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Isolation of a novel dehydrin gene from *Codonopsis lanceolata* and analysis of its response to abiotic stresses

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Dehydrins (DHNs) compose a family of intrinsically unstructured proteins that have high water solubility and accumulate during late seed development at low temperature or in water-deficit conditions. They are believed to play a protective role in freezing and drought-tolerance in plants. A full-length cDNA encoding DHN (designated as *ClDhn*) was isolated from an oriental medicinal plant *Codonopsis lanceolata*, which has been used widely in Asia for its anticancer and anti-inflammatory properties. The full-length cDNA of *ClDhn* was 813 bp and contained a 477 bp open reading frame (ORF) encoding a polypeptide of 159 amino acids. Deduced ClDhn protein had high similarities with other plant DHNs. RT-PCR analysis showed that different abiotic stresses such as salt, wounding, chilling and light, triggered a significant induction of *ClDhn* at different time points within 4-48 hrs post-treatment. This study revealed that *ClDhn* assisted *C. lanceolata* in becoming resistant to dehydration. [BMB reports 2008; 41(4): 338-343]

INTRODUCTION

Plants have developed defensive strategies against various stresses that arise from frequent environmental fluctuations to which they are exposed. Drought and low temperatures are the most severe factors limiting plant growth and yield. More than 100 genes have been shown to be responsive to such conditions and they are believed to function either during the physiological protection of cells from water-deficiencies or temperature-changes or in the regulation of gene expression (1-3).

DHNs are proteins that are known to accumulate in vegetative plant tissues under stress conditions, such as low temperature, drought, or salt-stress (2, 4-6). These proteins have been categorized as late embryogenesis abundant (LEA) proteins (7, 8).

DHNs have been subdivided into five classes according to the presence of highly conservative segments: $YnSK_2$, Kn, KnS, SKn and Y_2Kn. The K-segment (EKKIGIMDKIKEKLPG) is a conserved 15-mer lysine-rich sequence characteristic of DHNs, which may be present in one or several copies (5). The K-segment can form an amphiphathic α-helix structure that may interact with lipid components of bio-membranes and partially denatured proteins like chaperones (6, 9). The S-segment consists of contiguous serine residues in the centre of the protein, which may be phosphorylated. They are involved in nuclear transport through their binding to nuclear localization signal peptides (6). The Y-segment with the consensus sequence DEYGNP, shares some similarities to the nucleotide-binding site of chaperones in plants and bacteria (5, 10). Another conserved domain contained in many DHNs is φ-segment (repeated Gly and polar amino acids), which interacts with and stabilizes membranes and macromolecules, preventing structural damage and maintaining the activity of essential enzymes (11).

DHNs have been found in the cytoplasm (12), nucleus (12, 13), mitochondria (14), vacuole (15), and chloroplasts (16). They are known to associate with membranes (17, 18), proteins (19) and excess salt ions (15, 20).

Several DHN genes have been isolated and characterized from different species, including *cor47*, *erd10* and *erd14* from *Arabidopsis thaliana*; *Hsp90*, *BN59*, *BN115* and *Bnerd10* from *Brassica napus*; *cor39* and *wcs19* from *Triticum aestivum* (bread wheat); and *cor25* from *Brassica rapa subsp. Pekinensis* (21). Many studies have reported a positive correlation between the accumulation of DHN transcripts or proteins and tolerance to freezing, drought, and salinity (12, 17, 22-24). Moreover, modulation of transcripts by light has been reported for many DHN-encoding genes in drought- or cold-stressed plants (25-28). Although the biochemical functions and physiological roles of DHNs are still unclear, their sequence characterizations and expression patterns suggest that they may play a positive role in plant-response and adaptation to abiotic stress that leads to cellular dehydration. Indeed, many studies have indicated that transgenic plants with DHNs have a better stress-tolerance, recovery or re-growth after drought and freezing stress than that of the control (8, 29, 30).

