African Journal of Biotechnology Vol. 10(46), pp. 9225-9232, 22 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1196
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Expression of a SK2-type dehydrin gene from *Populus euphratica* in a *Populus tremula* × *Populus alba* hybrid increased drought tolerance

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Accepted 22 June, 2011

A dehydrin gene, isolated from cDNA library established from the root of *Populus euphratica*, was determined from sequence analysis to be an SK2-type dehydrin (*Pedhn)*. To investigate the function of *Pedhn*, it was expressed via the CaMV 35S promoter in transgenic *Populus tremula* × *Populus alba*. The *Pedhn* transgenic lines demonstrated higher water retention capacities in excised leaves of transgenic lines than that of wild type, and the rate of water loss in the leaves of transgenic lines were slower than that of wild type controls under drought stress. Higher water retention capacity and reduced water loss suggest that the transgenic lines would exhibit enhanced drought tolerance. Consistently, the seedlings of transgenic lines did have significantly enhanced drought tolerance when compared with that of the wild type controls under drought challenge, which indicated that expression of *Pedhn* could be used to genetically modify poplar to improve drought tolerance.

Key words: Dehydrin, transgenic poplar, *pedhn*, drought tolerance.

INTRODUCTION

Drought stress has a significant effect on the development and growth of plants by limiting plant growth and productivity. Plant adaptation to drought stress and the mechanism of drought tolerance has attracted significant interest from researchers in the world. Dehydrin, discovered in the 1980's, was found to confer protection to plant against water deficit (Mundy and Chua, 1988). Dehydrins are group II (D-11 family) of the late embryogenesis abundant (LEA) proteins that accumulate in the later stages of embryogenesis when the water content in seeds declines (Close, 1997). Dehydrins, ranging in size from 9 to 200 kD, are one kind of glycine and lysine-rich proteins, lacking cysteine and tryptophan, which are highly hydrophilic and heat-stable, maintaining stability even in boiling water (Close, 1997; Allagulova et al., 2003). Dehydrin proteins contain several identifiable sequence motifs: Y-segments with the consensus motif (T/VDEYGNP) of various permutation and number located near the N-terminus, S-segments consisting of 5 to 7 serine amino acid residues and K-segments with an EKKGIMDKIKEKLPG motif near the C-terminus (Allagulova et al., 2003). Accordingly, they are classified into five subclasses (YnSK2, Kn, SKn, Y2Kn and KnS) depending on the number of Y-, S- and K-segments that they individually contain (Mundy and Chua, 1988). Specifically, most of the SKn-type dehydrins, either SK2- or SK3-type, contribute to tolerance to drought and cold stresses in plant (Mundy and Chua, 1988; Hara et al., 2003).

Dehydrin genes are expressed at elevated levels in plants exposed to drought, cold or salinity stress (Lee et al., 2005). Dehydrin proteins are one kind of intrinsically unstructured proteins. Because of their disordered state, they readily bind to other macromolecules, such as proteins, membranes, RNA and DNA (Waterer et al., 2010). It is postulated that the interaction of dehydrins

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with cellular macromolecules protects them from the dehydration damage that is commonly associated with drought, heat or cold stress events (Zhu et al., 2000).

Expression of DHN genes in transgenic plants has shown a positive correlation between the accumulation of dehydrin transcripts or proteins and the tolerance to freezing, drought and salinity (Hara et al., 2003; Choudhury et al., 2007; Rorat et al., 2006). Much dehydrin research has been directed at herbaceous species, but reports concerning dehydrins in woody plants are not numerous. Two SK2-type dehydrin genes have been isolated from Populus, such as Podhn from *Populus alba* × *Populus tremula* (Bae et al., 2009) and *Peudhn1* from *Populus euramericana* (Caruso et al., 2002), whose expression was proved to be enhanced by drought, salt and cold. To the best of our knowledge, there are no reports on the expression of these genes to investigate whether it would enhance drought tolerance in transgenic tree.

