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Sequence variability and expression pattern of the dehydrin gene family in *Populus alba* × *P. tremula* var. *glandulosa*

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Abstract

The dehydrin (*Dhn*) genes occur as multi-gene families in the plant genome and are suggested to play a protective role in cold and drought tolerance. Here, we have identified 10 unique dehydrin genes (*PoDhn*) from poplar (*Populus alba* × *P. tremula* var. *glandulosa*), 6 of which have the full ORF. The *PoDhn1* encodes for SK_2-type, *PoDhn2* and *PoDhn7* for Kn-type, *PoDhn3* for K_3S-type, and *PoDhn5* and *PoDhn6* for the Y_3SKn-type dehydrins, respectively. Results of gene expression analysis demonstrated that most of the *PoDhn* genes were expressed under normal growth conditions and the transcription level of the *PoDhns* increased by abiotic stress treatment. In particular, *PoDhn2* and *PoDhn7* transcripts increased dramatically by both cold and drought treatment and *PoDhn5* was up-regulated by only drought stress. These results may be useful in further studies of *PoDhn* genes, including investigations into the mechanisms underlying gene expression, the nature of their variation, and their physiological functions.

Keywords: *PoDhn*; poplar; dehydrin; drought; cold; abiotic stress.
Abbreviations: Dhn- dehydrin; PoDhn- poplar dehydrin; EST- expressed sequence tag; ABA- abscisic acid; ORF- open reading frame.

Introduction

Drought and low temperature are the most severe environmental stresses that limit plant growth and yield. Many plants have developed strategies to adapt to abiotic stresses that they are exposed to. More than 1,000 genes have been shown to be responsive to drought and low-temperature conditions (Seki et al., 2001; Tommasini et al., 2008). The genes induced under these stress conditions are believed to function in the physical protection of cells against water deficiencies or temperature changes (Bray, 1993, 2002; Shinozaki and Yamaguchi-Shinozaki, 2000). The dehydrins (Dhns) are the most prominent proteins with regards to abiotic stress tolerance processes (Close, 1996; 1997; Hara et al., 2003). The most distinctive feature of the dehydrins is their conserved lysine-rich 15-amino acid referred to as the K-segment (Close, 1996). The S- and Y-segments are also found in dehydrin. On the basis of the number and order of the conserved segments, dehydrins can be classified into different sub-classes such as Y_nSK_n, SK_n, K_n and K_nS (Close, 1996; 1997). Dehydrins are distributed throughout a wide range of photosynthetic organisms, including both higher and lower plants (Close, 1997; Kosova et al., 2007). Expression of the most *Dhn* genes were induced by various environmental factors such as cold, frost, drought, salinity and heat (Kosova et al., 2007; AL-Ghumaiz and Motawei, 2011). Several studies applying overexpression and ectopic expression of the *Dhn* gene demonstrate that dehydrin increases tolerance to low temperature, drought and high salinity stress (Brini et al., 2007; Munoz-Mayor et al., 2011). The dehydrins are believed to function as stabilizers of membrane structure and proteins. Protective interactions with membranes can occur via the K-segment, which although intrinsically unstructured, assumes an amphipathic helical structure when bound to membranes (Koag et al., 2003). The dehydrins have also been suggested to function as water attractants in cells with low water potential, and hence also perform a function in osmotic potential regulation (Nylander et al., 2001). Additionally, a phloem iron-transport protein (ITP) from castor bean (*Ricinus communis*) was identified as a KS-type dehydrin (Kruger et al., 2002). Arabidopsis ERD14, a SK_2 dehydrin, and celery VCaB45, a dehydrin-like vacuolar protein, bind calcium in a phosphorylation-dependent manner and have chaperone activity (Heyen et al., 2002; Alsheikh et al., 2003; Kovacs et al., 2008). These findings demonstrate that the dehydrin structural type may exert a specific effect, and that some of the dehydrins may function as ion-sequesters. Poplar is a woody perennial plant which is exposed to drought and cold stress cycles during its lifespan. Therefore, poplar plants must sustain arid and freezing temperature conditions during the winter season, and harbor adaptive mechanisms that help them survive in these types of stressful environments. Here, we attempt to identify the dehydrin gene family from the poplar genome, and compare the sequence variation and expression pattern of each of the dehydrin genes.

