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Differential expression of a poplar SK_2-type dehydrin gene in response to various stresses

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Dehydrins are group II, late embryogenesis abundant proteins that act putatively as chaperones in stressed plants. To elucidate the function of dehydrins in poplar, we isolated the SK_2-type dehydrin gene *Podhn* from *Populus alba* × *P. tremula* var. *glandulosa* suspension cells and analyzed its expression following treatments of abiotic stress, wounding and plant growth regulator. Sequence homology and phylogenetic analyses indicate *Podhn* encodes an acidic dehydrin (p*I* 5.14, 277 amino acids, predicted size 25.6 kDa) containing two lysine-rich "K-segments" and a 7-serine residue "S-segment", both characteristic of SK_2-type dehydrins. Southern blots show *Podhn* genes form a small gene family in poplar. *Podhn* was expressed in all tissues examined under unstressed conditions, but most strongly in cell suspensions (especially in the stationary phase). Drought, salt, cold and exogenous abscisic acid (ABA) treatments enhanced *Podhn* expression, while wounding and jasmonic acid caused its reduction. Therefore, *Podhn* might be involved in ABA or stress response. [BMB reports 2009; 42(7): 439-443]

INTRODUCTION

Trees are sessile organisms with long lifespans that expose many to wide diurnal, seasonal and stochastic fluctuations in environmental variables such as periods of drought and various climatic stresses. Therefore, survival and reproduction is dependent upon an array of tolerance mechanisms that have evolved to help plants to overcome and adjust to stressful conditions throughout their growth and development. Such mechanisms involve a wide range of proteins and other molecules including dehydrins, which respond to dehydration caused by both drought and cold stresses (1, 2).

Dehydrins are Group II (D-11 family), late embryogenesis abundant (LEA) proteins that accumulate in the later stages of embryogenesis when the water content in seeds declines (3). However, they are also induced in vegetative tissues in response to drought and cold stresses (4, 5) where they appear to function in various cellular compartments including the cytoplasm, nucleus and mitochondria (6). 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 amino acid residues and K-segments with an EKKGIMD KIKEKLPG motif near the C-terminus (3). Accordingly, they are classified into five subclasses (Y_nSK_2, K_n, SK_n, Y_2K_n, and K_nS) depending on the number of Y-, S- and K-segments they individually contain (1). Specifically, most of the SK_n-type dehydrins are either SK_2- or SK_3-type and contribute to tolerance to drought and cold stresses (1, 7). Although SK_n-type dehydrins from crops and herbaceous plants have been investigated in a number of studies (1, 3), little attention has been paid to their role in trees, which are often exposed to both cold and drought stresses at various times in their life cycles.

In this study we isolated and characterized an SK_2-type dehydrin genomic sequence and its corresponding cDNA from a hybrid poplar (*Populus alba* × *P. tremula* var. *glandulosa*), followed by analyzing its expression in response to drought, salt, low temperature and plant hormones.

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RESULTS AND DISCUSSION

Isolation and structural characterization of the *Podhn* gene

A full-length EST clone derived from a cDNA library prepared from suspension cells of *Populus alba* × *P. tremula* var. *glandulosa* was selected with more than 50% homology to previously described plant dehydrins. As shown in Fig. 1, the cDNA is 1,077 bp long and has a 681 bp open reading frame (ORF) with an 103 bp upstream sequence and a 279 bp downstream sequence. The cDNA ORF starts at nucleotide position 104 and ends at position 788, encoding a protein of 227 amino acid residues with a predicted molecular mass of ca. 25.7 kDa and a p*I* of 5.13. According to the predicted amino acid sequence of the protein the most abundant amino acid residues are glutamate, lysine and glutamine, constituting 28%, 27% and 21% of the total, respectively, while cysteine or tryptophan residues are absent. Almost half (48.5%) of the predicted amino acid residues are polar with hydrophilic character, as supported by a mean hydropathy value of -1.6. An