In this paper, we isolated one SK2-typed dehydrin gene (*Pedhn*) from *Populus euphratica*, which is famous for its high drought tolerance. The *Pedhn* coding sequence was expressed in transgenic *P. tremula* × *P. alba* driven by CaMV 35S promoter in order to assess the effect of this gene on improving drought tolerance.

MATERIALS AND METHODS

Plant materials and isolation of dehydrin gene

A cDNA library was established from the root of *P. euphratica*. The 5'-single pass sequences were determined. One clone homologous to known plant dehydrins gene was selected by searching public databases using BLASTX. The whole cDNA were amplified by PCR and sequenced.

Construction of expression cassette

GUS encoding sequence was excised from the binary pBI121 vector (Clontech). *Pedhn* encoding cDNA was inserted downstream of the CaMV 35S promoter to create the expressing vector, pBI-Pedhn (Figure 1).

Poplar transformation and regeneration

The tissue culture seedlings of a common clone of *P. tremula* × *P. alba* were obtained from the tree growing in the field, using the bud as original explant. The seedlings were then micro-propagated on Murashige and Skoog medium (Murashige and Skoog, 1962) and used for *Pedhn* gene transformation. The vector of pBI-Pedhn was introduced into *Agrobacterium tumefaciens* LBA4404 by freezing-thawing methods. The internodes of the cultured seedlings of *P. tremula x P. alba* were cut into segments of 0.5 to 1 cm and placed on pre-culture medium (Murashige and Skoog medium containing NAA 10 μmol/L and 2ip 5 μmol/L) in a climate chamber at 25°C with a 16 h photoperiod provided by white fluorescent light at 50 $\mu mols^{-2} m^{-1}$ for 2 days. Pre-cultured segments were rinsed in diluted *A. tumefaciens* (OD600 =0.3 to 0.4) containing actosyringone (100 to 200 μmol/L) for 0.5 h. After co-culture on pre-culture medium in the dark for 2 days at 25°C , the segments were placed on callus-induced medium (pre-cultural medium containing carbencillin (Carb) 250 mg/L) at 25°C with a 16 h photoperiod for 10 to 15 days. The segments were then transferred to selection medium (callus-induction medium containing kanamycin, 50 mg/L) at 25°C with a 16 h photoperiod for 15 to 20 days. After shoot differentiation, the induced microshoots were excised and cultured on root-induction medium (Murashige and Skoog medium with half of the macro elements concentration with Km, 50 mg/L) for 2 to 3 weeks. Only one shoot was isolated from each stem explants to ensure that the transgenic lines were derived from independent transformation events. Both the translines and wild control were then micro-propagated by regeneration from the shoot nodal explants. Finally, the rooted transgenic plantlets were transferred into pot in the greenhouse.

PCR and southern assay of transgenic plants

Total genomic DNA extracted by SDS method was used as template for PCR assay. Two oligo-nucleotides, 5’- TCTAGA GGATCCACTAGTTCTAG-3’ (plus strand) and 5’-AGAAC ACAAGAATACTGCCTGCTGC-3’ (minus strand); the former corresponds to CaMV 35S promoter and the other corresponds to 3’ end of *Pedhn* cDNA, were used for PCR amplification. About 100 μg total genomic DNA was extracted from transgenic and untransformed plants with CTAB method and these DNA samples were completely digested with combination of Sac I and BamH I. After fractionation on a 0.8% agarose gel, the DNA fragments were transferred to nylon membrane. The southern hybridization was performed with digoxigenin labeled Pedhn as probe (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche Applied Science). The washing condition was 2 × 15 min in 0.5 × SSC containing 0.1% SDS at 65°C.