Results and Discussion

Identification of the *Dhn* genes from *Populus alba* × *P. tremula* var. *glandulosa*

We identified 6 genes encoding putative dehydrin from the *P. tricocarpa* genome and 13 ESTs from the genus *Populus*, such as *P. tremula* and *P. tremula* × *P. tremuloides* (Supplementary Table 1). A total of 19 *Dhn* primer sets were designed and used to amplify the *Dhn* gene in the genome of *Populus alba* × *P.*

tremula var. *glandulosa*. The *Dhn* gene candidates and primer sequences are summarized in Supplementary Table 1. A primer set from *P. tricocarpa* and three primer sets from poplar ESTs did not amplify any PCR products from the genomic DNA of the *Populus alba* × *P. tremula* var. *glandulosa*. The PCR products were subcloned and their sequence was determined, with the exception of a PCR product amplified from *P. tricocarpa* genomic DNA. A total of 14 putative *Dhn* genes were successfully amplify from the *Populus alba* × *P. tremula* var. *glandulosa* (Supplementary Table 1). Sequence analysis of these PCR products demonstrates that two PCR products did not contain the K-segment and were considered not to be dehydrin. Some PCR products overlapped with other PCR products; for example, ID13 and ID18 overlapped with ID3. This is why the three clones were considered the same clone. Similarly, the PCR product ID14 overlapped with ID7 (Supplementary Table 1). Finally, we identified 9 unique *Dhn* candidates from *Populus alba* × *P. tremula* var. *glandulosa*, and named them *PoDhn1*, *PoDhn2*, *PoDhn3*, *PoDhn5*, *PoDhn6*, *PoDhn7*, *PoDhn8*, *PoDhn9* and *PoDhn10*. The result of the sequence analysis of PCR products from the *PoDhn9* primer sets demonstrate that two different clones were amplified. These two clones had similar sequences to each other and were named *PoDhn9-1* and *PoDhn9-2* (Fig. 1C). A total of 10 *PoDhn* genes are characterized in Table 1. Among 10 *PoDhn*s, six *PoDhn*s clones encoded a full-length open reading frame, but other clones harbored 5'- or 3'-truncated DNA. The sequences of the identified *PoDhn*s were deposited in the Genbank database, under accession numbers HM626468 to HM62677. The *Dhn* genes are present as a multigene family in the genomes of higher plants (Close, 1997; Kosova et al., 2007). The *Populus alba* × *P. tremula* var. *glandulosa* is a tetraploid land plant and may contain more *Dhn* homologues in the genome than those identified in this study.

Characterization of the poplar dehydrins

The characteristics of the polypeptides deduced from the 10 *PoDhns* are summarized in Table 1 and Fig. 1. The typical features of the dehydrins can be described via the "YSK" shorthand (Close, 1996). The *PoDhn1* encodes for an acidic SK_2-type Dhn of 228 amino acids (25.9 kDa, pI, 5.01) (Fig 1A), which was isolated and reported previously (Bae et al., 2009).

