Gene: X 5 (2020) 100033

Contents lists available at ScienceDirect

Gene: X

journal homepage: www.journals.elsevier.com/gene-x

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

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ARTICLE INFO

Keywords:
Abiotic stress
Dehydrin
Alternative splicing
Bermudagrass
ROS

ABSTRACT

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 YSK_2-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 (*Cynodondactylon* × *C. transvaalensis* 'Tifway') has been developed to produce a highly desirable turf quality with limited irrigation (Hanna, 1998), and it exhibits more drought tolerance with greater maintenance of photosynthetic processes, water retention and antioxidant defenses compared to common bermudagrass (*C. dactylon* 'C299') (Hu et al., 2009; Hu et al., 2010; Zhao et al., 2011;). Drought stress increases the production of reactive oxygen species (ROS), which can destroy plant cell structures. Plants have developed complex antioxidant systems to eliminate ROS. The antioxidant system includes 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 (O^{2-}) by dismutation to form H_2O_2, and CAT splits H_2O_2 into H_2O and O_2 (Scandalios, 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 antioxidative defense system (Foyer and Halliwell, 1976).

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

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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https://doi.org/10.1016/j.gene.2020.100033
Received 4 February 2020; Received in revised form 25 March 2020; Accepted 15 April 2020
Available online 22 April 2020

protective proteins (dehydrin), and oxidative stress defense (Zhou et al., 2014). Hu et al. (2010) had identified multiple dehydrins (DHNs) expressed in 'Tifway' and 'C299' under drought stress, and the expression levels of a 40-kDa dehydrin were positively correlated with drought tolerance in bermudagrass. These transcript and protein analyses agree and suggest that dehydrins may protect bermudagrass leaves from water loss. Drought tolerance mechanisms have been associated with the accumulation of protective proteins such as dehydrins and other late-embryogenesis abundant (LEA) proteins protecting plant cells from dehydration (Close, 1997). Although the mechanisms whereby dehydrins and LEA proteins protect plant cells are still ambiguous, it is reported that some dehydrins and LEA proteins can act as chaperones to protect other protein and membrane structure (Hara et al., 2001; Bravo et al., 2003).

DHNs, group II LEA proteins, are major proteins expressed in response to water-limited environments (Close, 1997). DHNs contain five subclasses, Kn, KnS, 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., 2009), and prevention of protein or membrane aggregation (Kovacs et al., 2008). Many *DHN* genes in plants were found to undergo alternative splicing (Fernandez-Caballero et al., 2012; Yang et al., 2012; Vazquez-Hernandez et al., 2017). Alternative splicing is an important modulator of gene expression that can increase DHN variability and regulate it's mRNA levels (Laloum et al., 2018). Although alternative splicing of *DHN* is now widely known to occur, its biological role in plant stress response and tolerance is not yet completely understood. It was proposed that alternative splicing might provide a mechanism to introduce changes in various functional attributes of a single *DHN* gene (Tompa,2012). Understanding the functions of these genes at the molecular level may help us to understand why 'Tifway' is able to survival harsh environments.

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/gorf/). 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 DNAstar5.0 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 PONDR software (http://www.pondr.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.

Table 1
Primers used for PCR amplification.

Primer	Nucleotide sequence (5′-3′)	Function
3A1	GCGAACAGTCCGTGATAACTGTCTGTCA	3′ RACE; outer
3A2	TCGTGTAACATGATAAGATGGTCAGCCA	3′ RACE; inner
5S1	ACCATCTTATCATGTTACACGAACGTCG	5′ RACE; outer
5S2	GAACGTCGTGACAGACAGTTATCACGGA	5′ RACE; inner
qRT-S	TCGTCTGAGGATGATGGC	Real-time PCR
qRT-A	TGTCCTTGCTGACCGTAG	Real-time PCR
18S-S	GTGACGGGTGACGGAGAATT	Real-time PCR
18S-A	GACACTAATGCGCCCGGTAT	Real-time PCR
ORF-S	ATGGAGCACCAGGGACAGTACGGC	Amplification of ORF
ORF-A	TGTCCATGATGCCCTTCTTCTCGC	Amplification of ORF
Dehydrin_CS	AAGGATCCATGGAGCACCAGGGACAGTA	Amplification of ORF for expression
Dehydrin_CA	AACTAGTGTGCTGGCCGGGGAGCTT	Amplification of ORF for expression

2.3. Recombinant expression of CdDHN4

The complete ORF of *CdDHN4*, with the inclusion of *Bam*HI and *Spe*I 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 (T_0) were selected using 100 mg L^{-1} glyphosate (BBI, Markham, Ontario, Canada), and homozygous T_3 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, H_2O_2 levels, $O_2\cdot^-$ 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).