RT-PCR analysis to assess the expression of *Pedhn* in transgenic lines

Both the translines and wild controls were transferred to pot and grown in green house. Total RNA was extracted with TRIzol reagent (Invitrogen) from the new leaves of both translines and wild controls. Genomic DNA was removed by using RNase-Free DNase (QIAGEN) at room temperature for 30 min. RNA integrity and absence of DNA were checked by running on an agarose gel. Then, DNAase treated RNA was re-quantify by measuring OD260. We used equal amounts of RNA for reverse transcription, which was performed with PrimeScript® One Step RT-PCR Kit Ver.2 (TaKaRa). The first strand of cDNA was used as template to perform RT-PCR to assess the expression of *Pedhn* in transgenic lines. To avoid the interference of endogenous dehydrin genes, two special primers were designed for RT-PCR. One oligo-nucleotides exactly upstream of the starter condon ATG of *Pedhn* in pBI-Pedhn was used as sense primer, another oligo-nucleotides corresponding to 3’ end of *Pedhn* cDNA was used as the antisense primer. The sequences of the primers were 5’-CGCCACCGCGGG AATTCGATTCG-3’ and 5’-CCTTCTCTTCTTCGGTCTTGGG-3’, respectively.

Assay of excised leaf water retention capacity

The seedlings of wild controls and transgenic lines were grown in greenhouse, watered every 1 to 2 days. After one week, the third leaf (fully expanded) from the top of each seedling was cut off for the determination of excised leaf water retention capacity as follows: the cut leaves were weighed immediately as the weight (Wf) at zero time. The leaves were then put in an oven at 35°C and weighed every 30 min as the weight (Wn) of the corresponding

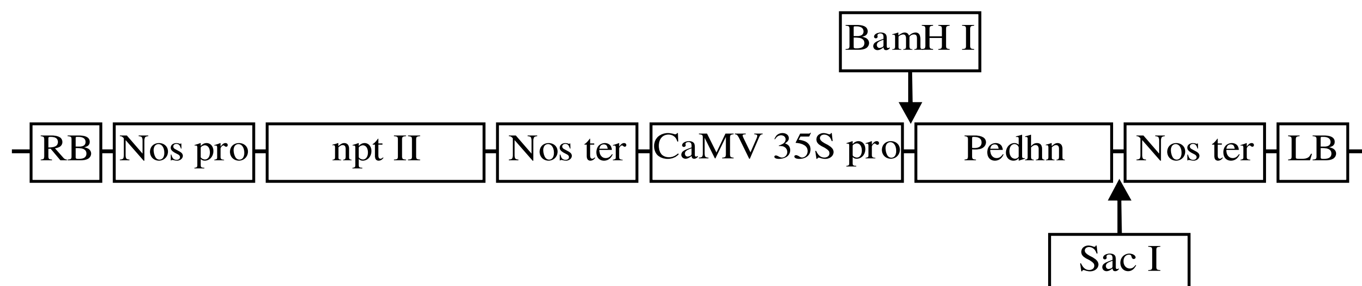

Figure 1. Map of *Pedhn* expression cassette in pBI-Pedhn. LB and RB: Left and right borders of Agrobacterium T-DNA; Nos pro, Nos promoter; Nos ter, Nos terminator; nptII, the neomycin phosphotransferase II; CaMV 35S pro, CaMV 35S promoter; BamH I and Sac I are two restriction enzymes beside *Pedhn*.

time. After 6 times of weighing, the leaves were dried up and weighed as dry weight (Wd). The relative water percentage (RWP) at each time during oven-drying was obtained as follows: RWP (%) = (Wn – Wd) / Wf × 100%; n is the time when the leaves wereweighed during drying. The excised leaf water retention capacity was analyzed by the rate of change of the average relative water percentage during the oven-drying. For each of line and wild control, three leaves from different seedlings were measured, respectively. The averages of excised leaf water retention capacities of the three leaves for each lines and wild control were used for analysis.

Measurement of leaf relative water content under drought stress

After rooting, the regenerated culture seedlings of both wild control and translines were continually grown in the root-induction medium in flask (Murashige and Skoog medium with half of the macro elements concentration with Km, 50 mg/L) for about two weeks. The seedlings were taken out and the roots were washed with water carefully. Each seedling was placed vertically by inserting through one ports of about 1.5 cm in a 2 cm thick foam board. The board was floated in a container full with PEG 6000 (polyethylene glycol 6000) solution of 15% so that the roots of the seedlings were immersed in the solution. The PEG 6000 solution of 15% was refreshed every day. The fully expanded leaves at the similar position were selected and removed from the plant at 2nd, 4th, 6th and 8th day, respectively. The collected leaves were weighed immediately as fresh weight (FW) and then crosscut into small sections of about 0.5 cm. The leaves sections were immersed in deionized water at 4°C overnight. Then, their rehydration weight (RW) was determined immediately after the extra water was soaked up with absorbent paper. Finally, the leaves sections were dried completely at 70°C, and weighed to obtain dry weight (DW). Relative water content (RWC) was determined as follows: RWC (%) = (FW-DW) / (RW-DW) × 100%. Each measurement was done using 3 different seedlings per transline and for the wild-type control which was repeated three times.