The *PoDhn2* encodes for a K_2-type Dhn of 625 amino acids (70.3 kDa, pI 5.95). The *PoDhn7* also encodes for a K_2 type dehydrin of 593 amino acids (66.9 kDa, pI 6.19), which exhibits a high degree of sequence homology with PoDhn2. The PoDhn2 and PoDhn7 harbor two K-segments in the C-terminal region, but no Y-segment or S-segment. In addition to the K-segment, the amino acid sequence analysis revealed that these PoDhn polypeptides harbor two different repeat segments (Fig. 1B). The first segment consists of the following 40 amino acid sequence, MIPAYKKTEDGPPSPAETAV-HPTETPLEPEKKSYFEQAKG (40 AA segment), found in the N-terminal region. The 40 AA segment was repeated 5 times without intervening sequences in the PoDhn2 polypeptide, but the segment was repeated 4 times in PoDhn7 (Fig. 1B). The second segment consists of the following 29 amino acid sequence, EPEEKRGFFDQAKERTPGFKKTEE-VSPRR (29 AA segment), and is present between the 40 AA segment and the K-segment. The second segment is repeated 6 times in the PoDhn2 and repeated 8 times in PoDhn7. PoDhn2 and PoDhn7 are of the K_n type without the S-segment and has the largest molecular weight in dehydrins identified from the poplar genome. The number of K-segments is shown to be variable in K_n dehydrins. The molecular weight of the K_n-type dehydrin is related to the number of K-segments. For example, TdDHN9.6, a K_2-type dehydrin from *Triticum durum*, is a polypeptide consisting of 93 amino acids (Labhilili et al. 1995), but barley Dhn5, K_9-type dehydrin harboring the nine K-segment is a polypeptide consisting of 575 amino acids (Choi et al., 1999). The PoDhn2 and PoDhn7 have high molecular weights but harbor only two K-segments. Instead of the K-segments, PoDhn2 and PoDhn7 harbor two different segments, which contribute to an increase in molecular weight. Analysis of the secondary structure of the PoDhn2 and PoDhn7 demonstrate that hydrophilic domains are repeated in regular patterns, which are the results of repetition of the 40 AA segment and 29 AA segment (data not shown). These segments may play a role in cold or desiccation tolerance, such as the K-segment. Analysis of the amino acid sequence showed that PoDhn10 also shows a significant degree of amino acid sequence homology with PoDhn2, but that the 3'-terminal region was truncated (Fig. 1B). *PoDhn3* encodes for a basic K_3S-type polypeptide of 186 amino acids (19.8 kDa, pI, 9.6). PoDhn9-2 has the highest amino acid sequence homology to PoDhn3, although several amino acids at the N-terminal region were missing (Fig. 1C). PoDhn8 and PoDhn9-1 also exhibits high amino acid sequence homology with PoDhn3, but the S-segment was not detected in the C-terminal region of their polypeptide (Fig. 1C). *PoDhn5* and *PoDhn6* encode for a Y_3SK_2-type polypeptide of 183 amino acids (19.6 kDa and pI, 6.46) and 133 amino acids (18.9 kDa and pI, 5.03), respectively. PoDhn6 was relatively similar to PoDhn5 except that it contained just one K-segment in the C-terminal region (Fig. 1D).

Comparison of the dehydrin from poplar and barley

The *Dhn* gene family has been extensively studied in barley (Choi et al., 1999; Zhu et al., 2000). In order to characterize the PoDhn, we compared the amino acid sequence of the Dhn polypeptides from barley (HvDhn) and poplar (PoDhn), and then constructed a phylogenetic tree (Fig. 2). The PoDhn1 is very similar to HvDhn8, which is an acidic barley SK_3-type dehydrin and is involved in freezing tolerance (Choi et al., 1999; Danyluk et al., 1998). The phylogenetic tree shows that K_n type PoDhn, Podhn2 and PoDhn7, made a branch with SK_2-type PoDhn1 (Fig. 2). PoDhn5 and PoDhn6 were similar to barley HvDhn11, which is the Y_2SK_2 type. The popular K_nS-type dehydrins, PoDhn3 and PoDhn8, were similar to barley HvDhn13, which is a KS-type polypeptide (Rodriguez et al., 2005). Fig. 2 shows that YSK_n type dehydrin including YSK_2 and YSK_3-type are a major group in the barley genome. Close (1996) suggested that the YSK_2-type dehydrins, HvDhn3 and HvDhn4, are major components in desiccation-tolerant barley, a monocotyledonous crop plant. However the YSK_2-type dehydrins are not detected in the poplar genome. Instead of the YSK_n type, the K_n- and K_nS- type dehydrins are more common in the poplar genome. These results indicate that the poplar dehydrin alleles are different from those in monocotyledonous crop plants.

