Protective functions of alternative splicing transcripts (CdDHN4-L and CdDHN4-S) of CdDHN4 from bermudagrass under multiple abiotic stresses

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A B S T R A C T

Dehydrins (DHNs) play critical roles in plant adaptation to abiotic stresses. The objective of this study was to characterize DHNs in bermudagrass (Cynodon spp.). CdDHN4 gene was cloned from bermudagrass ‘Tifway’. Two CdDHN4 transcripts were detected due to alternative splicing (the nonspliced CdDHN4-L and the spliced CdDHN4-S) and both the CdDHN4-S and CdDHN4-L proteins are YSK2-type DHNs, the Φ-segment is present in CdDHN4-L and absent in CdDHN4-S. Transgenic Arabidopsis thaliana expressing CdDHN4-L or CdDHN4-S exhibited improved tolerance to salt, osmotic, low temperature and drought stress compared to the wild type (WT). The two transgenic lines did not differ in salt or drought tolerance, while plants expressing CdDHN4-S grew better under osmotic stress than those expressing CdDHN4-L. Both transgenic lines exhibited reduced content of malondialdehyde (MDA) and reactive oxygen species (ROS); and higher antioxidant enzymatic activities than the wild type plants under salt or drought stress. CdDHN4-S exhibited a higher ROS-scavenging capacity than CdDHN4-L.

1. Introduction

Abiotic stresses whatever cold, drought or salinity can cause plant water loss. However, plants have evolved many defense mechanisms to prevent intracellular water loss. Perennial grass species, such as bermudagrass (Cynodon spp.), are planted in areas of limited water in many parts of the globe. Many perennial grass species are valuable sources of germplasm for studying drought tolerance mechanisms, because of their wide range of genetic variation (Taliaferro, 1995; Hanna, 1998; Wu et al., 2007; Jewell et al., 2012). Triploid hybrid bermudagrass (Cynodon dactylon × C. transvaalensis ‘Tifway’) has been developed to produce a highly desirable turf quality with limited irrigation (Hanna, 1998), and it exhibits improved tolerance to salt, osmotic, low temperature and drought stress compared to the wild type (WT). Our previous study using the subtraction suppression hybridization technique screened differentially expressed genes (DEGs) between a drought-tolerant hybrid bermudagrass genotype ('Tifway') and a drought-sensitive common bermudagrass genotype ('C299') under drought conditions; this work identified 36 DEGs more highly expressed in ‘Tifway’, including genes related to stress signaling, dehydration enzymatic antioxidants and nonenzymatic antioxidants (Gill and Tuteja, 2010). Among enzymatic antioxidants, superoxide dismutase (SOD) and catalase (CAT) represent the first line of antioxidant defense (Van Breusegem et al., 2001). SOD removes the superoxide anion (O2−) by dismutation to form H2O2, and CAT splits H2O2 into H2O and O2 (Scandalias, 1997). Nonenzymatic antioxidants includes ascorbic acid (AsA) and glutathione (GSH). AsA is a powerful ROS scavenger that can donate electrons in many enzymatic and nonenzymatic reactions (Gill and Tuteja, 2010). GSH plays a vital role in maintaining a reduced state in cells to eliminate the effects of oxidative stress induced by ROS (Meyer, 2008). Moreover, GSH can regenerate AsA via the AsA-GSH cycle, mediating functions in the antioxidant defense system (Foyer and Halliwell, 1976).

Abbreviations list: AsA, ascorbic acid; CAT, catalase; DEGs, differentially expressed genes; DHN, Dehydrin; DR, disordered region; ETR, electron transport rate; MDA, malondialdehyde; GSH, glutathione; IDP, intrinsically disordered protein; LEA proteins, late-embryogenesis abundant proteins; PAM, pulse-amplitude modulation; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; ORF, open reading frame

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DHNs, group II LEA proteins, are major proteins expressed in response to water-limited environments (Close, 1997). DHNs contain five subclasses, K, Kn, YnKn, SKn and YnSkn, based on the number of conserved K-, S- and Y-segment motifs in the protein sequence. All DHNs have at least one K-segment (Koag et al., 2009). DHN sequences are rich in polar and charged amino acids, making DHN highly hydrophilic and heat stable (Hughes and Graether, 2011). DHNs are intrinsically disordered proteins (IDPs), which stay flexible and have no well-defined folded structure, even in their native environment (Tompa, 2002). Functional analyses of DHN in other plant species have been performed, revealing that the protective functions of these IDPs are closely related to their various structures. The protective functions of DHN primarily include protection against water loss (Tompa et al., 2006), ion and nucleic acid binding capabilities (Hara et al., 2001; Bravo et al., 2003).