Thus far, there are no reports on isolation of the DHN gene from the oriental medicinal plant *Codonopsis lanceolata*. This plant belongs to the family of *Campanulaceae* (bellflower family), which contains many famous oriental medicinal plants such

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as *Platycodon grandiflorum* (Chinese bellflower or balloon flower), *Codonopsis pilosula* and *Adenophora triphylla* (nan sha shen). The roots of these plants have been used as herbal drugs to treat bronchitis, cough, spasm, macrophage-mediated immune responses and inflammation, and has also been administered as a tonic (31). *C. lanceolata* grows in North-eastern china, Korea, and far eastern Siberia. Despite their medicinal importance, little genomic study of this plant has been carried out. In this study, we characterized an Y_2SK_2 type DHN gene from *C. lanceolata* and analyzed its expression in response to various abiotic stresses.

RESULTS AND DISCUSSION

Isolation and characterization of the full length cDNA of the *ClDhn* gene

As part of a genomic project to identify genes in the medicinal plant *C. lanceolata*, a cDNA library consisting of about 1,000 cDNAs was previously constructed. A cDNA encoding a dehydrin (DHN), designated *ClDhn* was isolated and sequenced. The sequence data of *ClDhn* has been deposited in GenBank under accession number AB126059. As shown in Fig. 1, *ClDhn* is 813 bp in length and it has an open reading frame (ORF) of 477 bp nucleotide with an 87-nucleotide upstream sequence and a 248-nucleotide downstream sequence. The ORF of *ClDhn* starts at nucleotide position 88 and ends at position 565.

ClDhn encodes a precursor protein of 159 amino acids residues with no predicted signal peptide at the N-terminal. The calculated molecular mass of the protein is approximately 16.7 kDa with a predicated isoelectric point of 6.87. In the deduced amino acid sequence of ClDhn protein, the total number of negatively charged residues (Asp + Glu) amounted to 21 while the total number of positively charged residues (Arg + Lys) was 20. In addition, transmembrane helix prediction (TMHMMv2.0) did not identify any transmembrane helices in the deduced protein, implying that the protein did not function in the membrane but might function within the cytosolic or nuclear compartment.

Homology analysis

A GenBank Blastp search revealed that *ClDhn* had the highest sequence homology to the carrot (*Daucus carota*) *DHN* (BAD86644) with 51% identity and 61% similarity. *ClDhn* also shared homology with ginseng (*Panax ginseng*) *DHN5* (ABF48478, 50% identity and 60% similarity), wild potato (*Solanum commersonii*) *DHN* (CAA75798, 50% identity and 58% similarity), robusta coffee (*Coffea canephora*) *DHN1α* (ABC55670, 47% identity and 55% similarity), grape (*Vitis vinifera*) *DHN* (ABN79618, 47% identity and 57% similarity), American beech (*Fagus sylvatica*) *DHN* (CAE54590, 46% identity and 56% similarity), tobacco (*Nicotiana tabacum*) *DHN* (BAD13498, 45% identity and 56% similarity), sunflower (*Helianthus annuus*) *DHN* (CAC80719, 45% identity and 52% similarity), and soybean (*Glycine max*) *DHN* (AAB71225, 44% identity and 52% similarity). The *DHNs* showing the highest similarities were Y_2SK_2 type *DHNs* except grape (*Vitis vinifera*) *DHN* (YSK_2 type) (32). Thus *ClDhn* might belong to Y_2SK_2 type *DHNs* based on the two Y-segments, one S-segment, and two K-segments present in its amino acid sequence. Phylogenetic analysis of ten of the plant *DHNs* were carried out using the Clustal X program (Fig. 2). Fig. 3 is a sequence alignment result of *ClDhn* and other closely related *DHNs*.

