constructed a phylogenetic tree. The close relationship between *Pmdhn* and pinus DHNs, such as *P. sylvestris*, *P. mugo*, *P. hwangshanensis* and *P. pinaster* was found (Fig. 2). These findings indicated that the DHN proteins in *Pinus* species remained largely unchanged during evolution.

Table 1: Analysis of the Pmdhn protein sequence with BLAST

Name	Max score	Total score	Query cover	E value	Ident	Accession
Pinusresinosa	384	433	94%	3e-132	96%	FJ415453
Pinussylvestris	281	328	94%	6e-92	92%	FJ201497
Pinusmugo	397	447	94%	2e-137	98%	HQ108523
Piceaabies	168	217	77%	4e-48	71%	EF522169
Corylusmandshurica	43.5	43.5	32%	0.045	48%	JF433379
Jatrophacurcas	45.8	128	64%	3e-11	45%	GU325779
Arabidopsis thaliana	54.7	101	93%	2e-05	31%	X90959
Populustrichocarpa	31.6	62.0	62%	6e-06	37%	XM_002307732

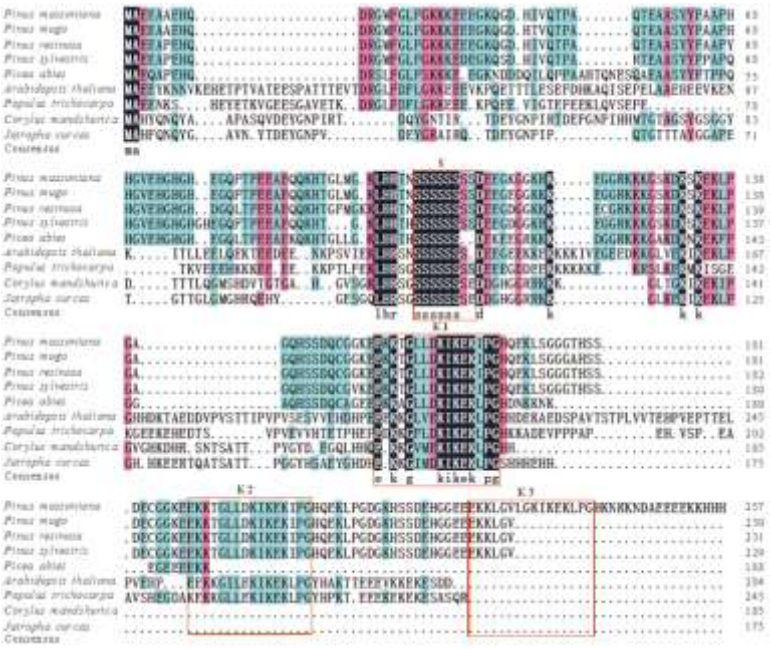

Fig. 1: Alignment of the Pmdhn (KF910087) protein sequence with sequences from *Pinus resinosa* (FJ415453); *Pinus sylvestris* (FJ201497); *Pinus mugo* (HQ108523); *Picea abies* (EF522169); *Corylus mandshurica* (JF433379); *Jatropha curcas* (GU325779); *Arabidopsis thaliana* (X90959); and *Populus trichocarpa* (XM_002307732)

The *Pmdhn* Expression during *P. Massoniana* Drought Treatment

To understand how *Pmdhn* is transcribed under drought stress, RNA from *P. massoniana* roots, stems and leaves was extracted and analyzed the pattern of *Pmdhn* expression. A single 191-bp band was generated by RT-PCR that it can be used for analysis of expression levels. *Pmdhn* expression was found in roots, stems and leaves, and the expression levels in roots and stems were higher than in leaves (Fig. 3).

qRT-PCR was used to study the expression pattern of *Pmdhn* under drought stress conditions using the expression level of *Pmdhn* under well-watered conditions as reference. The results of the qRT-PCR analysis demonstrated the inducibility of the *Pmdhn* transcript in drought treatments. Based on the analysis of *Pmdhn* expression levels using control 18S, an initial up-regulation, followed by a down-regulation at day 15, was observed in roots and stems. The expression levels of *Pmdhn* at day 15 were significantly higher than in other drought stress stages ($P < 0.05$). The *Pmdhn* expression levels in leaves kept rising under drought stress, but the rising rates appeared not so sharp from 5 to 25 day (Fig. 3). These results revealed that *Pmdhn* was induced by drought stress not only in leaves and stem but also in roots.