2. Materials and methods

2.1. Isolation and cloning of CdDHN4

Total RNA was extracted from bermudagrass 'Tifway' using TRizol Reagent (Invitrogen, USA), and mRNA enrichment was performed using the Oligotex –dT30(super) mRNA Purification Kit (TaKaRa, Japan). The full-length cDNA of CdDHN4 was obtained using the SMARTer® RACE cDNA Amplification Kit (Clontech, USA) following the manufacturer’s instructions. The primers for RACE were designed based on the partial sequence of CdDHN (Table 1). Because the EST of CdDHN is very similar to that of barley DHN4, we named it CdDHN4 (Zhou et al., 2014). The PCR products were cloned into pMD 19-T (TaKaRa) and sequenced.

2.2. Sequence analysis of CdDHN4

Open reading frame (ORF) identification of CdDHN4 was conducted using ORFfinder (http://www.ncbi.nlm.nih.gov/projects/orffinder/). For genomic characterization, the CdDHN4 gene was amplified by PCR using the ORF primers (Table 1). The cDNA and deduced amino acid sequence of CdDHN4 were analyzed using DNAstrider software. CdDHN4 nucleotide sequence homology searches were conducted by the BLASTx algorithm. Multiple alignments were performed with ClustalX 2.0 software. A phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MAGA5.1 software. The protein disordered regions (DRs) were identified using POND software (http://www.pond.com/index). CdDHN4 was amplified from genomic DNA using ORF primers (Table 1), and an alignment of the CdDHN4 gDNA and cDNA sequences was performed to identify introns and exons.

2.3. Recombinant expression of CdDHN4

The complete ORF of CdDHN4, with the inclusion of BamHI and SpeI sites, was PCR-amplified with primers Dehydrin_CS and Dehydrin_CA (Table 1). The full-length cDNA of DHN4 was cloned into the binary vector PHB (with the glyphosate resistance gene bar) at an expression site driven by the 35S promoter, forming the 35S:DHN construct. Wild type A. thaliana were used for gene transformation. Transgenic plants (T0) were selected using 100 mg L⁻¹ glyphosate (BBI, Markham, Ontario, Canada), and homozygous T3 lines were used for phenotypic analysis after confirmation by genomic PCR.

2.4. Abiotic stress tolerance assays

Seeds of A. thaliana (Col-0) and 8 selected homozygous transgenic lines were sterilized for 15 min in 7% bleach with 0.05% Tween-20, followed by incubation at 4 °C for 3 days and plating on 1/2 MS medium. Plants were grown for 5 days at 22 °C with a photoperiod of 16 h.

For cold stress treatment, 5-day-old seedlings were cultured on MS medium in a low temperature incubator (8 °C / 0 °C, day/night). For salt stress and osmotic treatments, 5-day-old seedlings were subjected to salt or osmotic stress by including NaCl (100, 150 or 200 mM) or mannitol (300, 400 or 500 mM), respectively, in the 1/2 MS medium. Some seedlings (21-day-old) were transferred to soil and irrigated with NaCl (100, 150 or 200 mM), mannitol (300, 400 or 500 mM) or polyethylene glycol 6000 (PEG; 12, 18 or 24%). Treated seedlings were monitored by obtaining images every 3 days, and rosette size and root elongation were measured using ImageJ software (rsb.info.nih.gov/ij).

2.5. Detection of stress physiological indexes

SOD, CAT and POD activities, H₂O₂ levels, O₂•⁻ inhibition activity, hydroxyl radical (OH•) generation activity, and AsA, GSH and MDA contents detection were measured using biological assay kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer’s instructions according to Yang et al. (2019) and Zhang et al. (2015).

| Table 1 | Primers used for PCR amplification. |
|---------|-----------------------------------|
| Primer  | Nucleotide sequence (5’-3’)        | Function             |
| 3A1     | GGAAACAGTTCGTTAATGGTAACCTGTCGCA  | 3’ RACE; outer       |
| 3A2     | TGCTGTAAAGCTAGAAGATTGGTTCGACCGCA | 3’ RACE; inner       |
| SS1     | ACCATCTCTAGGCTTACGGAACTGGGCGC    | 5’ RACE; outer       |
| SS2     | GAACTGCTGACAGACAGGTCACAGGTTCA   | 5’ RACE; inner       |
| qRT-S   | TGGCTCTGAGGATGGTGGCC            | Real-time PCR        |
| qRT-A   | TGGCTCTGAGGATGGTGGTA            | Real-time PCR        |
| 18S-S   | GTGACGCGGTCGAGGCGGAGATT        | Real-time PCR        |
| 18S-A   | GACACTAATGGGCAGCGGTATG          | Real-time PCR        |
| ORF-S   | ATGGAGCAGCGAGGGCAGTGGC          | Amplification of ORF|
| ORF-A   | TGGCTGAGGATGGTGGCTCTCTCGGC     | Amplification of ORF|