Fig. 1. Sequence analysis of *Podhn*. (A) Nucleotide and deduced amino acid sequences of *Podhn* (GenBank Accession Number DQ856592). Predicted amino acids are shown in one letter code. Nucleotide numbers are shown on the left and amino acid numbers on the right. The putative NLS is underlined with a double line, the S-segment with a single line, the K-segment in italics and the poly-proline cluster in bold. An intron is shown in lowercase letters while the *Hin*dIII and *Nco*I sites are boxed. Arrows indicate primer binding sites for genomic PCR amplification. (B) Similarity tree showing the relationships among *Podhn* and other plant dehydrins. Alignments of the deduced amino acid sequences were performed using Clustal W and the tree was generated by the neighbor-joining method using percentage identities. Each type of dehydrin is indicated in parenthesis.

S-segment consisting of seven serine residues and two K-segments was found near the C-terminus, yet no Y segment was detected. Therefore, the characteristics of this poplar dehydrin are consistent with the general characteristics of dehydrins as described by Close *et al*. (3). In that study it was found most dehydrins have low levels of hydrophobic amino acid residues, lacking both cysteine and tryptophan while (as mentioned above) containing various permutations and numbers of well-conserved Y-, S- and K-segments.

The cDNA clone we isolated in the present study, designated *Podhn* (Poplar dehydrin), appears to encode a SK_2-type dehydrin containing an S-segment located between residues 87 and 93 upstream of two K-segments (named K-1 and K-2 and located at residues 149-163 and 195-209, respectively, near the carboxy terminus). The homology of the K-segments found in this study is higher than 80% when compared to conserved K-segments. There are also a poly-proline cluster located at residues 171-176 between the K1-and K2-segments and a lysine motif consisting of six residues (*KKKKKK*) at residues 103-108, which is speculated to function as a nuclear targeting signal sequence (NTS) (8). A search for transmembrane helices using TMHMMv2.0 did not detect any transmembrane helix in *Podhn,* suggesting it functions in the cytosol or nucleus rather than in membranes (9).

Fig. 2. Genomic Southern blot analysis of the *Podhn* gene in poplar. Genomic DNA (10 μg) digested with *Eco*RI (E), *Hin*dIII (H) or *Xba*I (X) was fractionated by electrophoresis in an 1.0% agarose gel. The gel was blotted onto a nylon membrane and hybridized with ^{32}P-labeled full-length *Podhn* cDNA.

Homology analysis

Podhn has its highest sequence homology (95%) to the SK_2-type dehydrin Peudhn1, which belongs to another *Populus* species, *P. euramericana,* located in a different section of the genus (10). Podhn also shows 59% and 50% sequence homology to two SK_3-type dehydrins, DHN24 of *Solanum sogaradinum* (7) and Cadhn of *Capsicum annuum* (11), respectively.

Phylogenetic analysis of YSK_2-type and SK_n-type dehydrins indicate Podhn clusters together with SK_n-type dehydrins, especially with SK_2-types (Fig. 1B). Thus, the results of both the homology and phylogenetic analyses suggest Podhn is an SK_2-type dehydrin.

Genomic organization of the *Podhn* gene

The copy number of the *Podhn* gene was investigated by Southern blotting with a full-length *Podhn* cDNA probe. It was found digestion of genomic DNA with either *Eco*RI or *Hin*dIII yielded four bands that hybridized with the probe, while digestion with *Xba1* yielded only three bands (Fig. 2). We speculated this difference was potentially due to the presence of a restriction site in an intron in the S-segment, since many dehydrin genes contain such an intron (12-14). Therefore, to detect possible *Eco*RI*, Hin*dIII or *Xba*I restriction sites we determined the nucleotide sequences of the PCR-amplified DNA fragments of *Podhn.* The results confirmed both the presence of an 112 nt intron between residues 91 and 92 within the S-segment (residues 87 to 93) as well as the absence of any *Eco*RI, *Hin*dIII or *Xba*I restriction sites (Fig. 1A). After detection of a *Nco*I site in the intron, this enzyme was used to cut the *Podhn* into two DNA fragments that were later used as probes. Southern blotting with the two probes gave the same pattern as shown in Fig. 2 (data not shown). Therefore, 2 to 4 copies of the *Podhn* gene appear to be present in the poplar genome since an *Hin*dIII cutting site was detected in the untranslated region.