Overexpression of *Pmdhn* Improves Drought Resistance in *A. thaliana*

To investigate whether *Pmdhn* was involved in drought-resistance responses, plant expression vector and generated transgenic *A. thaliana* overexpressing *Pmdhn* driven by the CaMV35S promoter was constructed. The diagram of the expression vector pBI121-*Pmdhn* is shown in Fig. 4a. In the experiment, a total of six transgenic lines (D1, D3, D5, D7, D8 and D10) in the T_1 generation using akanamycin segregation analysis were obtained. The *Pmdhn* in transgenic *A. thaliana* was determined using RT-PCR,

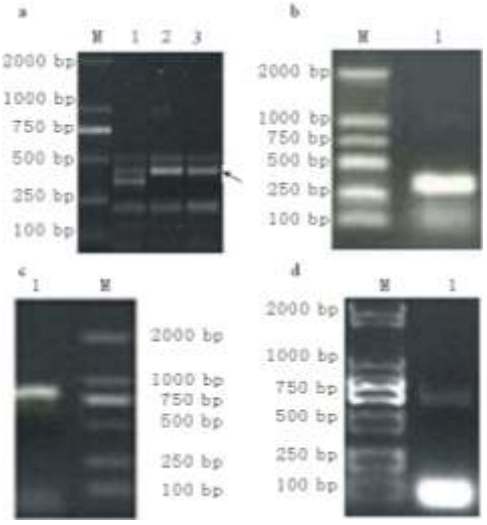

Fig. 1S: Agarose gel electrophoresis results. a. Fragment products. M: DL2000, 1-3: PCR products; b.5′-RACE product.M:DL2000, 1: 5′-RACE product; c. 3′-RACE product. M: DL2000, 1: 3′-RACE product;d. *Pmdhn* cDNA Product. M: DL2000, 1: *Pmdhn* cDNA product

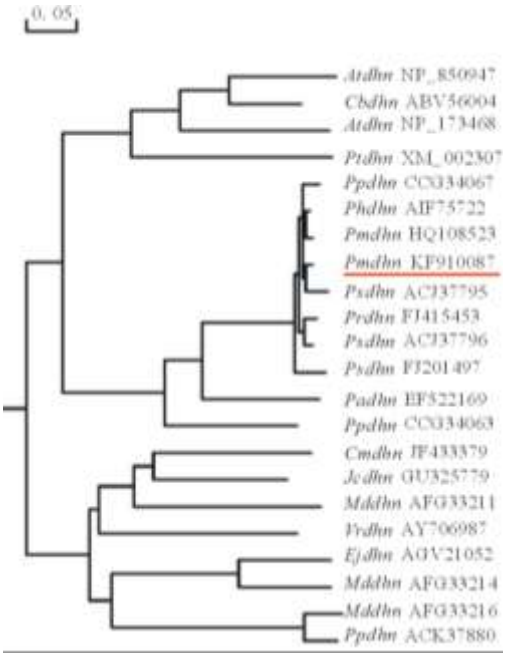

Fig. 2: Phylogenetic relationship of *Pmdhn* with other closely related dehydrin proteins. The accession numbers of the protein sequences used for building the phylogenic tree are provided (see chart)

and the results showed that *Pmdhn* existed in all transgenic lines, but not in WT plants (Fig. 4b). Considering the limited number of seeds and genetic stability, we continued to produce the T_2 generation for further studies under drought stress conditions.

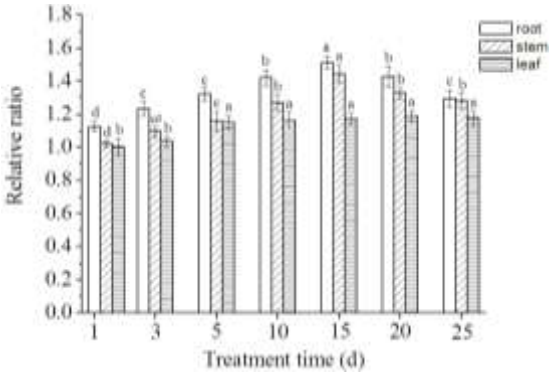

Fig. 3: The transcriptional levels of *Pmdhn* using the 18S control in *P. massoniana* under drought stress compared to normal condition. Data are mean ±SD of triplicate experiments. The different normal letters indicate significant difference after drought stress at 0.05 levels determined by one-way analysis of variance (ANOVA)

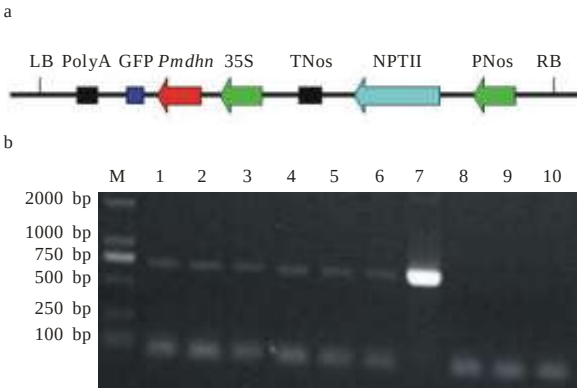

Fig. 4: Plant expression vector construction of *Pmdhn* and PCR analysis of putative *Pmdhn* transgenic *A. thaliana*. a. Diagram of expression vector of pBI121- *Pmdhn*; b.PCR amplifications from DNA of the T_1 generation of selected transgenic *A. thaliana*. M: DL2000; 1-6: Transgenic *A. thaliana* 7: pBI121-*Pmdhn* 8-9: wild-type 10:ddH_2O