Jiancheng Bioengineering Institute, China following the manufacturer’s instructions according to Yang et al. (2019) and Zhang et al. (2015).
2.6. Photochemical efficiency and photosynthesis capability measurement

Photosynthetic capability was measured according to the method of Yang et al. (2014). In brief, 2-week-old seedlings were tested by withholding water treatment for 15 days. Chlorophyll fluorescence was measured using a pulse-amplitude modulation (PAM) chlorophyll fluorometer (Heinz-Walz-GmbH, Effeltrich, Germany). To measure the maximum quantum yield of PSII, plants were dark-adapted for 30 min. Fv/Fm was recorded during a saturating photon pulse (4000 μmol·m⁻²·s⁻¹) using a whole plant. Fv/Fm was calculated as follows: Fv/Fm = (Fm -Fo)/Fm (Genty et al., 1989). Images of fluorescence parameters were created using the machine. The rapid light curves were drawn as a plot of the electron transport rate (ETR), where ETR = the effective quantum yield (ΦPSII) × Photon flux density (PFD) × 0.5 × leaf absorptivity coefficient (Kalaji et al., 2014).

2.7. Statistical analysis

SAS 9.1.3 software (SAS Institute Inc., Cary, NC) was used for statistical analysis. One-way ANOVA was performed for quantitative data, followed by the LSD multiple range test to detect significant differences (P < 0.05). Different capital letters A-D were used to indicate significant differences between the same genotypic samples across different treatment conditions, and different lowercase letters (a-d) represent significant differences between different genotypes under the same treatment condition.

To comprehensively analyze the abilities of the three genotypes to eliminate ROS, we defined a-d as indexes in multiple comparisons to show the comprehensive ROS-eliminating abilities of different genotypes in response to the same stress treatment (named EL-ROS), and assigned each ability a score from 1 to 4 corresponding to a-d (a larger value indicates a stronger ability to eliminate ROS). We also defined A-D as the indexes in multiple comparisons to show the comprehensive effects of ROS under different stress treatments for the same genotype (named EF-ROS) and assigned the effects a score ranging from 1 to 4 corresponding to A-D (a smaller value indicates a greater ROS effect).

3. Results

3.1. Isolation and characterization of the CdDHNF4 gene in bermudagrass

Two CdDHNF4 cDNAs were isolated from ‘Tiway’; the full-length ORFs of CdDHNF4-L and CdDHNF4-S were 543 and 495 bp and encoded 180 and 164 amino acid residues, respectively (Fig. 1A; GenBank accession: KJ000690). BLASTx analysis revealed that they are highly conservative to the dehydrin DHN3 from Hordeum vulgare and Aegeilops tauschii (GI: 118487 and 475601478, respectively), dehydrin WZY1-1 from Triticum aestivum (GI: 17980974), dehydrin-LEA2-like protein from Lophopyrum elongatum and Cleistogenes songorica (GI: 2970213 and 288300160), and dehydrin from Zea mays (GI: 18964) (Fig. 1B). Analysis of the protein sequences indicated that CdDHNF4-L and CdDHNF4-S have calculated isoelectric points of 8.78 and 8.81 and molecular masses of 18.19 and 16.73 kDa, respectively. Further analysis showed that the CdDHNF4 proteins contain a Y-segment (T/VDEYGNP), a single S-segment (SSSSSSS) and two K-segments (EKKGIMDKIKEKLPG) (Fig. 1B). Additionally, phylogenetic tree analysis suggested that CdDHNF4s clusters with A. thalitana Skn-type DHNs, including At5g664400 (YSK2) and At3g50980 (SK2).

Genomic sequence analysis showed that CdDHNF4 consists of two exons and one intron and that exon2 undergoes alternating splicing. The produced YSK2-type CdDHNF4s include two splice variants (the nonspliced CdDHNF4-L and the spliced CdDHNF4-S) (Fig. 2); these variants differ by 16 amino acid residues in the Φ-segment, which connects the 2 K-segments and represents the structural difference between CdDHNF4-L and CdDHNF4-S. Moreover, in silico predictions suggested the intrinsically disordered structure of both proteins (Fig. 2). Using the PONDR VSL1 algorithm, we found that the scores of the particular amino acids were mostly above 0.5 (the threshold separating ordered from disordered regions in proteins) indicating that CdDHNF4s are mostly disordered proteins.