Identification of drought tolerance of transgenic seedlings

Both the transgenic and control plantlets were transferred to the pot and grown in green house with watering every 1 to 2 days. After 10 days, the watering was stopped. The growth and development of each seedling was observed to identify drought resistance of the transgenic and control plants at the 10th and 20th day, respectively.

RESULTS

Sequence analysis of *Pedhn*

A full-length EST clone derived from a cDNA library prepared from the root of *P. euphratica* was found to have high homology to dehydrin genes in plants, sharing 96% homology with Podhn (GenBank: DQ856592.1) from *P. alba* × *P. tremula* and 97% homology with Peudhn1 (GenBank:AJ300524.4) from *P. euramericana* at nucleic acid level, respectively. The *P. euphratica* cDNA sequence was designated as *Pedhn*. As shown in Figure 2, the open reading frame (ORF) encoding sequence of *Pedhn* was about 684 bp long, encoding a protein of 227 amino acid residues. An S-segment consisting of seven serine residues (located at 87-93) and two K-segments (located at 149-164 and 195-209, respectively) were found near the C-terminus, yet no Y segment was detected. Therefore, according to Campbell and Close (1997), this *Pedhn* gene from *P. euphratica* encodes a SK2-type dehydrin.

Analysis of transgenic plants and verification of expression of *Pedhn* in translines

For PCR assay, two oligonucleotides corresponding to *Pedhn* were designed and used as primers. Using total genomic DNA of transformed plants as template, specific DNA fragments were amplified with the size of about 800 bp, which was same as expected for the *Pedhn* fragment produced from that of pBI-Pedhn vector control (Figure 3). The results showed that the *Pedhn* sequence was integrated into the transgenic poplars.

A 800 kb DNA fragment is expected to be released from pBI-Pedhn with Sac I and BamH I restriction enzymes (Figure 1). Southern analysis (Figure 4) showed that several DNA fragments from Sac I and BamH I digested genomic DNA of transgenic plants and control were hybridized by labeled *Pedhn* cDNA. The band of about 800 bp, which was same as that in pBI-Pedhn digested with Sac I and BamH I, was not generated in the untransformed poplar (Figure 4, lane 2). It is obvious that