There was no difference in the phenotypes and root lengths between the WT and OP plants in petri dishes with the MS medium under normal conditions containing 0% PEG-6000 (Fig. 5a and b). The average root length of the WT plants was 4.05 cm and of the six OP lines 4.21, 4.15, 4.14, 4.53, 4.11 and 3.82 cm, respectively. No statistical significant difference was found between the control and the treatment groups (Fig. 5b). Under drought stress in MS medium containing 3.5% PEG-6000, the difference in phenotypes between the WT and the OP plants was minimal, while the root lengths of the OP seedlings were longer than of the WT lines (Fig. 5a and b). The average root length of the WT plants was 2.93 cm, and 3.64, 3.98, 3.10, 3.86, 3.96 and 3.63 cm, for the six OP lines, respectively corresponding to an average increase of 1.24, 1.36, 1.06, 1.32, 1.35 and 1.24 times, respectively.

Fig. 2S: The nucleotide and deduced amino acid sequences of *Pmdhn*. The S fragment is indicated by the shadow; K fragments are underlined; and the stop codon is marked with an asterisk

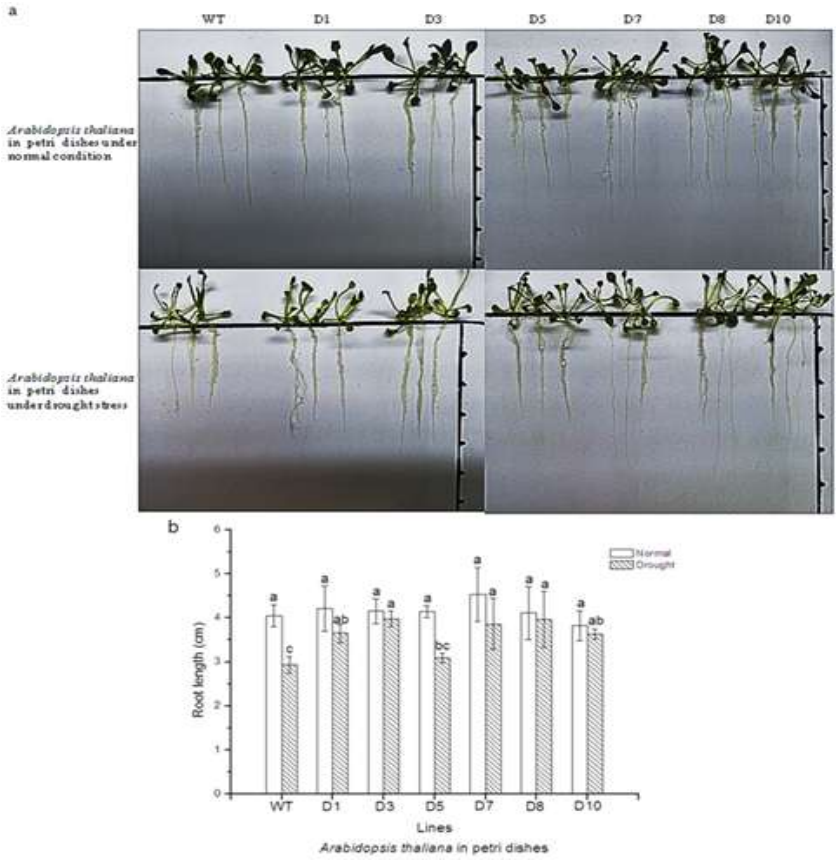

Fig. 5: Analysis of root lengths of wild-type (WT) and transgenic *A. thaliana* under normal (0% PEG-6000) and drought stress (3.5% PEG-6000) conditions. a. Root developments of wild-type (WT) and transgenic *A. thaliana* (D1, D3, D5, D7, D8, D10) with 0% PEG-6000 or 3.5% PEG-6000 (treated) for 15 days. b. Analysis of root lengths of wild-type (WT) and transgenic *A. thaliana*

These results suggested that the root length increased in the transgenic *A. thaliana* under drought stress. The difference in root length between the transgenic (D1, D3, D7, D8, D10) and the wild group was statistically significant ($P<0.05$) (Fig. 5b). The result demonstrated that under drought stress, the roots of the transgenic *A. thaliana* could develop better than of the WT *A. thaliana*, suggesting that the *Pmdhn* gene may participate in the drought process.

To evaluate whether *Pmdhn* is expressed in the T_2 of transgenic *A. thaliana*, the transcriptional levels of *Pmdhn* were assessed under normal and drought conditions. The results of RT-PCR analysis showed that *Pmdhn* can be transcribed (Fig. 6). Compared to the normal treatment, the transcriptional levels of six *Pmdhn* lines were enhanced under drought condition. The Southern blot was undertaken to determine the number of copies of the *Pmdhn* gene present in the transgenic *A. thaliana* genome. The results showed that two copies of *Pmdhn* were present in transgenic *A. thaliana* of D1 and D3 lines, but not in the wild-type *A. thaliana* (Fig. 7). Meanwhile, GFP analysis of the roots in D3 line showed that green fluorescence was excited by blue light in the dark (Fig. 8), which also indicated that *Pmdhn* can be transcribed in transgenic *A. thaliana*.