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 $\mu mol \cdot m^{-2} \cdot s^{-1}$) 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 CdDHN4 gene in bermudagrass

Two *CdDHN4* cDNAs were isolated from 'Tifway'; the full-length ORFs of *CdDHN4-L* and *CdDHN4-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 homologous to the dehydrin DHN3 from *Hordeum vulgare* and *Aegilops 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 *CdDHN4-L* and *CdDHN4-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 CdDHN4 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 CdDHN4s clusters with *A. thaliana* SKn-type DHNs, including At5g66400 (YSK_2) and At3g50980 (SK_2).

Genomic sequence analysis showed that *CdDHN4* consists of two exons and one intron and that exon2 undergoes alternative splicing. The produced YSK_2-type *CdDHN4s* include two splice variants (the nonspliced *CdDHN4-L* and the spliced *CdDHN4-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 CdDHN4-L and CdDHN4-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 CdDHN4s are mostly disordered proteins.

3.2. Heterologous expression of CdDHN4s in Arabidopsis enhances tolerance to multiple abiotic stresses

From each *CdDHN4*-overexpressing *Arabidopsis* strain, 8 transgenic lines were selected for phenotypic observation (Supplementary Fig. S1). Both *CdDHN4-L* and *CdDHN4-S* transgenic plants exhibited good seed setting under normal growth conditions and better tolerance to drought stress compared to wild type plants. Subsequently, the *CdDHN4-L* transgenic plant lines 2, 3 and 7, and *CdDHN4-S* lines 2, 3 and 5 were selected based on their similar *CdDHN4* expression levels. *CdDHN4-L* and *CdDHN4-S* homozygous transgenic lines (T_3) were used for abiotic stress tolerance assays and physiological tests.

3.2.1. Overexpression of CdDHN4s improves plant tolerance to salt stress

Plant fresh weight, rosette size and root length were not significantly different between the *CdDHN4* transgenic and wild type (WT) lines under normal growth conditions (Fig. 3). Under salt stress, 5-day-old WT plants grew abnormally and exhibited stress 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 *CdDHN4* 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 *CdDHN4-L* and *CdDHN4-S* plants decreased by 47.6% and 43.1% compared to WT, respectively.

Under salt stress, the content of MDA, H_2O_2 and OH· were reduced in transgenic plants compared to WT specimens, through the difference did not reach statistical significance (Fig. 8). The $O_2 \cdot^-$ inhibition activity in transgenic *A. thaliana* was increased compared to WT under salt stress, especially in the *CdDHN4-S* line, which was significantly higher than the WT ($P < 0.05$). The content of AsA, one of nonenzymatic antioxidants, was significantly increased in both transgenic genotypes, compared to WT plants.

3.2.2. Overexpression of CdDHN4s improves plant tolerance to drought and osmotic tress

Two-week-old WT seedlings deprived of water began to die and were unable to recover; however, most of the *CdDHN4-L* and *CdDHN4-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 refresh weights of the seedlings showed significant differences between transgenic seedlings and WT after treatment with 18% PEG ($P < 0.05$) (Fig. 4C, D). *CdDHN4-S* seedlings exhibited reduced leakage compared to CdDHN4-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 *CdDHN4-L* and *CdDHN4-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 *CdDHN4-S* transgenic plants during drought stress (Fig. 5). These results indicate that *CdDHN4s*-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