Fig. 1. Nucleotide sequence and deduced amino acid sequence of a *ClDhn* cDNA isolated from *C. lanceolata*. Numbers on the left represent nucleotide positions. The deduced amino acid sequence is shown in a single-letter code below the nucleotide sequence. The asterisk denotes the translation stop signal. Amino acids in two double boxes represent the Y-segment and amino acids in a single box the S-segment, respectively. The two underlined sequences represent the K-segments.

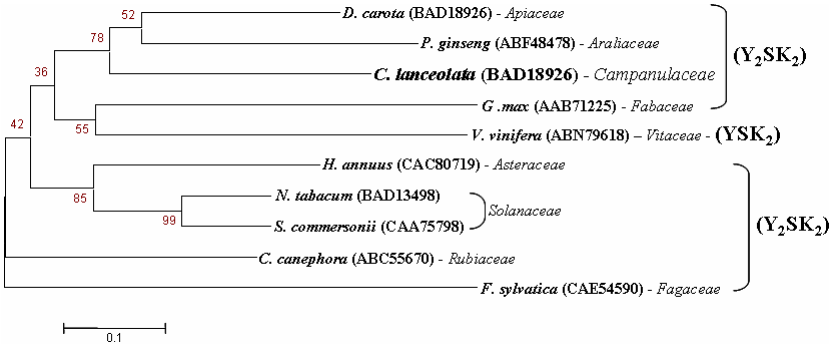

Fig. 2. A phylogenetic tree based on DHN amino acid sequence, showing the phylogenetic relationship between *ClDhn* and other plant *DHNs*. The tree was constructed using the Clustal X method (Neighbor-joining method) and a bar represents 0.1 substitutions per amino acid position.

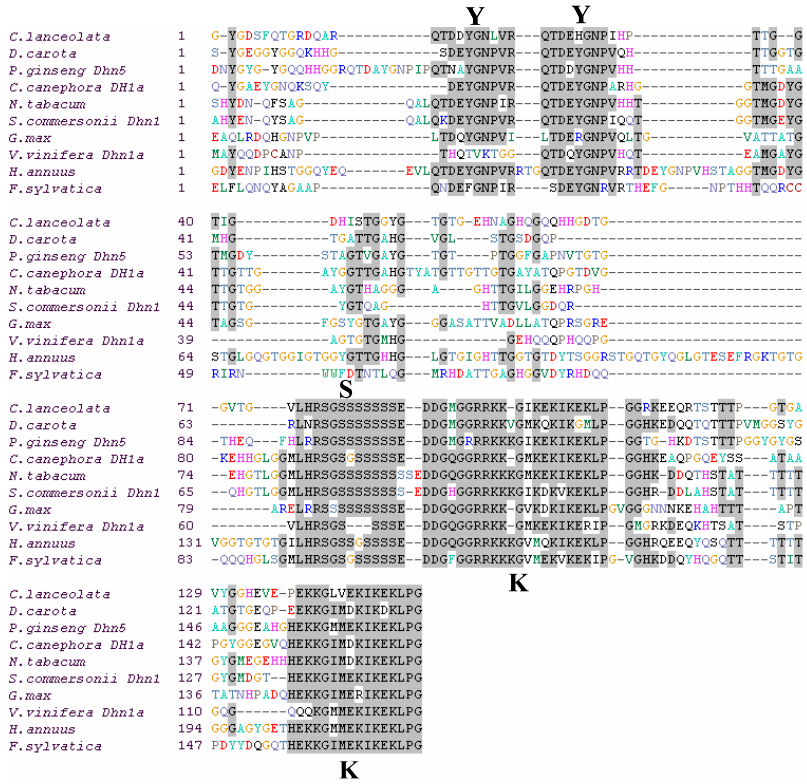

Fig. 3. Alignment of *ClDhn* with the most closely related *DHNs* from carrot (*Daucus carota*, BAD86644), ginseng (*Panax ginseng* DHN5, ABF48478), commerson's wild potato (*Solanum commersonii*, CAA75798), robusta coffee (*Coffea canephora*, ABC55670), grape (*Vitis vinifera*, ABN79618), American beech (*Fagus sylvatica*, CAE54590), tobacco (*Nicotiana tabacum*, BAD13498), sunflower (*Helianthus annuus*, CAC80719) and soybean (*Glycine max*, AAB71225). Gaps are marked with dashes. The conserved amino acid residues are shaded and Y-, S-, and K-segments are shown.