In the present study, the seedlings of the D3 line from the same growth period to further elucidate the water stress-resistance of transgenic *A. thaliana*. The rate of water loss in leaves of the WT (16.43%, 25.74%, 31.94% and 35.40% at 1, 2, 3, 4 h respectively) and of the transgenic plants (11.04, 18.87, 24.49 and 27.73% at 1, 2, 3, 4 h respectively) increased as a function of treatment time (Fig. 9a). The rate of water loss of the WT plants were higher than of the transgenic plants, indicating that water loss occurred faster in the WT plants than in the transgenic plants ($P<0.05$). In addition, an increase in proline content was observed in the OP and WT plants under drought stress, and the proline content of the transgenic plants increased by 7.79% and 15.79% respectively (Fig. 9b). The MDA content in the WT plants was higher and increased more rapidly than in the OP plants under drought stress, and the MDA content of the WT plants increased by 4.08 and 11.52% respectively (Fig. 9c). The contents of proline and MDA were statistically different ($P<0.05$) (Fig. 9b and c).

Discussion

Dehydrins (DHNs) are associated with the maintenance of plant protein structure under abiotic stress (Farrant *et al*., 2004; Liang *et al*., 2012). Previous studies have shown that K fragments can result in the formation of a double-α-helix, which is the foundation of the hydrophilic structure (Ismail *et al*., 1999). The S-segment leads to nuclear targeting through phosphorylation (Jensen *et al*., 1998). DHNs can prevent the excessive loss of intracellular water, the decrease of the membrane lipid bilayer spacing and maintaining the biological membrane structure (Danyluk *et al*., 1998; Allagulova *et al*., 2003).

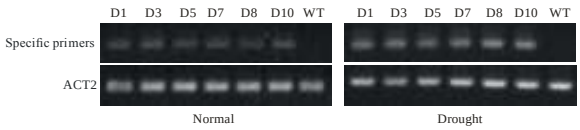

Fig. 6: RT-PCR of *Pmdhn* gene in transgenic and wild-type *A. thaliana* under normal and drought conditions

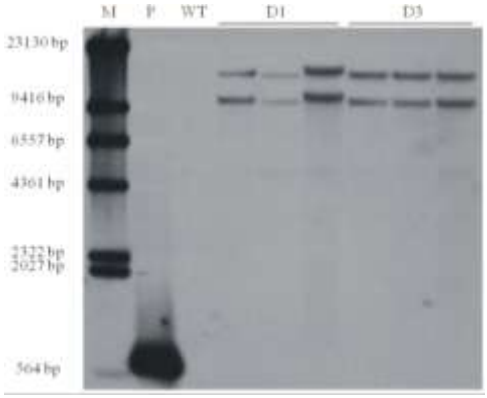

Fig. 7: Southern blot assay of T_2 transgenic *A. thaliana*. M: marker; P: positive control; WT: wild-type *A. thaliana*; D1: 3 randomly selected transgenic *A. thaliana* in D1 line; D3: 3 randomly selected transgenic *A. thaliana* in D3 line

Thus, DHNs can play an important role, similar to the chaperone-like activity, in preventing further degeneration of some denatured proteins (Close, 1996; Tompa and Kovacs, 2000).

In our study, a dehydrin cDNA has been isolated by RT-PCR using degenerate primers. It was found that multiple bands were amplified in the process of *Pmdhn* fragment cloning and all the bands were sequenced and found that only one of them is DHN sequence. Gene-specific primers with its high specificity and sensitivity are easily designed. As mixed bases are matched with degenerate positions, degenerate primers are more difficult to design (Langeveld *et al*., 1991; Colinet and Kummert, 1993; Zheng *et al*., 2008). Although, the target band was amplified by the primers with 1024-fold degeneracy (Rybicki and Hughes, 1990), there may be still great possibility of false positives due to the lower PCR specificity (Rybicki, 2001; Zheng *et al*., 2008). At the same time, the genome size of *P. massoniana* was about 27,400 Mbp (Fan *et al*., 2013), which will also increase the probability of amplifying unrelated sequences. Due to many impacting factors, the generation of non-specific amplifications in process of gene cloning appeared in previous studies (Lin *et al*., 2008; Liu *et al*., 2015).