Expression of the *PoDhn* genes

RT-PCR results show that the *PoDhn1* gene is expressed in leaf tissue under normal growth conditions, as well as abiotic stress conditions (Fig. 3). Bae et al. (2009) reported that the expression of *PoDhn1* was enhanced following abiotic stresses

Table 1. Characteristics of PoDhns from *P. tremula* x *P. alba* var. *glandulosa*

	Accession No.*	Amino acid No.	M.W. (kDa)	pI	Dhn type	comment
PoDhn1	HM626468	228	25.9	5.01	SK_2	-
PoDhn2	HM626472	625	703	5.93	K_n	-
PoDhn3	HM626471	186	19.8	9.60	K_3S	-
PoDhn5	HM626469	183	19.6	6.46	Y_3SK_n	-
PoDhn6	HM626470	133	18.9	5.03	Y_3SK_n	-
PoDhn7	HM626473	593	66.9	6.19	K_n	-
PoDhn8	HM626474	N/A**	N/A	N/A	N/A	5'- and 3'-truncated
PoDhn9-1	HM626476	N/A	N/A	N/A	N/A	5'-truncated
PoDhn9-2	HM626477	N/A	N/A	N/A	N/A	5'-truncated
PoDhn10	HM626475	N/A	N/A	N/A	N/A	3'-truncated

*Genebank (www.ncbi.nlm.nih), ** N/A, not available

such as drought, salt and cold. However, wounding and jasmonic acid induced a reduction of *PoDhn1* expression. The *PoDhn8* and *PoDhn9* gene transcripts were detected in the control condition at similar levels to the stress treated tissues. PCR amplification with the *PoDhn9* primer sets produced two different PCR products, which are named *PoDhn9-1* and *PoDhn9-2* (Fig. 1C, Fig. 3). The transcript of the *PoDhn3* was detected as several bands at the same level under control as well as stress conditions. In an effort to determine whether these different sizes of RT-PCR products originated from alternative splicing of the *PoDhn3* transcript or from a different dehydrin gene, we subcloned the PCR product and determined the sequence. The sequence analysis results indicate a repeat sequence and the PCR product size depends on the number of repeat sequences (data not shown). Among the *PoDhns*, *PoDhn2*, *PoDhn5* and *PoDhn7* responded to cold and drought stress. The expression of *PoDhn2* increased, particularly under cold treatment conditions. *PoDhn5* was detected only under drought conditions and *PoDhn7* was detected under drought and cold stress conditions (Fig. 3). *PoDhn6* and *PoDhn10* were not detected in the leaf samples under any stress conditions tested. This result suggests that the expression of *PoDhn6* and *PoDhn10* may be stress- or organ-specific under natural conditions. In order to determine the organ-specific expression pattern of the *PoDhn* genes, total RNA samples were prepared from open leaves and stems under cold stress conditions, and winter buds at field-grown plants. The RT-PCR result show that most *PoDhn* genes are expressed at the highest levels in winter buds, although their transcripts were detected in the stem and leaf (Fig. 4). In particular, abiotic stress responsive *PoDhn* genes, *PoDhn2*, *PoDhn5* and *PoDhn7* were expressed at the highest levels in winter buds (Fig. 4). *PoDhn5* was only expressed in the winter bud. The winter buds are exposed to arid and freezing temperature conditions during the winter season. Fig. 3 show that the *PoDhn5* transcripts were detected in the leaf tissues under drought stress conditions. These results demonstrate that the expression of *PoDhn5* is drought-specific and regulated via a pathway different from the pathway associated with the regulation of other *PoDhn*s. The poplar is a perennial tree, which is distributed in diverse habitats in the northern hemisphere. The poplar plants must sustain dry and freezing temperatures during the winter season. *PoDhn2*, *PoDhn5* and *PoDhn7* are abiotic stress-responsive and may contribute to drought and cold tolerance in poplar. The transcripts of *PoDhn6* and *PoDhn10* were not detected in tested tissues, although genomic DNAs were amplified (data not shown).