The differential expression of *ClDhn* in different organs of *C. lanceolata*

The expression patterns of *ClDhn* in different *C. lanceolata* organs were examined using RT-PCR analysis. Almost similar levels of *ClDhn*-mRNA expression were observed in leaves and roots, whereas *ClDhn* was expressed in slightly higher levels in the stems. (Data was not shown).

Expression of *ClDhn* in response to various stresses

Expression patterns of *ClDhn* under various conditions were examined using RT-PCR analysis. Fig. 4A showed the accumulation of *ClDhn*-mRNA in response to 100 mM ABA in MS agar. ABA is a hormone secreted when environmental conditions become dry. Expression of *ClDhn* was induced and reached a maximum level after 12 hrs, and then gradually decreased. When plants are submitted to dehydration the endogenous content of ABA increases, with ABA mediating the closure of the stomata. Several studies have identified ABA as a key hormone in the induction pathway of many inducible genes including *DHN*, in response to drought (33-36). 100 μM of ABA in spray induced DHN-levels in *Brassica napus* and increased its expression up to 48 hrs after treatment with ABA (37). 100 μM of ABA in MS agar induced *DHN*-levels in rice and cause a max-

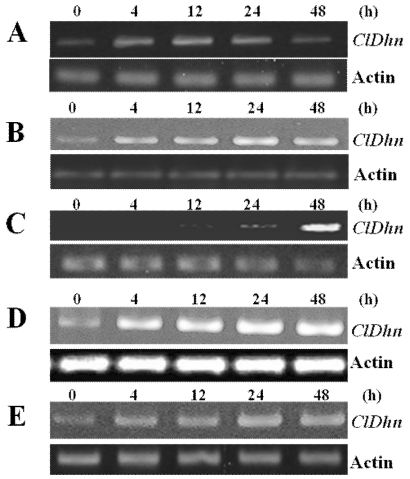

Fig. 4. RT-PCR analyses of the expressions of *ClDhn* gene in the leaves of *C. lanceolata* at various time points (h) post-treatment with various stresses: A, 100 mM ABA; B, 100 mM NaCl; C, wounding; D, chilling and E, light treatment. Actin was used as an internal control.

imum expression level at 1 hr post-treatment (10).

Fig. 4B shows the accumulation of *ClDhn* mRNA in response to salt stress (100 mM NaCl). *ClDhn* expression was induced at 4 hrs post-treatment and gradually increased until 48 hrs. In *Brassica napus*, 250 mM NaCl added in the nutrient medium induced DHN-expression and reached a maximum at 48 hrs post-treatment (37). The application of NaCl to soil brought on a progressive decrease of the pre-dawn leaf water potential, a decrease of stomatal-conductance and a growth-reduction. Osmotic potential increase during salt treatment could result from Na^+ or Cl^- absorption and from the synthesis of compatible compounds (38).

Under wounding stress, *ClDhn* gene transcription was induced at 4 hrs post-treatment and gradually increased until 48 hrs (Fig. 4C). Richard *et al*. (39) discussed that the cumulative effect of wounding on transcript accumulation could also be associated with greater water-loss through more open surfaces arising from the wounding treatment.

Under cold treatment, increase of *ClDhn* transcripts was observed at 4 hrs post-treatment and gradually increased until 48 hrs (Fig. 4D). Induction of DHN by low temperatures has been observed in numerous plants (17, 38). Overexpression of citrus DHN improved the cold tolerance in tobacco (18). Overexpression of multiple DHN genes in *Arabidopsis* resulted in accumulation of the corresponding DHNs to levels similar or higher than in cold-acclimated wild-type plants (24). Another example showed that overexpression of the acidic DHN WCOR410 could improve freezing tolerance in transgenic strawberry leaves (29).