In this study, the S segment of *Pmdhn* related to the nuclear localization signal and participates in transporting the dehydrated protein into the cell nucleus (Close, 1996), contained eight Ser residues and formed a phosphorylated Ser cluster. In addition, *Pmdhn* contains three K-fragments,

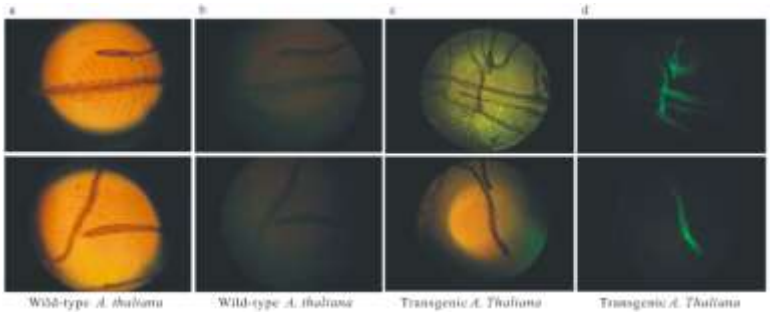

Fig. 8: GFP analysis on roots of wild-type and transgenic *A. thaliana* by fluorescence microscopea.c. The photographs were taken in the bright light b.d. In the dark field for green fluorescence excited by blue light

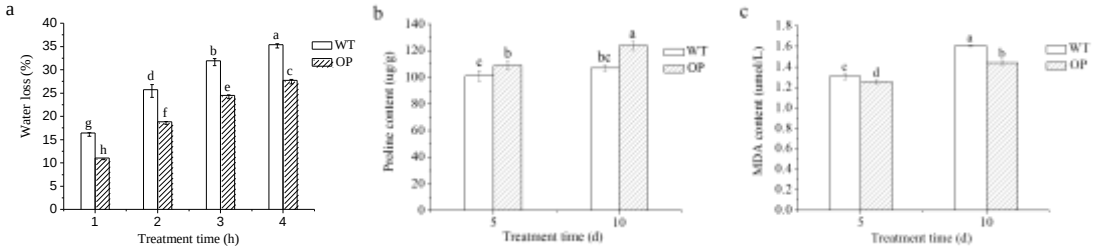

Fig. 9: Effects of *Pmdhn* overexpression under drought stress. a. Water loss in wild-type (WT) and transgenic *A. thaliana* (OP); b. Proline content of WT and OP under drought stress; c. MDA content of WT and OP under drought stress

and each K fragment sequence had three or four different amino acids and contained an extra amino acid (T or L) as compared to the theoretical K fragment (EKKGIMDKIK EKLPG). Surprisingly, the extra amino acid was always found in other Pinaceae plants, which could be the result of different species evolution, or some single nucleotide replacement or structure modification.

Plant drought resistance is a complicated quantitative trait, and environmental stress adaptation in plants is achieved by changing physiology and biochemistry through up-regulated or down-regulated gene expression (Talamè *et al*., 2007). *CsDHN1* and *CsDHN2* isolated from *Camellia sinensis* were observed to be initially up-regulated, followed by down-regulation at 48 h (Paul and Kumar, 2013). *AmDHN* could be induced by PEG-6000 and drought treatments, and the expression of *AmDHN* could be improved under water deficit conditions. At the same time, the drought resistances of transgenic plants overexpressing *AmDHN* were improved (Sun *et al*., 2013). In addition, the drought-related DHNs *PaDhn1* and *PaDhn6* were strongly up-regulated in stem bark and current-year needles in response to drought (Eldhuset *et al*., 2013).

Under drought stress conditions, the *Pmdhn* expression levels in roots and stems increased continuously for the first 15 days and then tapered down slowly. This phenomenon indicated that *Pmdhn* was induced by water deficit in the initial period. Afterwards, *Pmdhn* can no longer adapt for drought stress degree ascending and days prolonging, and then showed a downward trend. The *Pmdhn* expression levels in leaves kept rising under drought stress, but the rates of rising was small.

No apparent changes in phenotype in normal or drought conditions were found. The roots in the OP plants had same length as those of the WT plants under normal conditions. However, roots in transgenic plant were longer than of the WT plants under drought stress, indicating that *Pmdhn* improved the drought resistance in transgene plants. The Southern bolt experiment showed that two copies of *Pmdhn* were present in transgenic *A. thaliana* of D1 and D3 lines, and the GFP analysis of the roots in D3 line also indicated that *Pmdhn* can be transcribed in transgenic *A. thaliana*.

The water balance is favorable to the plant growth and the plant adaptation to drought stress. The accumulation of proline as the main compatible solute can maintain physiological activity (Mahajan and Tujeta, 2005; Ober *et al*., 2005) and balance the redox status of the cell during stress (Lehmann *et al*., 2010). Plants can increase proline concentrations during drought for protecting cells from oxidative damage (Alia *et al*., 2001). In our results, the *Pmdhn* over-expression lowered the water-loss rate and increased the proline content, indicating that *Pmdhn* enhanced the drought resistance by limiting the symplastic water transport to prevent fast wilting and by quickly accumulating proline in transgenic *A. thaliana* to keep the stability of the biological macromolecular structure (Popisilova *et al*., 2011). This phenomenon has also been reported in other species (Lehmann *et al*., 2010). MDA is the final decomposed product of lipo-peroxidation and the MDA content can reflect the level of damage (Xiang *et al*., 2008). In this study, it was found that the MDA contents in the OP and WT plants both increased during the drought

treatment, but the MDA contents were low in the transgenic plants and high in the WT plants, suggesting that the transgenic *A. thaliana* could markedly alleviate the lipid peroxidation caused by the water deficit and protect the stability of the membranes (Xiang *et al*., 2008). These findings suggested that *Pmdhn* may be involved in the drought stress and the overexpression of *Pmdhn* might enhance drought resistance in transgenic *A. thaliana*.