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1     ATGGCTGAGGGAAACAAGAGTCATGGGTACGAGACCAAAGTTGGTGAAGAGAGTGGTGCT
1      M  A  E  G  N  K  S  H  G  Y  E  T  K  V  G  E  E  S  G  A
61    GTTGAGACCAAGGATCGCGGGTTGTTTGATTTCCTGGGGAAGAAAGAAGAAGAGAAGCCT
21     V  E  T  K  D  R  G  L  F  D  F  L  G  K  K  E  E  E  K  P
121   CAAGAGGAGGTGATTGTTACTGAATTTGAAGAGAAACTTCAGGTTTCTGAACCCGAGACT
41     Q  E  E  V  I  V  T  E  F  E  E  K  L  Q  V  S  E  P  E  T
181   AAAGTAGAGGAAGAGCACAAGAAAACAGAGGAAGAGGAGAAGAAACCTACTCTCTTTGGG
61     K  V  E  E  E  H  K  K  T  E  E  E  E  K  K  P  T  L  F  G
241   AAACTCCATCGATCAGGCAGCAGTTCCAGCTCTTCTAGTGACGAGGAGGAAGGTGACGAT
81     K  L  H  R  S  G  S  S  S  S  S  S  S  D  E  E  E  G  D  D
301   GAAGAGAAAAAGAAGAAGAAGAAGGAAAAGAAGTCATTGAAAGAGGAGATGAAGATGTCA
101    E  E  K  K  K  K  K  K  E  K  K  S  L  K  E  E  M  K  M  S
361   GGAGAGAAAGGAGAGGAGAAGGAACACGAGGATACTAGTGTTCCTGTCGAGGTAGTCCAT
121    G  E  K  G  E  E  K  E  H  E  D  T  S  V  P  V  E  V  V  H
421   ACAGAAACACCCCATGAACCAGAGGAGAAGAAGGGTTTCCTTGACAAAATCAAGGAGAAA
141    T  E  T  P  H  E  P  E  E  K  K  G  F  L  D  K  I  K  E  K
481   TTGCCAGGACATAAGAAAGCTGACGAGGTCCCCCCTCCTCCTCCAGCTCCTGAACATGTT
161    L  P  G  H  K  K  A  D  E  V  P  P  P  P  P  A  P  E  H  V
541   TCCCCTGAAGCTGCAGTCTCCCATGAAGGATCAGATGCCAAGGAGAAGAAAGGACTACTC
181    S  P  E  A  A  V  S  H  E  G  S  D  A  K  E  K  K  G  L  L
601   GAGAAGATCAAGGAGAAGTTACCTGGGTACCACCCCAAGACCGAAGAAGAGAAGGAGAAA
201    E  K  I  K  E  K  L  P  G  Y  H  P  K  T  E  E  E  K  E  K
661   GAAAAGGAGAGTGCTTCCCAGTAG
221    E  K  E  S  A  S  Q  *
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Figure 2. Nucleotide and deduced amino acid sequences of *Pedhn*. Predicted amino acids are shown in one letter code. Nucleotide numbers and amino acid numbers are shown on the left. The putative NLS (nuclear localization signal) is underlined with a double line, the S-segment is shown with a single line, the K-segment is shown in bold italics and the poly-proline cluster is in bold.

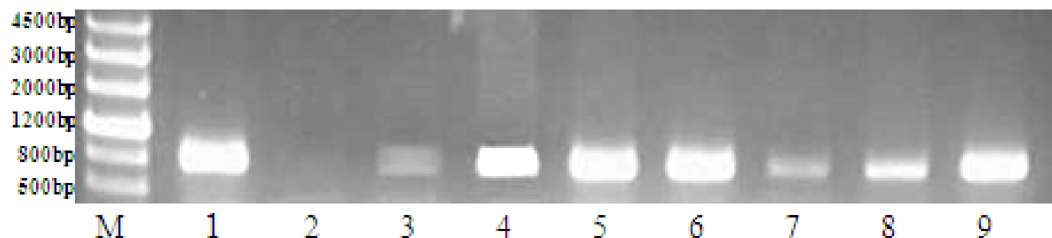

Figure 3. PCR assay of seven independent transgenic lines. M, DNA molecular weight marker;1, pBI-Pedhn; 2, wild type control; 3 to 9, transgenic lines 1, 2, 4, 14, 15, 30 and 31.

the other signal bands in both transgenic and untransformed plants, except that of 800 bp, came from endogenous dehydrin genes. The result demonstrated convincingly that Pedhn had been integrated into the genomic DNA of transgenic lines.

In RT-PCR analysis, about 750 bp band was amplified from translines, while it was not generated in the RT-PCR of the wild control. The DNA fragment corresponding to the sense primer which is exactly upstream of the start condon of *Pedhn* in the pBI-Pedhn vector, could be transcribed when *Pedhn* was expressed under the drive of CaMV 35S promoter. Only the transgene of *Pedhn* could be amplified. Figure 5 indicates that the *Pedhn* was expressed in translines 2, 4, 14 and 31.