Fig. 4E shows that *ClDhn* gene expression was induced by light stress and increased continuously until 48 hrs post-treatment. Natali *et al*. (40) showed that the G-box (CACGTGGC), a motif found in the promoter region of many light regulated genes, was found in the DHN gene promoter of helianthus and that DHN was responsive to light stress (41).

In conclusion, we isolated a new dehydrin gene (*ClDhn*) from *C. lanceolata* and characterized its expression in response to various stresses. *ClDhn* was induced by various stresses related to water-deficiency (ABA, salt, wounding and cold) and was induced by light, similar to other *DHN* genes isolated from other plants.

MATERIALS AND METHODS

Plant materials

Codonopsis lanceolata were grown *in vitro* on MS medium supplemented with 3% sucrose and 0.8% agar under the 16 hrs light and 8 hrs dark period. Its growth was maintained by regular subculture every 4 weeks. Abiotic stress studies were carried out on plants that were subcultured for one month. To analyze gene expression in different organs, samples were collected from leaves, roots and stem of *C. lanceolata* plants.

Sequence analyses

The full-length *ClDhn* gene was analyzed using the softwares BioEdit, Clustal X, Mega 3 and other databases listed below; NCBI (http://www.ncbi.nlm.nih), SOPMA (http://npsa-pbil.ibcp/npsaautomat.pl?page=npsopma.html).

Stress assays

To investigate the response of the *ClDhn* gene to various stresses, the third leaves with petioles from *C. lanceolata* were used. For treatment with ABA (100 mM) and NaCl (100 mM), leaf samples were incubated in media containing each compound at 25°C for 48 hrs. For mechanical wounding stress, excised leaves were wounded with a needle puncher (42). Chilling stress was applied by exposing the leaves to a temperature of 4°C (43). To investigate the *ClDhn* gene-expressions in light, leaves were incubated under an electrical lamp with a light intensity of 24 mol m-2 s-1 for 48 hrs. All treatments were carried out on MS media with or without the treatment solution (ABA, NaCl). All treated plant materials were immediately frozen in liquid nitrogen and stored at -70°C until further analysis.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from various whole plant tissues (leaves, stem, roots) of *C. lancolata* using the Rneasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR (reverse transcriptase-PCR), 800 ng of total RNA was used as a template for reverse transcription using oligo (dT) primer (0.2 mM) (INTRON Biotechnology, Inc., South Korea) for 5 mins at 75°C. The reaction mixture was then incubated with AMV Reverse Transcriptase (10 U/μl) (INTRON Biotechology, Inc., South Korea) for 60 mins at 42°C. The reaction was inactivated by heating the mixture at 94°C for 5 mins. PCR was then performed using a 1 μl aliquot of the first stand cDNA in a final volume of 25 μl containing 5 pmol of specific primers for coding of *ClDhn* gene (forward, 5'-AAA GAG AGA GAA AAT

GGC AGG TTA C-3'; reverse, 5'-GGA GTA GTT GTT GAA GTT CTC TGC T-3') were used. As a control, the primers specific to the *C. lanceolata* actin gene were used (forward, 5'-CAA GAA GAG CTA CGA GCT ACC CGA TGG-3'; reverse, 5'-CTC GGT GCT AGG GCA GTG ATC TCT TTG CT-3'). PCR was carried out using 1 μl of taq DNA polymerase (Solgent Co., South Korea) in a thermal cycler programmed as follows: an initial denaturation for 5 mins at 95°C, 30 amplification cycles [30 s at 95°C (denaturation), 30 s at 53°C (annealing), and 90 s at 72°C (polymerization)], followed by a final elongation for 10 mins at 72°C. Actin was used as an internal control to normalize each sample for variations in the amount of RNA used.

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