Tissue expression profile of *Podhn*

To characterize the tissue-specific expression of *Podhn*, total

RNA was extracted from leaves, stems, roots and floral buds of whole poplar plants, and from suspension cells 14 days after subculturing for northern analysis. The results revealed that while *Podhn* was expressed in all the tissues examined, it was expressed most strongly in suspension cells and most weakly in stems and floral buds (Fig. 3A). Therefore, this SK_n-type dehydrin gene seems to lack any tissue-specific expression pattern, which coincides with previous studies indicating these dehydrins are expressed differentially depending on the growth conditions and plant species rather than tissue. For instance *OsDhn1*, an SK_3-type dehydrin in rice, is expressed in callus, seeds, leaves and shoots (15); *peudhn1*, an SK_2-type dehydrin from *P. euramericana,* is reportedly expressed in both leaves and (less strongly) roots (10); and *PgDhn1*, an SK_4-type dehydrin from white spruce (*Picea glauca*), is expressed in various tissues, including the petiole, stem, roots and buds (5).

Expression of *Podhn* during cell growth

Cultured plant cells can have high degrees of genetic and physiological homogeneity and thus have potential in the large-scale commercial production of various secondary metabolites (16). Cell culture systems are also useful in examining responses of cells to changes in culture conditions, cell division and growth. To examine changes in *Podhn* expression during the growth cycle of suspension cells, both the total RNA content and fresh weight of the suspension cells were monitored after sub-culturing in fresh medium. The observed changes in fresh weight are shown in Fig. 3B. Cells remained in an initial lag phase until entering an exponential growth phase from day 8 to day 16. Cells then appeared to grow very slowly until day 22, whereupon their growth completely ceased. During this growth cycle, cellular levels of *Podhn* mRNA changed substantially, rising rapidly upon entering the exponential growth phase at day 8 until day 24. The mRNA level in the late stationary phase (day 24) was 2.8-fold higher than in the lag phase (day 4) (Fig. 3C). The reason why the *Podhn* level was so high in the late stationary phase remains unknown, but may be related to nutrient deficiency in the culture medium and osmotic stress as the cells enter the apoptotic cell death phase (16). Nevertheless, more detailed analysis is needed to explain why *Podhn* mRNA increases steadily from the early exponential phase until the late stationary phase of the cell culture cycle.

Effects of abiotic stress, wounding and plant growth regulators on *Podhn* expression

To examine the effects of various stresses and abscisic acid

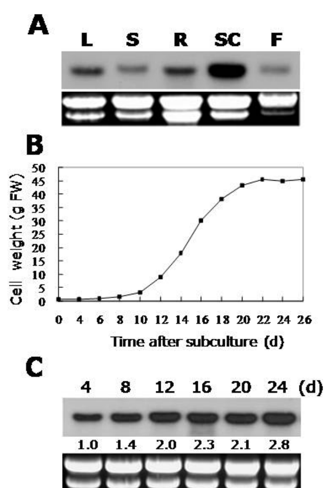

Fig. 3. *Podhn* expression in various tissues of poplar cells during normal growth in suspension culture. (A) Northern analysis of total RNA extracted from mature leaves (L), stems (S), roots (R), flowers (F) and cell suspension culture (SC). (B) Growth kinetics of poplar cells in suspension culture. After sub-culturing, fresh cellular mass was determined every 2 days for 26 days. (C) Northern analysis of total RNA extracted from the cells at the indicated times. Full-length *Podhn* cDNA was used as the probe and ethidium- bromide stained ribosomal RNA served as a loading control. *Podhn* expression levels represented as relative values when compared with those of 4-day cultured cells were determined by computer-based densitometry, as described in *Materials and Methods*.