Measurement of excised leaf water retention capacity

The tanslines 2, 14 and 31 were selected for the measurement of excised leaf water retention. Water retention capacity in excised leaf reflects the tolerance of plants against water-deficit stress. The water retention capacity curves of the three transgenic poplar lines (2, 14 and 31) and non-transgenic control leaf are shown in Figure 6. The relative water percentage of fresh leaves of the transgenic plants and non-transgenic plants were

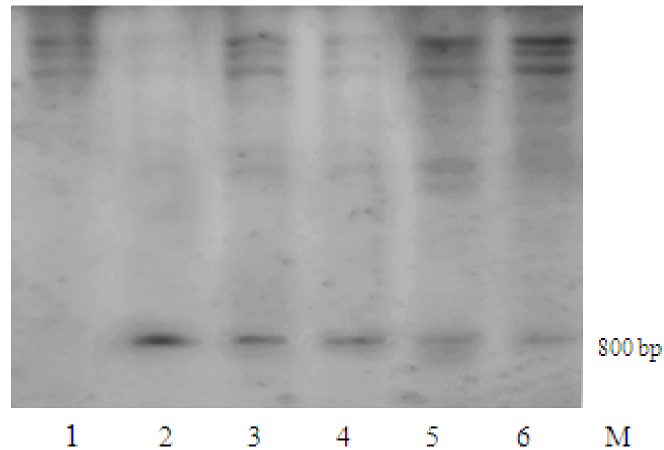

Figure 4. Southern blotting of the four transgenic lines with *Pedhn* as probe. M, DNA molecular weight; 1, wild type control; 2, pBI-Pedhn vector; 3 to 6, transgenic lines 2, 4, 14 and 31.

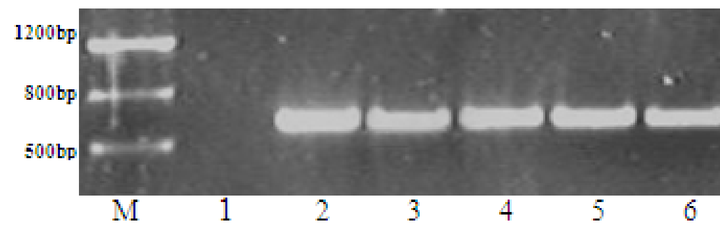

Figure 5. RT-PCR analysis to assess the expression of *Pedhn* in transgenic lines. M, DNA molecular weight; 1, wild type control; 2, pBI-Pedhn vector; 3 to 6, transgenic lines 2, 4, 14 and 31.

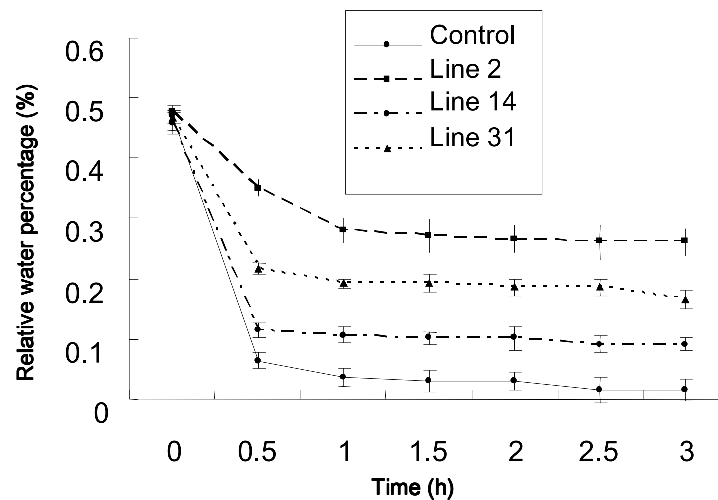

Figure 6. Water retention capacity of the wild type and transgenic lines. Bar at each point represents standard error.

almost the same at the beginning and all decreased over the drying time. The average relative water percentage decreased dramatically at the first hour of treatment but stabilized thereafter. However, the water percentage in