Based on these results, we expect that *PoDhn6* and *PoDhn10* may be expressed in different tissues, such as the seeds or roots. Some dehydrin genes, such as *Dhn12*, showed tissue-specific expression in barley (Choi and Close, 2000). In summary, nine poplar dehydrin genes were identified herein and could be classified into four groups--SK_2-, K_n-, K_3S- and Y_3SK_n-type dehydrin--on the basis of their amino acid sequence homology. YSK_2-type Dhns, which are major Dhn in the barley genome, were not detected in the poplar genome. The K_n-type Dhn, PoDhn2 and PoDhn7 contain two repeat segments, which are not found in other plants, including barley. *PoDhn2* and *PoDhn7* transcript levels were dramatically increased by both cold and drought conditions, while *PoDhn5* responded only to drought stress. These three *PoDhn* genes may contribute to drought and cold tolerance in the poplar plant. The results presented in this study provide a foundation that may facilitate further studies of the *Dhn* genes, including the nature and evolution of sequence variation, as well as the cause-and-effect relationships that occur between specific alleles of the *Dhn* genes and stress-tolerance traits in perennial plants.

Materials and Methods

Plant materials

The poplar trees (*Populus alba* × *P. tremula* var. *glandulosa*) used in this study were obtained from the Korea Forest Research Institute and cultured in the field. One year old branches were cut and rooted in soil pot. Poplars in the soil pot were cultured under field condition.

Abiotic stress and ABA treatment

In order to study the expression analysis of the *PoDhn* genes, soil pot grown poplar plants were transferred to a 25℃ growth room under artificial light of 200 μmole photons $m^{-2}s^{-1}$, provided by cool-white fluorescent lamps with a 16:8 photoperiod. For the dehydration treatment, whole poplar stems were cut and transferred onto a clean paper towel, their fresh weight was reduced to 60% at room temperature, and the leaf tissues were snap-frozen in liquid nitrogen. To determine the effects exerted by cold stress conditions, the plants were moved to a growth chamber at 5Ċ for 3 hours, and leaf tissues were harvested. For the plant stress hormone ABA, 100 μM ABA solution was sprayed onto the poplar plants in the soil pot at room temperature, and leaves were harvested at 3 hours after treatment.

Fig 1. Comparison of the amino acid sequences of poplar dehydrins. Amino acid residues are designated via single-letter codes. Dashes indicate places in which a sequence has been expanded to allow for optimal sequence alignment. The Y-, S- and K-segments are boxed. Alignments of the deduced amino acid sequences were conducted with the CLUSTAL X program. A, Amino acid sequence of the PoDhn1. B, Comparison of the amino acid sequence and repeat sequence in K_n type dehydrins, PoDhn2, PoDhn7 and PoDhn10. The 40 amino acid repeat segments were bold-faced and the 29 amino acid repeat segments were underlined. The circle and bracket number indicate repeated 40 AA and 29 AA segments, respectively. C, Comparison of amino acid sequences of the K_nS type PoDhns, PoDhn3, PoDhn8, PoDhn9-1 and PoDhn9-2. D, Comparison of amino acid sequence of the Y_nSK_n type dehydrin, PoDhn5 and PoDhn6.