3.2. Heterologous expression of CdDHNF4s in Arabidopsis enhances tolerance to multiple abiotic stresses

From each CdDHNF4-overexpressing Arabidopsis strain, 8 transgenic lines were selected for phenotypic observation (Supplementary Fig. S1). Both CdDHNF4-L and CdDHNF4-S transgenic plants exhibited good seed setting under normal growth conditions and better tolerance to drought stress compared to wild type plants. Subsequently, the CdDHNF4-L transgenic plant lines 2, 3 and 7, and CdDHNF4-S lines 2, 3 and 5 were selected based on their similar CdDHNF4 expression levels. CdDHNF4-L and CdDHNF4-S homozygous transgenic lines (T₃) were used for abiotic stress tolerance assays and physiological tests.

3.2.1. Overexpression of CdDHNF4s improves plant tolerance to salt stress

Plant fresh weight, rosette size and root length were not significantly different between the CdDHNF4 transgenic and wild type (WT) lines under normal growth conditions (Fig. 3). Under salt stress, 5-day-old WT plants grew abnormally and exhibited stressed symptoms in response to all salt treatments, while transgenic lines still grew in 100 mM and 150 mM salt media but began to die in 200 mM salt media (Fig. 3A). Additionally, plant fresh weight, rosette size and root length of 5-day-old CdDHNF4 transgenic plants were increased compared to WT under higher salt concentration treatments. For 3-week-old seedling of all three lines, significant differences were observed between transgenic plants and WT after treatment with 150 mM NaCl for 15 days (P < 0.05) (Fig. 3E, H). The relative electrolyte leakage of CdDHNF4-L and CdDHNF4-S plants decreased by 47.6% and 43.1% compared to WT, respectively.

Under salt stress, the content of MDA, H₂O₂ and OH⁻ were reduced in transgenic plants compared to WT specimens, through the difference did not reach statistical significance (Fig. 8). The O₂⁻ inhibition activity in transgenic A. thalitana was increased compared to WT under salt stress, especially in the CdDHNF4-S line, which was significantly higher than the WT (P < 0.05). The content of AsA, one of nonezymatic antioxidants, was significantly increased in both transgenic genotypes, compared to WT plants.

3.2.2. Overexpression of CdDHNF4s improves plant tolerance to drought and osmotic stress

Two-week-old WT seedlings deprived of water began to die and were unable to recover; however, most of the CdDHNF4-L and CdDHNF4-S seedlings exposed to the same conditions exhibited regrowth upon watering (Fig. 4A). Seedlings of all three lines showed serious stress damage in response to high concentrations of PEG (Fig. 4B), gradually withering after treatment with 24% PEG. Plant fresh weights of the seedlings showed significant differences between transgenic seedlings and WT after treatment with 18% PEG (P < 0.05) (Fig. 4C, D). CdDHNF4-L and CdDHNF4-S seedlings exhibited reduced leakage compared to CdDHNF4-L seedlings and WT under drought stress (Fig. 4E).

The Fv/Fm values of WT plants exhibited a significant decline under drought conditions, while Fv/Fm values of CdDHNF4-L and CdDHNF4-S transgenic plants were clearly higher than WT (P < 0.05) (Fig. 5). Furthermore, the rapid light-response curve (RLC) showed the electron transport rate (ETR) decreased obviously in WT, and increased in CdDHNF4-S transgenic plants during drought stress (Fig. 5). These results indicate that CdDHNF4s-overexpressing plants exhibit excellent photosynthetic capability.

Upon examining the lipid peroxidation, ROS, and antioxidant system activity, we found that under drought stress, MDA content was significantly lower in transgenic plants than in WT specimens (P < 0.05) (Fig. 8). SOD and POD activities in transgenic plants were
significantly higher compared to WT, and the O$_2$ − inhibition activity in CdDHN4-S transgenic A. thaliana was significantly increased compared to the other two genotypes under drought treatment.

On highly osmotic media, plant morphology was not significantly different between CdDHN4 transgenic plants and WT Arabidopsis (Fig. 6A–D). Although 3-week-old seedlings of all genotypes grown in soil exhibited stress characteristics after 3 days under osmotic stress conditions, the CdDHN4 plants displayed less severe stress symptoms than WT plants (Fig. 6E–G). Plant fresh weight and rosette size were significantly different between CdDHN4 transgenic plants and WT Arabidopsis in response to 400 mM mannitol treatment ($P < 0.05$) (Fig. 6F,G). The relative electrolyte leakage of all plants increased from 9.2% to nearly 80%, with the value for CdDHN4-S plants being significantly lower than for CdDHN4-L and WT plants ($P < 0.05$) (Fig. 6H). Additionally, the increase in H$_2$O$_2$ content in WT plants was approximately 173.5–243.6% after 400 mM mannitol treatment, which was significantly higher than that of transgenic plants ($P < 0.05$) (Fig. 8).