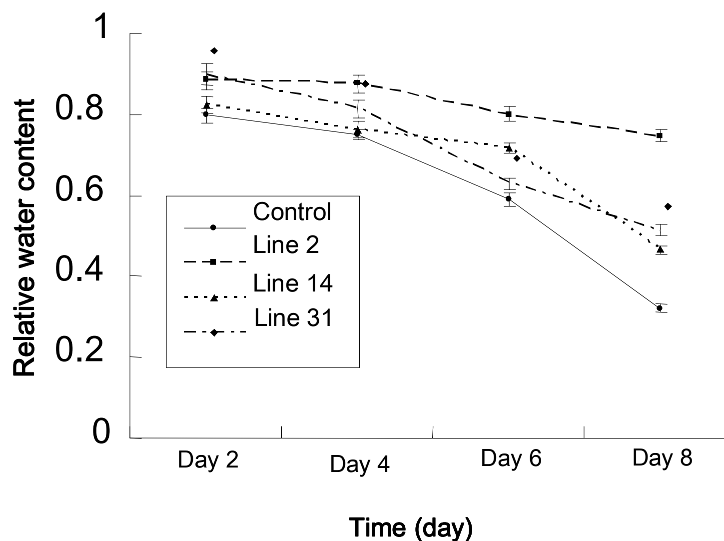

Figure 7. Relative water content of wild-type and transgenic plants under drought stress. Bar at each point represents standard error.

the transgenic leaves remained higher than that of the non-transgenic control. At 3 h, the relative water percenttages were about 29% for line 2, 17% for line 31, 10% for line 14 and only 4% for the non-transgenic control, respectively. It was shown that transgenic line leaves had a higher water holding capacity than wild type. Increased water retention capacity in transgenic lines indicated an enhanced tolerance to drought stress.

Measurement of relative water content under drought stress

Leaf relative water content is a parameter to assess water status in the plant, which is an indicator of how water uptake by the roots matches vegetative water loss under drought stress. Leaf relative water contents of three transgenic lines and the non-transgenic control at different time points under drought condition are shown in Figure 7. At the second day of drying, there was little difference among relative water contents of leaves of transgenic and control plants. Over the drying time, the leaf relative water contents all decreased both in transgenic lines and control, but it decreased more quickly in the control than in the three transgenic lines. At the 8th day of treatment, the average leaf relative content in the control was about 32%, while those in transgenic lines 14, 31 and 2 were about 48, 50 and 73%, respectively. The results displayed that the water in transgenic lines was lost more slowly under drought condition than those of non-transgenic control.

Considering all the data, this indicates that the drought tolerance of transgenic lines with the expression of Pedhn gene was improved as compared to the non-transgenic control.

Observation of drought tolerance of transgenic plant

Plants of transgenic lines 2, 14 and 31 and the non-transgenic control were transplanted into pots and grown in greenhouse. After 2 weeks, watering was stopped to compare the growth under drought stress. There were no significant differences between the transgenic line and control during the first several days but differences appeared at the 10th day (Figure 8), with the transgenic lines showing greater vigor than the control. On the 20th day, the entire non-transgenic plants had withered, while the transgenic lines continued to grow, but wilted and had yellow-green leaves. This showed that the drought tolerance of transgenic lines increased significantly in transgenic lines.

DISCUSSION

Although there has been much effort to improve plants for abiotic stress resistance by traditional breeding, success has been limited, especially for trees. Genetic modification by transferring drought-resistant genes into trees had been considered to be one possible effective means to improve the drought-resistant tree plants in the tree breeding (Harfouche et al., 2010; Li et al., 2008). The effects of dehydrin in drought stress tolerance in plants has led to widespread concern, and the research has also made considerable progress (Rorat et al., 2006; Bae et al., 2009; Caruso et al., 2002; Rorat, 2006). Poplar has proven to be a valuable model plant for tree genetic research. Although dehydrin genes have been reported to be isolated from different species of Populus (Bae et al., 2009; Caruso et al., 2002), there had been no research on its application in genetic modification to