Conclusion

In summary, this study demonstrated the feasibility of cloning a new DHN gene, *Pmdhn*, from *P. massoniana* that was induced by drought stress. This was also confirmed that transgenic *A. thaliana* overexpressing *Pmdhn* can improve plant drought resistance. Further studies which overexpress *Pmdhn* are needed to further characterize and functionally analyze the *Pmdhn* gene in *P. massoniana*.

Acknowledgments

The authors would like to kindly thank financial support from the National Natural Science Foundation of China (31260183) and the 863 Program of China (2011AA10020301). Also, deep thank Fuhua Fan for valuable advice at various stages of the project.

References

Alia, J.M., P. Mohanty and J. Matysik, 2001. Effect of proline on the production of singlet oxygen. *Amino Acids*, 21: 195–200

Allagulova, C.R., F.R. Gimalov, F.M. Shakirova and V.A. Vakhitov, 2003. The plant dehydrins: structure and putative functions. *Biochem.* (*Mosc.*), 68: 945–951

Campbell, S.A. and T.J. Close, 1997. Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytol.*, 137: 61–74

Chen, H.H. and G.J. Ding, 2004. Study on planting density effects for Masson pine plantation. *Sci. Sily. Sin*. (*Chin.*), 40: 92–98

Chen, H.H., G.J. Ding, H.H. Wen and Y. Lu, 2011. Effects of planting density on growth and economic benefit of Masson pine plantation. *For. Res*. (*Chin.*), 24: 470–475

Close, T.J., 1996. Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. *Physiol. Plant.*, 97: 795–803

Colinet, D. and J. Kummert, 1993. Identification of a sweet potato feathery mottle virus isolate from China (SPFMV-CH) by the polymerase chain reaction with degenerate primers. *J. Virol. Methods*, 45: 149–159

Danyluk, J., A. Perron, M. Houde, A. Limin, B. Fowler, N. Benhamou and F. Sarhan, 1998. Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *Plant Cell*, 10: 623–638

Ding, G.J., Z.C. Zhou and Z.R. Wang, 2006. *Cultivation and Utilization of Pulpwood Stand for Pinus massoniana*. China Forestry Publishing House, Beijing, China

Du, D.L., Q.X. Zhang, T.R. Cheng, H.T. Pan, W.R. Yang and L.D. Sun, 2013. Genome-wide identification and analysis of late embryogenesi s abundant (LEA) genes in *Prunus mume*. *Mol. Biol. Rep*., 40: 1937–1946

Dure, L., M. Crouch, J. Harada, T.H.D. Ho, J. Mundy, R. Quatrano, T. Thomas and Z.R. Sung, 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol. Biol*., 12: 475–486

Eldhuset, T.D., N.E. Nagy, D. Volařík, I. Børja, R. Gebauer, I.A. Yakovlev and P. Krokene, 2013. Drought affects tracheid structure, dehydrin expression, and above- and belowground growth in 5-year-old Norway spruce. *Plant Soil*, 366: 305–320

Fan, F.H., B.W. Cui, T. Zhang, G. Qiao, G.J. Ding and X.P. Wen, 2014. The temporal transcriptomic response of *Pinus massoniana* seedlings to phosphorus deficiency. *PLoS ONE*, 9: e105068

Fan, F.H., X.P. Wen, G.J. Ding and B.W. Cui, 2013. Isolation, identification, and characterization of genomic LTR retrotransposon sequences from masson pine (*Pinus massoniana*). *Tree Genet. Genom.*, 9: 1237–1246

Farrant, J.M., C. Bailly, J. Leymarie, B. Hamman, D. Come and F. Corbineau, 2004. Wheat seedlings as a model to understand desiccation tolerance and sensitivity. *Physiol. Plant.*, 120: 563–574

Han, W.P. and G.J. Ding, 2012. The responses of water stress on germination of *Pinus massoniana* seeds from different provenance. *Seed* (*Chin.*), 31: 81–86

Han, W.P., G.J. Ding and B. Bao, 2012. Physiological and ecological responses of *Pinus massoniana* seedling from different provenances to drought stress. *J. Central South Univ. For. Technol.* (*Chin.*), 32: 25–29

Hara, M., 2010. The multifunctionality of dehydrins: an overview. *Plant Signal. Behav*., 5: 1–6

He, P.Y., G.J. Ding and H.H. Chen, 2011. Comparison on soil fertilities of Masson pine plantations of different generations. *For. Res.* (*Chin.*), 24: 357–362