3.2.3. Overexpression of CdDHN4s improves plant tolerance to low temperature stress

CdDHN4 isoform expression significantly improved plant growth and seed germination under low temperature (LT) conditions. Five-day-old WT seedlings grew and developed very slowly under LT stress. The root growth rate for CdDHN4-L and CdDHN4-S plants was approximately 103% higher than in WT plants under LT stress (Fig. 7A, C, D). CdDHN4 transgenic seedlings of 2-week-old plants also exhibited faster growth and development than did WT plants (Fig. 7B). Plant fresh weight was significantly different between transgenic and control groups ($P < 0.05$) (Fig. 7E), and relative electrolyte leakage results indicated that CdDHN4-S seedlings suffered less stress damage compared to CdDHN4-L and WT seedlings (Fig. 7J). Furthermore, CdDHN4s promoted seed germination and hypocotyl development under LT conditions (Fig. 7G). The rate of seed germination in WT Arabidopsis was <15% and the hypocotyl of germinating seeds did not elongate under LT conditions in the dark; however, rates of seed germination in CdDHN4-L and CdDHN4-S were approximatively 50% and hypocotyl elongation was obvious (Fig. 7H, I).

3.2.4. Comparative analysis of the functional roles of CdDHN4-S and CdDHN4-L

To comprehensively assess the ability of the CdDHN4 isoforms to perform ROS scavenging in transgenic plants, we combined multiple comparisons and assigned numerical values to different letters (Fig. 8). We compared the EF-ROS (the comprehensive effects of ROS) of different stresses: 63.5 under good conditions, 45 under 400 mM mannitol treatment, 45.5 under 150 mM NaCl, and 44.5 under 18% PEG. Results revealed that the effects of ROS under 18% PEG treatment are the greatest, followed by 400 mM mannitol, and then 150 mM NaCl. Under good conditions, the effects of ROS are the lowest. The EL-ROS index, which reflects ROS-eliminating ability, was calculated as 45 for CdDHN4-L transgenic plants, 47.5 for CdDHN4-S, and 44.5 for WT. Data demonstrated that CdDHN4-S has the highest ROS-scavenging capacity, followed by CdDHN4-L, with the ROS-scavenging capacity of WT being the lowest among the three genotypes.

4. Discussion

Dehydrins are IDPs that accumulate in plants during the late stages of embryogenesis and in response to abiotic stresses (Hernández-Sánchez et al., 2014). In this study, we isolated two stress-responsive CdDHN4 transcripts, CdDHN4-S and CdDHN4-L, from the drought-tolerant hybrid bermudagrass genotype ‘Tifway’. BLASTx analysis revealed that both dehydrins are highly homologous to the dehydrin
DHN3 from barley (GI: 118487), and dehydrin-LEA2 like protein from *Lophopyrum elongatum* (GI: 2970213). Phylogenetic tree analysis demonstrated that both *CdDHN4* and *CdDHN4-*2 respond to salt stress, hinting that *CdDHN4* may have the same function (Gulick and Dvořák, 1992). The structural characteristics of nonspliced (*CdDHN4-L*) and spliced (*CdDHN4-S*) sequences. Colored boxes indicate the positions of the Y, S, K and Φ domains, and the black box represents the alternatively spliced sequence of *CdDHN4*. In silico prediction of the disordered regions of *CdDHN4-L* and *CdDHN4-S*. Predictions were performed with the PONDR VSL1 algorithm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Both *CdDHN4-S* and *CdDHN4-L* can be classified as YSK2-type DHNs. They have the same conserved segments, including one Y-segment, one S-segment and two K-segments, and they are splice variants of the same genomic sequence (spliced, *CdDHN4-S*; nonspliced, *CdDHN4-L*). There are 48 additional nucleotides in exon 2 of *CdDHN4-L* that do not exhibit typical intronic structure, and they encode the Φ-segment that accounts for the structural difference between proteins *CdDHN4-S* and *CdDHN4-L*. The Φ-segments are poorly conserved in sequence and length, however, these segments are important for maintaining the unstructured state (Hughes and Graether, 2011; Lv et al., 2018). Additionally, the *CdDHN4-L*s both localize to the cytoplasm, and a small amount infiltrates the plant cell nucleus (Supplementary Fig. S2), hinting they may play roles in both the cytoplasm and nucleus.