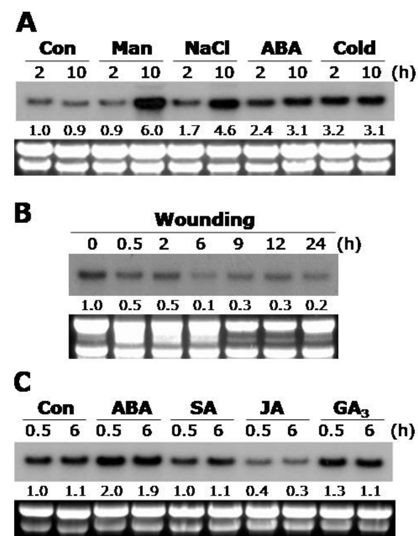

Fig. 4. *Podhn* expression under various treatment conditions. (A) *Podhn* expression in suspension cells in response to mannitol treatment (Man, 250 mM), NaCl (150 mM), ABA (25 μM) and cold (2°C) for 2 and 10 h. Untreated control cells (Con) were incubated for the same time periods. (B) *Podhn* expression in leaf tissues in response to wounding. Ca. 1 cm cuts were made with sterile scissors along the edges of leaves from 1-year-old poplar plants. Total RNA was extracted from the trimmed leaves at the indicated times. (C) *Podhn* expression in suspension cells in response to treatment with plant growth regulators including ABA (20 μM), SA (20 μM), JA (10 μM) and GA_3 (20 μM) for 0.5 and 6 h. *Podhn* expression levels repesented as relative values when compared to those of untreated controls were determined by computer-based densitometry, as described in *Materials and Methods.*

(ABA) on *Podhn* expression, poplar suspension cells were treated separately with 250 mM mannitol, 150 mM NaCl, 25 μM ABA and low temperature (2°C). Northern analysis of RNA sampled from the cells revealed that all treatments increased *Podhn* expression (Fig. 4A). Changes in gene expression induced by each of the treatments over time were also compared. While ABA treatment or low temperature caused 2-3 fold increases in *Podhn* mRNA levels within 2 hrs, mannitol and NaCl induced increases in mRNA levels more slowly (after 10 hrs) and of greater magnitude (ca. 4 to 6 fold). The increases induced by treatment with mannitol (which is known to induce osmotic stress by decreasing water availability in plant cells; 17, 18) and NaCl are consistent with the previously reported effects of salt and PEG6000 on *Peudhn1* expression (10), which indicated the level of SK_2-type dehydrins in woody species increases in response to increased cellular osmotic pressure. ABA treatment and low temperatures also cause increases in the level of an SK_2-type dehydrin (*BpuDhn2*) in birch (13). Our results indicate SK_2-type dehydrins in woody species are likely upregulated *via* an ABA-dependent signaling pathway in response to low temperature, drought or salt stresses (19, 20). However, whether the ABA-mediated pathway or other stress-specific pathways are solely responsible for increases in *Podhn* expression in response to low temperature, drought or salt remains to be elucidated. In contrast, the expression level of *Podhn* began to decrease as early as 30 min after wounding and did not recover to pre-wounding levels for up to 24 h (Fig. 4B).

The effects of applying various hormones-ABA, jasmonic acid (JA), salicylic acid (SA) and gibberellic acid (GA_3)-for either 30 min or 6 h on the expression level of *Podhn* were also examined. As shown in Fig. 4C, Northern blot analysis revealed *Podhn* transcript levels increased only in response to ABA while decreasing in response to JA (Fig. 4C). The latter finding is interesting since JA regulates the expression of numerous defense genes, with cells accumulating JA upon becoming wounded or infected by pathogens (19). While the SK_4-type dehydrin *PgDhn1* has been shown to be up-regulated by either wounding or JA treatment (5), the present study shows SK_2-type *Podhn* was downregulated by the same treatments. Therefore, SK_2-and SK_4-type dehydrins appeared to respond differently to wounding and pathogen infection. Further, given that *PgDhn1* in *Picea glauca* is also reportedly up-regulated by drought and low temperature (5), we speculate all SK_n-type dehydrins are induced in response to drought and low temperature stresses yet differ in their response to wounding and pathogen infection stresses.