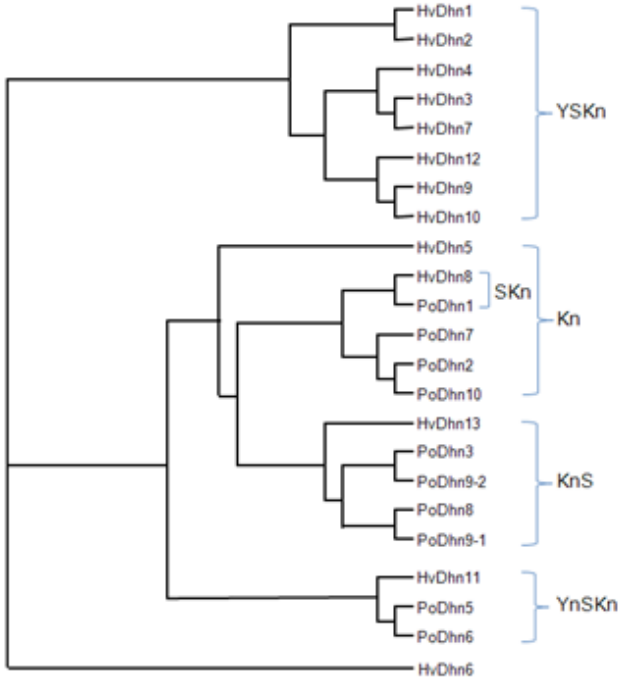

Fig 2. The phylogenetic tree of the dehydrin family member isolated from poplar and barley. The tree was constructed via the neighbor-joining method. Genbank accession numbers of the barley HvDhn1 to HvDhn11 are AAF01689 to AAF01699, HvDhn12 is AAD38400, and HvDhn13 is AAT81473.

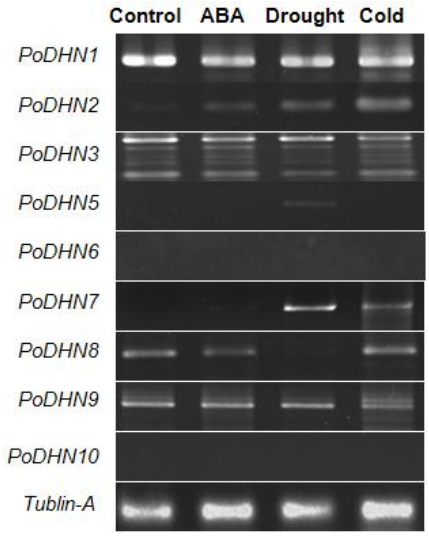

Fig 3. Expression pattern of the *PoDhn* genes in leaf tissues of *Populus tremula* × *Populus alba* var. *glandulosa*. Total RNA was isolated from the leaves under drought conditions, cold-treated at 5°C, or ABA treated, and RT-PCR was performed with gene-specific primer sets (Supplementary table 1). Most of the *PoDhn* genes were expressed under normal growth conditions as well as abiotic stress. In particular, *PoDhn2* and *PoDhn7* transcripts increased dramatically by both cold and drought treatment and *PoDhn5* was up-regulated by only drought stress. Transcript of the *PoDhn6* and *PoDhn10* were not detected in the leaf tissue. The *Tublin-A* gene was used as an internal control. The RT-PCR products were separated by 1.0% agarose gel electrophoresis.

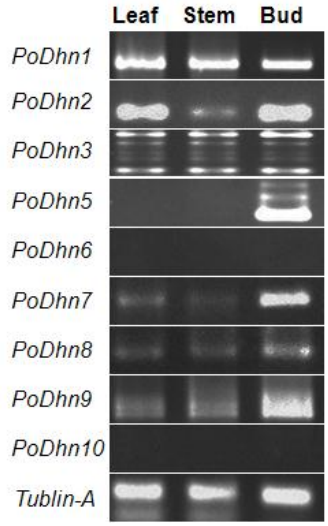

Fig 4. Expression of the *PoDhn* genes under different tissues of *Populus alba* × *P. tremula* var. *glandulosa*. Leaf and stem samples were harvested from pot-grown plants cold-acclimated at 5°C for 10 hours. Winter buds (Bud) were harvested from field grown plants. Most of *PoDhn* genes were expressed in leaf, stem and winter bud. But transcript of the *PoDhn5* was in only winter bud, and the *PoDhn6* and *PoDhn10* were not detected in the tested tissues. The *Tublin-A* gene was used as an internal control. RT-PCR was performed with gene specific primers, and PCR products were separated by 1% agarose gel electrophoresis. The *tublin-A* gene was used as an internal control.