Dehydrins have crucial roles in regulating plant stress tolerance and have been extensively studied in many plant species for decades (Graether and Boddington, 2014). The dehydrin gene *PpDHNA* from *Physcomitrella patens* was proven to protect plants from salt and osmotic-stress (Saavedra et al., 2006); *CarDHN* from the arctic chickweed plant enhances tolerance to salt, osmotic and freezing stress in tobacco plants (Hill et al., 2016). Overexpression of four dehydrin genes (*PmLEA10, PmLEA19, PmLEA20*, and *PmLEA29*) from *Prunus mume* in tobacco enhances tobacco tolerance to cold and drought (Fei et al., 2017).

In this study, transgenic plants overexpressing *CdDHN4-L* or *CdDHN4-S* were subjected to abiotic stress tolerance assays and physiological tests. Transgenic plants were exposed to salt, drought, osmotic, and low temperature stress. Under salt stress, *CdDHN4-L* and *CdDHN4-S* transgenic plants showed better stress tolerance than WT, especially when the irrigation water contained 150 mM NaCl. After 3 weeks without water, *CdDHN4-L* transgenic plants in soil could still recover when watered again, while WT were not able to recover, suggesting that *CdDHN4-L*s may enable plant cells to resist drought. Although 5-day-old *CdDHN4-L* transgenic seedlings displayed no differences compared to WT *Arabidopsis* after 15 days in high osmotic media, 3-week-old transgenic seedlings in soil showed some tolerance to osmotic stress. Under LT conditions, *CdDHN4-L* transgenic plants show better growth and had higher seed germination rates compared to WT. In addition, when exposed to LT in the dark, the seed germination rates of both *CdDHN4-L* transgenic plants reached approximately 50%, and the hypocotyl elongation of transgenic plants reached approximately 60 mm, which was far higher than WT plants. All experimental evidence demonstrated that both *CdDHN4-L* and *CdDHN4-S* enhance transgenic plants tolerance to salt, drought, osmotic and low temperature stress.

When comparing the protective effects of *CdDHN4-S* and *CdDHN4-L*, we found that they differ slightly. Our previous results showed that, when the NaCl concentration was less than or equal to 100 mM, the *CdDHN4-L* transgenic plants exhibited better growth than *CdDHN4-S* transgenic plants (*P < 0.05*) (Lv et al., 2018). In this work, we observed similar results: when the NaCl concentration was 100 mM, *CdDHN4-L* plants grew better than *CdDHN4-S* plants, though this difference was not statistically significant. Under osmotic stress of 400 mM mannitol, the relative electrolyte leakage of *CdDHN4-S* was significantly lower.
than that of CdDHN4-L, and phenotypic data, fresh weight and rosette size of CdDHN4-S were slightly higher. Under LT, the fresh weight of CdDHN4-L was significantly higher than CdDHN4-S. After treatment with 12 or 18% PEG, fresh weight and rosette size of CdDHN4-L plants were higher than CdDHN4-S, suggesting that CdDHN4-L transgenic plants are more tolerant to drought than CdDHN4-S plants (Fig. 4). These

![Image](image_url)

**Fig. 3.** Effects of overexpression of CdDHN4s on *A. thaliana* growth under salt stress. (A) Phenotypes of wildtype and CdDHN4s-overexpressing *A. thaliana* under normal and salt stress conditions. Five-day-old seedlings were transferred to salt media and grown for 15 days. (B) Root growth status of wildtype and CdDHN4s-overexpressing *A. thaliana* under normal and salt stress conditions. (C) Rosette size of wildtype and CdDHN4s-overexpressing *A. thaliana* under normal and salt stress conditions. (D) Phenotypic comparison of 3-week-old wildtype, CdDHN4-L and CdDHN4-S *A. thaliana* seedlings grown in a soil and vermiculite mixture (1:4) and irrigated with water containing 100 mM, 150 mM or 200 mM NaCl. Plants were photographed after 15 days. (E) Fresh weight and rosette size of plants under salt stress. (F) and (G) Relative electrolyte leakage of plants under salt stress. Bars represent means and standard errors of triplicate measurements, columns labeled with different capital letters indicate significant differences between the same genotypic samples under different treatment conditions, and columns labeled with different lowercase letters represent significant differences between different genotypic samples under the same treatment conditions ($P < 0.05$, least significant difference test).