MATERIALS AND METHODS

Plant materials and growth conditions

Poplar suspension cells were maintained by sub-culturing when they reached the stationary phase by transferring 0.4 g fresh cellular mass to 100 ml of liquid MS medium (21) containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.1 mg l^{-1} 1-naphthalene acetic acid and 0.01 mg l^{-1} 6-benzylaminopurine (22). The suspensions were maintained at 120 rpm on a gyratory shaker placed inside a culture room at 22 ± 1°C under dim (20 μmol m^2s^{-1}) cool-white fluorescent light. To analyze expression during normal growth cycles, cells were harvested every two days after sub-culturing for 26 days, followed by vacuum filtration through 3MM filter paper, weighing, liquid nitrogen freezing, and storage at 70°C for RNA isolation. For tissue-specific expression analysis, leaves, stems and roots were harvested from 1-year-old poplar plants growing in a nursery while flowers were harvested from approximately 25-year-old plants.

Construction of cDNA library and isolation of the dehydrin gene

Total RNA was extracted from a cell suspension 8 days after sub-culturing by the guanidine thiocyanate method (23), followed by purification of poly $(A)^+$ RNA from the total RNA by oligo (dT) column chromatography. A cDNA library was constructed using a ZAP-cDNA Gigapack III Gold Cloning kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. Using a ExAssist helper phage, the cDNA library was randomly excised *in vivo*, the plasmid DNAs were isolated and the 5'-single pass sequences were determined. Public databases were then searched using BLASTX to select clones homologous to known plant dehydrins. The selected cDNA clones were then sequenced and analyzed by Vector NTI advance 9.0 (Invitrogen, USA) and shown to encode an SK_2-type dehydrin.

PCR amplification was performed with genomic DNA using primers specific for the *Podhn* gene. Genomic DNA was extracted from the leaves of 1-year-old poplar plants growing in a nursery using a MagExtractor-Plant Genome kit (Toyobo, Osaka, Japan). The primers used were Podhn-s (5'-AAGTTCATATTGT GATTCTGATCAG-3') and Podhn-as (5'-CGTTCTATAGAACAC AATAATACT-3'). PCR amplification consisted of 35 cycles of 40 s denaturation at 94°C, 30 s annealing at 60°C and a 120 s extension at 72°C. The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using T7 and SP6 primers. Sequences of the PCR product were compared with those of *Podhn*.

Southern blot analysis

Ten μg of genomic DNA were digested with *Eco*RI, *Hin*dIII or *Xba*I restriction enzymes overnight. The DNA was then electrophoretically separated on an 1% agarose gel, transferred to a Hybond-XL nylon membrane (Amersham-Pharmacia, Piscataway, NJ) according to the capillary transfer method (24) and probed using labeled full-length *Podhn* cDNA. Labeling of the probe, hybridization and membrane washing were done as described by Lee *et al*. (25).

Stress treatments and northern blot analysis

To mimic drought-induced osmotic stress, four-day-old suspension cultures were supplemented with mannitol or NaCl. To assess their response to the drought-associated hormone ABA,

replicate suspensions were treated with the hormone. To examine the effects of cold stress, suspension cells were incubated in Erlenmeyer flasks at ca. 2°C in ice on a gyratory shaker. In addition, the effects of other plant growth regulators were tested by supplementing cells with SA, ABA, JA or GA_3. Replicate flasks (n = 3) were harvested for each case. Amounts and duration of the treatments are indicated in the figure legends. A wounding treatment was also applied to the leaves of 1-year-old poplar plants by making ca. 1 cm cuts along their edges with sterile scissors followed by leaf collection. All treated cells and leaves were immediately frozen in liquid nitrogen and stored at 70°C until analyzed. Total RNA was isolated from the samples using TRI Reagent (Molecular Research Center, Cincinnati, Ohio), followed by electrophoretically separating 10 μg portions of the RNA on 1.2% formaldehyde agarose gels and transfer to a Hybond-XL nylon membrane. Labeling of the probe, hybridization and membrane washing were done as described by Lee *et al.* (25). Signals were quantified by densitometric calculations using ImageJ 1.4 software (http://rsb.info.nih.gov/ij).

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