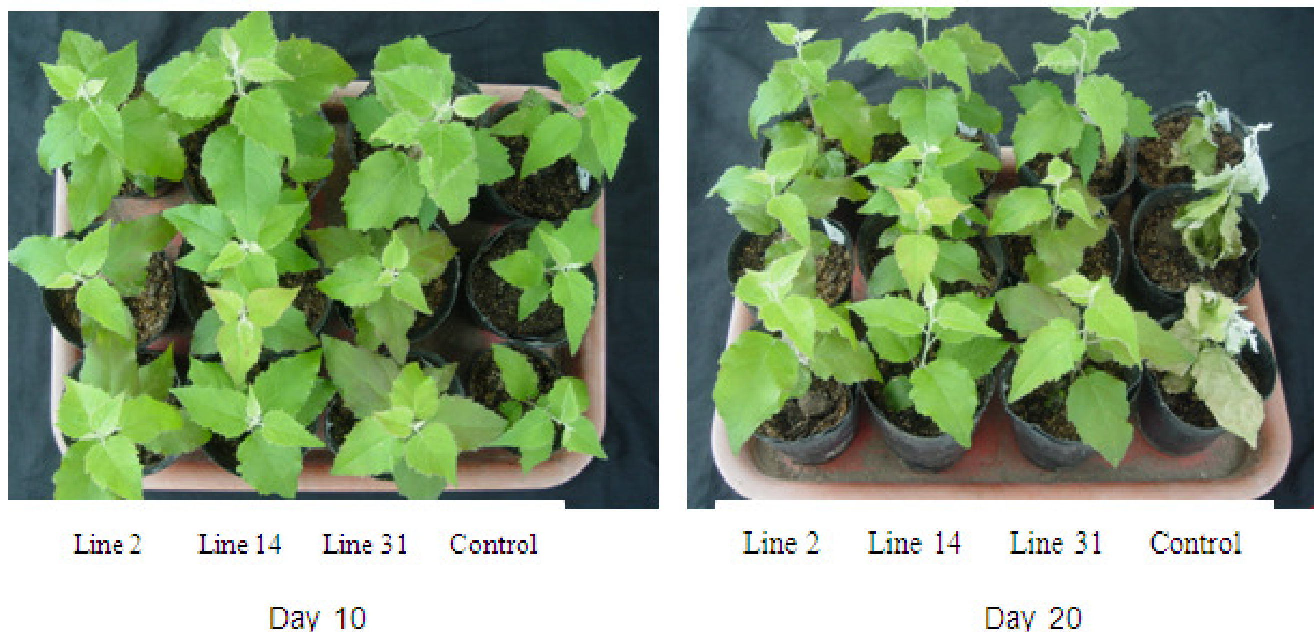

Figure 8. The seedlings of transgenic lines and the control at days 10 and 20 after stopping watering.

improve drought-resistant in poplar. In this paper, a dehyhrin cDNA was isolated from *P. euphratica*, a *Populus* species which is well known for its high drought tolerance. This dehydin gene appeared to be the SK2 type by sequence analysis. To verify whether itsexpression could enhance drought tolerance in transgenic trees and provide some evidence for its biological function in plants, *Pedhn* was expressed in transgenic *P. tremula × P. alba*. Relative water content under drought stress and water retention capacity could reflect tolerance of plant to water deficit stress. The results showed that both transgenic lines had higher water capacity retention and relative water content than non-transgenic controls under drought stress. Consistently, drought caused more severe damage to leaves of non-transgenic control plants than that of transgenic lines when the relative water contents were measured. We could presume that expression of dehydrin protected either the membrane of cells or the macromolecules in transgenic lines from shortage of water, resulting in enhanced water conservation capacity and reduced water loss rate under drought stress. Moreover, drought treatment on greenhouse grown trees showed that transgenic seedlings had an enhanced tolerance to drought.

Considering all these observations, the introduction of an expression cassette to cause the expression of *Pedhn* from *P. euphratica* appeared to improve water conservation capacity and decrease water loss rate under drought stress, resulting in the enhancement of drought tolerance in transgenic poplars. Since the poplar is a perennial plant, the transgenic poplars in this paper need to be observed continuously for several years in the field to confirm the enhanced drought tolerance. The results in this paper indicated that expression of *Pedhn* gene could be applied to improve drought tolerance in tree plants by genetical modification.

ACKNOWLEDGEMENTS

This work was supported by National Natural Sciences Foundation (Grant No. 30972392), the National Major Program on Transgenic Organisms (Grant No. 2009ZX08009-060B) and Hebei Province Natural Science Foundation(Grant No. C2007000732).

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