![Image](image_url)

**Fig. 4.** Effects of drought stress on *A. thaliana* wildtype and CdDHN4s transgenic plants. (A) Phenotypic comparison of 2-week-old wildtype, CdDHN4-L and CdDHN4-S *A. thaliana* grown in a soil and vermiculite mixture (1:4) under normal watering and recovery from 3 weeks of drought stress. (B) Phenotypic comparison of 3-week-old wildtype, CdDHN4-L and CdDHN4-S *A. thaliana* grown in a soil and vermiculite mixture (1:4) and irrigated with water containing 12%, 18% or 24% PEG. Plants were photographed after 10 days of treatment. (C) and (D) Fresh weight and rosette size of plants under PEG drought stress. (E) Relative electrolyte leakage of plants under PEG drought stress. Bars represent means and standard errors of triplicate measurements, columns with different capital letters indicate significant differences between the same genotypic samples under different treatment conditions, and columns with different lowercase letters represent significant differences between different genotypic samples under the same treatment condition ($P < 0.05$, least significant difference test).
differences might be related to the lack of Φ-segment in the CdDHN4-S protein compared to CdDHN4-L, which was partly discussed in Lv et al., 2018. However, additional investigation is required to fully understand the mechanism of CdDHN4.

ROS production is a primary consequence of multiple stresses. ROS severely damage cell components and prevent plant growth. Numerous publications have described how dehydrins alleviate oxidative damage by scavenging hydroxyl and peroxyl radicals or by binding metals in stressed plants (Xu et al., 2018). Many dehydrins, such as CuCOR19 and AtHIRD11, were reported to scavenge hazardous ROS in plant cells to prevent membrane lipid peroxidation and increase plant resistance to stress (Liu et al., 2017a, 2017b). These dehydrins contain many His, Arg, and other reactive amino acid residues that can scavenge ROS. For example, Gly, His, and Lys residues in CuCOR19 account for 15.8%, 12.9%, and 12.9% of total residues, respectively, which facilitates its high ROS-scavenging ability. CdDHN4-L contains glycine (18.23%), histidine (8.69%), and lysine (8.87%); and CdDHN4-S contains glycine (17.10%), histidine (8.64%), and lysine (8.88%). The high percentage of Gly, His, and Lys residues suggests that both CdDHN4 isoforms can scavenge hazardous ROS. Determining whether they bind free metal ions requires further study.

The experimental results obtained using CdDHN4s transgenic plants confirmed that both CdDHN4s scavenge ROS. Electrolyte leakage, an indicator of membrane damage caused by ROS under multiple stresses, was also examined. Results illustrated that under 150 mM NaCl treatment, the relative electrolyte leakage in CdDHN4 transgenic plants was significantly lower than in WT plants. Even under other NaCl concentrations, relative electrolyte leakage values in the transgenic plants were lower than in WT plants, though the difference was not significant. In response to LT, 400 mM mannitol or drought treatment, the relative electrolyte leakage values in CdDHN4-S transgenic plants were significantly lower than in WT. Under drought treatment, MDA content was significantly lower in both CdDHN4s transgenic plants than in WT. The H2O2 content of both CdDHN4s plants was significantly lower than in WT under 400 mM mannitol treatment. Furthermore, the CdDHN4-L transgenic plants exhibited higher O2•− inhibition activity than WT plants under salt or drought treatment. All data indicated that CdDHN4 enables plants to scavenge ROS, such as H2O2 and O2•−.

CdDHN4 proteins have the ability to protect enzymatic antioxidants and may contribute to the nonenzymatic antioxidant scavenging ROS. By transferring CdDHN4s into Arabidopsis, we found that the SOD and POD activity of CdDHN4s plants was significantly higher under drought treatment compared to WT, suggesting CdDHN4s have ability to protect SOD and POD, thereby enabling them to scavenge excess H2O2 and O2•− and help plants resist drought stress. The AsA content was significantly lower in WT than in transgenic plants under salt stress, revealing that CdDHN4s may enhance AsA generation. Comprehensively assessing all the data related to ROS, we conclude that CdDHN4s have protective effect against plant cell injury caused by ROS and that CdDHN4-L has higher ROS-scavenging capacity than CdDHN4-S.

Combined with what we published in Lv et al. (2018), we noticed that through alternative splicing, the single CdDHN4 generates two transcripts (CdDHN4-S and CdDHN4-L). In vivo experiments proved CdDHN4-L has higher protein protective ability than CdDHN4-S (Lv et al., 2018). In vivo experiments revealed that both CdDHN4-S and CdDHN4-L enhance the transgenic plants’ tolerance to abiotic stress, and CdDHN4-S has higher ROS-scavenging ability than CdDHN4-L. These findings indicate that these two transcripts exhibit distinct functional preference, supporting the hypothesis that alternative splicing provides various functional attributes to a single DHN to respond to abiotic stress in plants (Tompa, 2012).
Fig. 6. Effects of overexpression of CdDHN4s on Arabidopsis growth under osmotic stress.