Isolation of the *Dhn* genes from *Populus alba* × *P. tremula* var. *glandulosa*

To identify *Dhn* genes in the genome of *Populus alba* × *P. tremula* var. *glandulosa*, we searched the *Populus trichocarpa* genome sequence (http://genome.jgi-psf.org) by key word search and the Blastp program with a 15 amino acid sequence of the K-segment, EKKGIMDKIKEKLPG of the dehydrin. Additionally, we searched the ESTs database with the *Dhn* sequence identified from the *P. trichocarpa* genome and a *Dhn* from *Populus alba* × *P. tremula*var. *glandulosa* (Bae et al., 2009). From the *Dhn* sequence identified from ESTs database and the *P. trichocarpa* genome, poplar *Dhn* gene-specific primers were designed using the program Primer Premier 5.0 (www. Premierbiosoft.com). In order to isolate the poplar *Dhn* gene, genomic DNA purified from *Populus alba* × *P. tremula* var. *glandulosa* was applied to *Dhn* gene-specific PCR. PCR products were subcloned into pGEM-T-Easy vector (Promega, Madison, WI, USA) to determine the sequence. In order to purify the poplar genomic DNA, 100 mg of leaf tissue powder was mixed with 750 ul of DNA extraction buffer (50 mM Tri-HCl, 10 mM EDTA, 10 mM NaCl, 1% SDS, 10 mM β–mercaptoethanol) and incubated for 10 min in a 65℃ water bath. The reaction was mixed with 250 ul of 5M potassium acetate and centrifuged for 20 min at 10,000 g. Genomic DNA was precipitated from the supernatant by adding ethanol, and subsequently utilized as a PCR template.

Sequence analysis

The plasmid DNAs were purified using a QIAquick Plasmid Extraction Kit (Qiagen, Hilden, Germany) and sequenced at

GnC Bio (www.gncbio.kr). Sequence editing and amino acid sequence prediction of the cloned PCR product were carried out using the Sequencher program (Gene Code Corporation. Ann Arbor, MI. USA). The putative molecular weights and PI values of the deduced polypeptides were predicted using the DNASIS Max program (MiraiBio Inc. San Francisco, CA, USA). The alignments of the deduced amino acid sequence phylogenetic tree were conducted using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw).

Gene-specific RT-PCR

Total RNAs were prepared from plant tissues, using the Plant RNeasy kit (Qiagen, Hilden, Germany). The first-strand cDNAs were constructed from 2 μg of total RNA via reverse-transcription in 20 ㎕ reaction volumes, using oligo$(dT)_{17}$ primer and Superscript III reverse transcriptase, in accordance with the manufacturer's instructions (BRL Life Technologies, Carlsbad, CA, USA). The first-strand cDNA reaction was diluted by a factor of 5, after which 2 ㎕ of diluted cDNA was applied to a 50 ㎕ PCR-amplification reaction, containing PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), dNTPs, gene-specific primer (10 pmol ㎕/l), ExTaq DNA polymerase (Takara, Japan). PCR reactions were conducted for 35 cycles, each consisting of 30 seconds at 95℃, 30 seconds of 60℃, 90 seconds of 72℃, and 5 minutes of termination at 72℃. The annealing temperatures and PCR cycles were adjusted for some of the primer sets in order to optimize the PCR reactions.

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