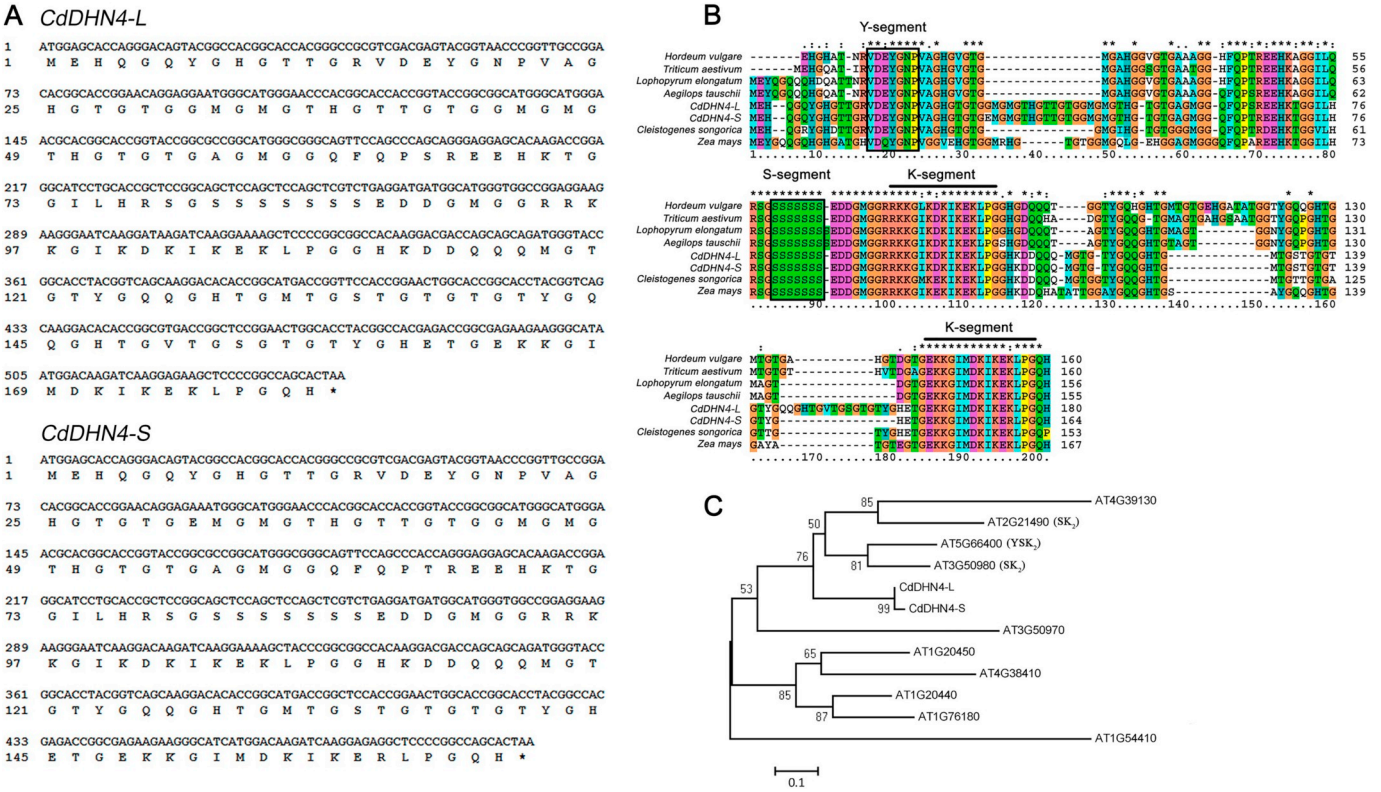

Fig. 1. Sequence analysis of *CdDHN4* and phylogenetic relationships among CdDHN4s and 10 dehydrin proteins from *A. thaliana*. (A) Nucleotide and deduced protein sequence of *CdDHN4s*. (B) Multiple sequence alignment of CdDHN4s with their homologous sequences. The amino acid sequences of Y- and S-segments are highlighted with boxes and K-segments with a bar. (C) The molecular phylogeny was constructed from a complete protein sequence alignment of DHNs by the neighbor-joining method with bootstrapping analysis (1000 replicates). The numbers beside the branches indicate bootstrap values.

significantly higher compared to WT, and the $O_2 \cdot^-$ 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_2O_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