(A) Phenotypes of wildtype and CdDHN4s-overexpressing A. thaliana under normal and osmotic stress conditions; five-day-old seedlings were transferred to high osmotic media and grown for 15 days. (B) Root growth of wildtype and CdDHN4s-overexpressing A. thaliana under normal and osmotic stress conditions. (C) Rosette size of wildtype and CdDHN4s-overexpressing A. thaliana under normal and osmotic stress conditions. (D) Root length of wildtype and CdDHN4s-overexpressing A. thaliana under normal and osmotic stress conditions. (E) Phenotypic comparison of 3-week-old wildtype, CdDHN4-L and CdDHN4-S A. thaliana seedlings grown in a soil and vermiculite mixture (1:4) and irrigated with water containing 300 mM, 400 mM or 500 mM mannitol. Plants were photographed after 5 days of treatment. (F) and (G) Fresh weight and rosette size of plants under osmotic stress. (H) Relative electrolyte leakage of plants under osmotic stress. Bars represent means and standard errors of triplicate measurements, columns labeled with different capital letters indicate significant differences between the same genotypic samples under different treatment conditions, and columns labeled with different lowercase letters represent significant differences between different genotypic samples under the same treatment conditions ($P < 0.05$, least significant difference test).
5. Conclusions

This study demonstrated that the CdDHN4 gene in the drought-tolerant hybrid bermudagrass genotype ‘Tifway’ produces two transcripts: CdDHN4-S and CdDHN4-L. Both CdDHN4-S and CdDHN4-L can be classified as YSK2-type DHNs. Both share the same genomic sequence. The alternatively spliced 48 nucleotides encode the Φ-segment that accounts for the structural difference between the CdDHN4-S and CdDHN4-L proteins. We analyzed transgenic Arabidopsis thaliana strains overexpressing CdDHN4-L or CdDHN4-S and found that both CdDHN4s improve multiple plant stress tolerances. Comprehensive assessment of the ROS-scavenging ability of the CdDHN4s indicated that both can scavenge ROS and that CdDHN4-S has higher ROS scavenging capacity than CdDHN4-L.

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CRediT authorship contribution statement

Di Zhang: Writing - original draft. Aimin Lv: Investigation. Tianchen Yang: Resources. Xiaoqing Cheng: Resources. Enhua Zhao: Resources. Peng Zhou: Writing - review & editing, Supervision.

Fig. 7. Effects of overexpression of CdDHN4s on Arabidopsis growth under low temperature stress. (A) Phenotypes of wildtype and CdDHN4s-overexpressing A. thaliana grown at room temperature (22 °C) and low temperature (8 °C day/0 °C night) for 3 weeks. (B) Phenotypic comparison of 2-week-old wildtype, CdDHN4-L and CdDHN4-S A. thaliana seedlings grown in a soil and vermiculite mixture (1:4) and cultured at room temperature (22 °C) and low temperature (8 °C day/0 °C night). (C) and (D) Rosette size and root length of wildtype and CdDHN4s-overexpressing A. thaliana at room temperature and low temperature. (E) and (F) Plant fresh weight and rosette size before and after low temperature treatment. (G) Seed germination status of wildtype and CdDHN4s-overexpressing A. thaliana at low temperature (4 °C) in the dark. (H) and (I) Seed germination rates and hypocotyl lengths of wildtype and CdDHN4s-overexpressing A. thaliana at low temperature in the dark. (J) Relative electrolyte leakage of seedlings under low temperature stress. Bars represent means and standard errors of triplicate measurements, columns labeled with different capital letters indicate significant differences between the same genotypic samples under different treatment conditions, and columns with different lowercase letters represent significant differences between different genotypic samples under the same treatment condition (P < 0.05, least significant difference test).
Fig. 8. Quantitative analyses of MDA, ROS, and the antioxidant system of 3-week-old A. thaliana wildtype and CdDHN4s transgenic plants under different stress conditions, comprehensive analysis of the ROS-eliminating abilities of the three genotypes, and the comprehensive effects of ROS under different stresses. Bars represent means and standard errors of triplicate measurements, columns with different capital letters indicate significant differences between the same genotypic samples in different treatment conditions, and columns with different lowercase letters represent significant differences between different genotypic under the same treatment condition (P < 0.05, least significant difference test).

Declaration of competing interest

There are no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2020.100033.

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