Hunault, G. and E. Jaspard, 2010. LEAPdb: a database for the late embryogenesis abundant proteins. *BMC Genom.*, 11: 221

Hundertmark, M. and D.K. Hincha, 2008. LEA (late embryogenesis abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genom.*, 9: 118

Ismail, A., A.E. Hall and T.J. Close, 1999. Purification and partial characterization of a dehydrin involved in chilling tolerance during seedling emergence of cowpea. *Plant Physiol*., 120: 237–244

Jensen, A.B., A. Goday, M. Figueras, A.C. Jessop and M. Pages, 1998. Phosphorylation mediates the nuclear targeting of the maize Rab17 protein. *Plant J*., 13: 691–697

Langeveld, S.A., J.M. Dore, J. Memelink, A.F. Derks, C.I. van der Vlugt, C.J. Asjes and J.F. Bol, 1991. Identification of potyviruses using the polymerase chain reaction with degenerate primers. *J. Gen. Virol*., 72: 1531–1541

Lehmann, S., D. Funck and L. Szabados, 2010. Proline metabolism and transport in plant development. *Amino Acids*, 39: 949–962

Li, H.S., 2006. *The Principle and Technology of Plant Physiology and Biochemistry Experiment*. Higher Education Press, Beijing, China

Liang, D., H. Xia, S. Wu and F.W. Ma, 2012. Genome-wide identification and expression profiling of dehydrin gene family in *Malus domestica*. *Mol. Biol. Rep*., 39: 10759–10768

Lin, H.L., N.Q. Ren, G.X. Zheng, K. Zhang and Z.J. Duan, 2008. Primers design of cloning aldehyde alcohol dehydrogenase fragment for bacteria B49. *J. Harbin Inst. Technol*. (*Chin.*), 40: 865–869

Liu, Y., X.F. He, J.X. Liu and Z. Ding, 2015. Cloning and prokaryotic expression of Dd-Gpx from *Ditylenchus* destructor. *Biotechnol. Bull.* (*Chin.*), 31: 131–139

Lorenz, W.W., F. Sun, C. Liang, D. Kolychev, H. Wang, X. Zhao, M.M. Cordonnier-Pratt, L.H. Pratt and J.F. Dean, 2006. Water stress-responsive genes in loblolly pine (*Pinus taeda*) roots identified by analyses of expressed sequence tag libraries. *Tree Physiol*., 26: 1–16

Mahajan, S. and N. Tuteja, 2005. Cold, salinity and drought stresses: an overview. *Arch. Biochem. Biophys*., 444: 139–158

Muñoz-Mayor, A., B. Pineda, J.O. Garcia-Abellán, T. Antón, B. Garcia-Sogo, P. Sanchez-Bel, F.B. Flores, A. Atarés, T. Angosto, J.A. Pintor-Toro, V. Moreno and M.C. Bolarin, 2012. Overexpression of dehydrin tas14 gene improves the osmotic stress imposed by drought and salinity in tomato. *J. Plant Physiol*., 169: 459–468

Ober, E.S., M. Le Bloa, C.J.A. Clark, A. Royal, K.W. Jaggard and J.D. Pidgeon, 2005. Evaluation of physiological treats as indirect selection criteria for drought tolerance in sugar beet. *Field Crops Res*., 91: 231–249

Paul, A. and S. Kumar, 2013. Dehydrin2 is a stress-inducible, whereas Dehydrin1 is constitutively expressed but up-regulated gene under varied cuesin tea [*Camellia sinensis* (L.) O. Kuntze]. *Mol. Biol. Rep*., 40: 3859–3863

Perdiguero, P., M.D.C. Barbero, M.T. Cervera, C. Collada and A. Soto, 2013. Molecular response to water stress in two contrasting Mediterranean pines (*Pinus pinaster* and *Pinus pinea*). *Plant Physiol. Biochem*., 67: 199–208

Perdiguero, P., C. Collada, M.D.C. Barbero, G.G. Casado, M.T. Cervera and A. Soto, 2012. Identification of water stress genes in *Pinus pinaster* Ait. by controlled progressive stress and suppression-subtractive hybridization. *Plant Physiol. Biochem*., 50: 44–53

Perdiguero, P., M. Venturas, M.T. Cervera, L. Gil and C. Collada, 2015. Massive sequencing of Ulmus minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. *Front. Plant Sci*., 6: 541

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*., 29: 2002–2007

Popisilova, J., D. Haisel and R. Vankova, 2011. Responses of transgenic tobacco plants with increased proline content to drought and/or heat stress. *Amer. J. Plant Sci*., 2: 318–324

Porebski, S., L.G. Bailey and B.R. Baum, 1997. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol. Biol. Rep*., 15: 8–15

Qiu, H., L. Zhang, C. Liu, L. He, A. Wang, H.L. Liu and J.B. Zhu, 2014. Cloning and characterization of a novel dehydrin gene, *SiDhn2*, from *Saussurea involucrat e* Kar. et Kir. *Plant Mol. Biol*., 84: 707–718

Rorat, T., B.M. Szabala, W.J. Grygorowicz, B. Wojtowicz, Z. Yin and P. Rey, 2006. Expression of SK_3-type dehydrin in transporting organs is associated with cold acclimation in Solanum species. *Planta*, 224: 205–221

Rybicki, E.P., 2001. *PCR Primer Design and Reaction Optimisation*, pp: 39–45. Molecular Biology Techniques Manual, South Africa

Rybicki, E.P. and F.L. Hughes, 1990. Detection and typing of maize streak virus and other distantly related geminiviruses of grasses by polymerase chain reaction amplification of a conserved viral sequence. *J. Gen. Virol*., 71: 2519–2526

Shekhawat, U.K.S., L. Srinivas and T.R. Ganapathi, 2011. *MusaDHN-1*, a novel multiple stress-inducible SK_3-type dehydrin gene, contributes affirmatively to drought- and salt-stress tolerance in banana. *Planta*, 234: 915–932

Shi, J.Y., G.J. Ding and X.F. Yuan, 2004. Studies on water parameters in *Pinus massoniana* seedlings of different Families. *Sci. Silv. Sin.* (*Chin.*), 40: 51–55

Shih, M.D., F.A. Hoekstra and Y.C. Hsing, 2008. Late embryogenesis abundant proteins. *Adv. Bot. Res*., 8: 211–255

Sun, J., L. Nie, G. Sun, J. Guo and Y. Liu, 2013. Cloning and characterization of dehydrin genefrom *Ammopiptanthus mongolicus*. *Mol. Biol. Rep*., 40: 2281–2291

Sunderlíková, V., J. Salaj, D. Kopecky, T. Salaj, E. Wilhem and I. Matusíková, 2009. Dehydrin genes and their expression in recalcitrant oak (*Quercus robur*) embryos. *Plant Cell Rep*., 28: 1011–1021

Szabala, B.M., S. Fudali and T. Rorat, 2014. Accumulation of acidic SK_3 dehydrins in phloem cells of cold- and drought-stressed plants of the Solanaceae. *Planta*, 239: 847–863

Talamè, V., N.Z. Ozturk, H.J. Bohnert and R. Tuberosa, 2007. Barley transcript profiles under dehydration shock and drought stress treatments: a comparative analysis. *J. Exp. Bot*., 58: 229–240

Tompa, P. and D. Kovacs, 2000. Intrinsically disordered chaperones in plants and animals. *Biochem. Cell Biol*., 88: 167–174

Wang, B.M., J.L. Guo, B.Y. Ye, L.P. Xu and Y.Q. Chen, 2008. Molecular cloning and analysis of pinosylvin synthase gene promoter from *Pinus massoniana*. *Subtrop. Agric. Res*. (*Chin.*), 4: 292–296

Wang, Y. and G.J. Ding, 2013. Influence of ectomycorrhiza on nutrient absorption of *Pinus massoniana* seedlings under water stress. *For. Res.* (*Chin.*), 26: 227–233

Xiang, J., Z.B. Chen, P. Wang, L.J. Yu and M.T. Li, 2008. Effect of $CaCl_2$ treatment on the changing of drought related physiological and biochemical indexes of *Brassica napus*. *Front. Agric*., 2: 423–427

Xu, X.H. and G.J. Ding, 2006. Physiological and biochemical responses of *Pinus massoniana* to low phosphorus stress. *Sci. Sivl. Sin.* (*Chin.*), 42: 24–28

Yang, Y., X. Sun, S. Yang, X. Li and Y. Yang, 2014. Molecular cloning and characterization of a novel SK_3-type dehydrin gene from *Stipa purpurea*. *Biochem. Biophys. Res. Commun*., 448: 145–150

Yuan, X., G. Ding and J. Shi, 2008. Effect of soil drought stress on water use efficiency of Masson pine of different species, *J. Zhejiang Normal. Univ.* (*Nat. Sci.*) (*Chin.*), 31: 328–331

Zhang, D.J., L.X. Qin, L. Li, Y. Rao, X.B. Li and W.L. Xu, 2013. Expression of Cotton *GhPRP5* Gene in *Arabidopsis* Enhances Susceptibility toABA and Salt Stresses. *Acta Agron. Sin.* (*Chin*.), 39: 563–569

Zhang, F.K., T. Pan, L. Sheng and K.S. Ji, 2014. Cloning and coding protein structure prediction on cDNA encoding cinnamyl alcohol dehydrogenase from *Pinus massoniana*. *Mol. Plant Breed*. (*Chin.*), 12: 638–645

Zheng, L., M.J. Gibbs and B.C. Rodoni, 2008. Quantitative PCR measurements of the effects of introducing inosines into primers provides guidelines for improved degenerate primer design. *J. Virol. Methods*, 153: 97–103

(Received 27 February 2017; Accepted 25 September 2017)