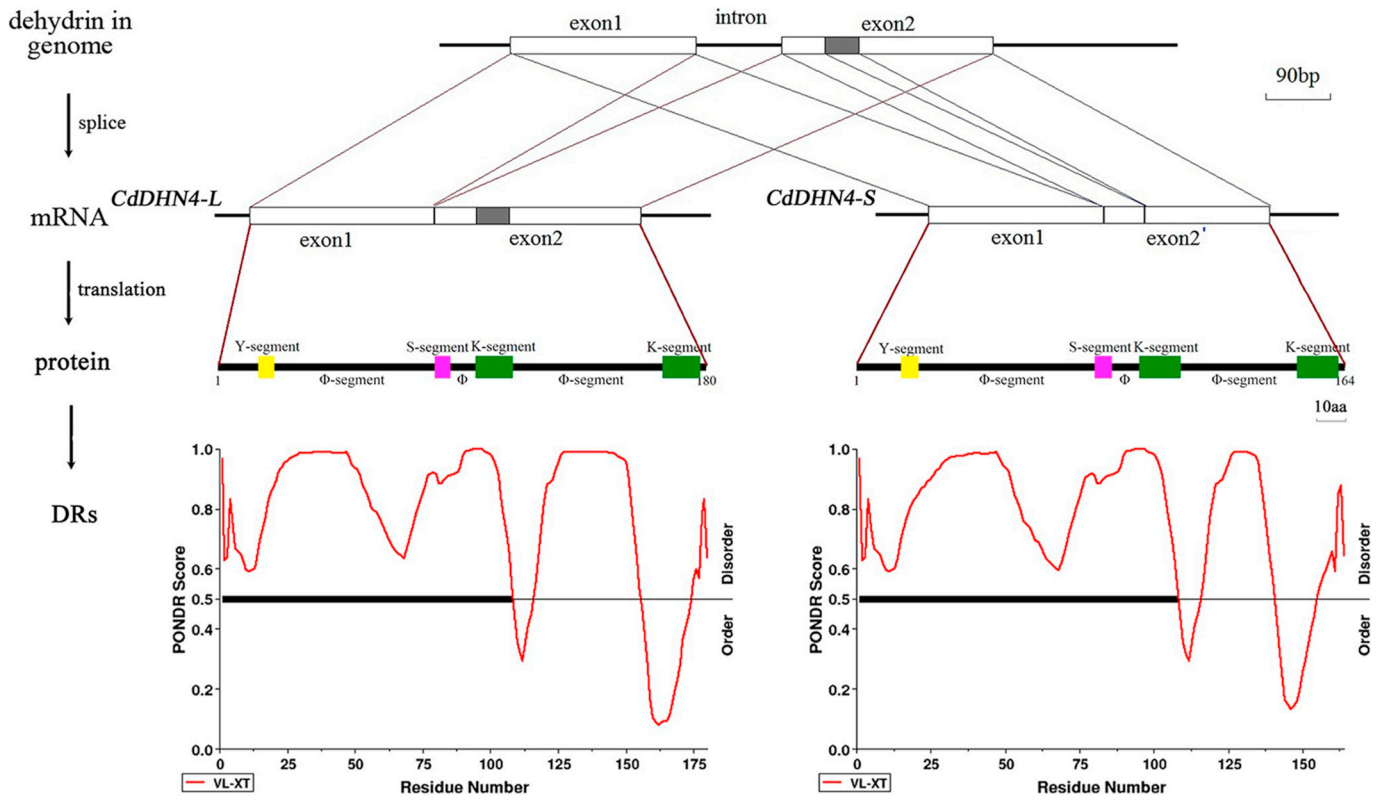

Fig. 2. Schematic representation indicating the structural characteristics of the CdDHN4 gene and its products.
The structural characteristics of nonspliced (*CdDHN4-L*) and spliced (*CdDHN4-S*) sequences. Colored boxes indicate the positions of the Y, S, K and Φ domains, and the black box represents 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.)

DHN3 from barley (GI: 118487), and dehydrin-LEA2 like protein from *Lophopyrumelongatum* (GI: 2970213). It has been reported that dehydrins GI: 118487 and GI: 2970213 respond to salt stress, hinting that *CdDHN4-S* and *CdDHN4-L* may have the same function (Gulick and Dvořák, 1992). Phylogenetic tree analysis demonstrated that both *CdDHN4s* clustered with the *A. thaliana* DHNs (At5g66400 (YSK_2) and At3g50980 (SK_2)). Evidence suggests that At5g66400 and At3g50980, named RAB18 and XERO1 respectively, may contribute protective effects to plants in response to salt, cold or osmotic stress (Lång and Palva, 1992).

Both *CdDHN4-S* and *CdDHN4-L* can be classified as YSK_2-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 CdDHN4s 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, *CdDHN4s* transgenic plants showed better stress tolerance than WT, especially when the irrigation water contained 150 mM NaCl. After 3 weeks without water, *CdDHN4s* transgenic plants in soil could still recover when watered again, while WT were not able to recover, suggesting that *CdDHN4s* may enable plant cells to resist drought. Although 5-day-old *CdDHN4s* 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, *CdDHN4s* plants grow better and had higher seed germination rates compared to WT. In addition, when exposed to LT in the dark, the seed germination rates of both *CdDHN4s* 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

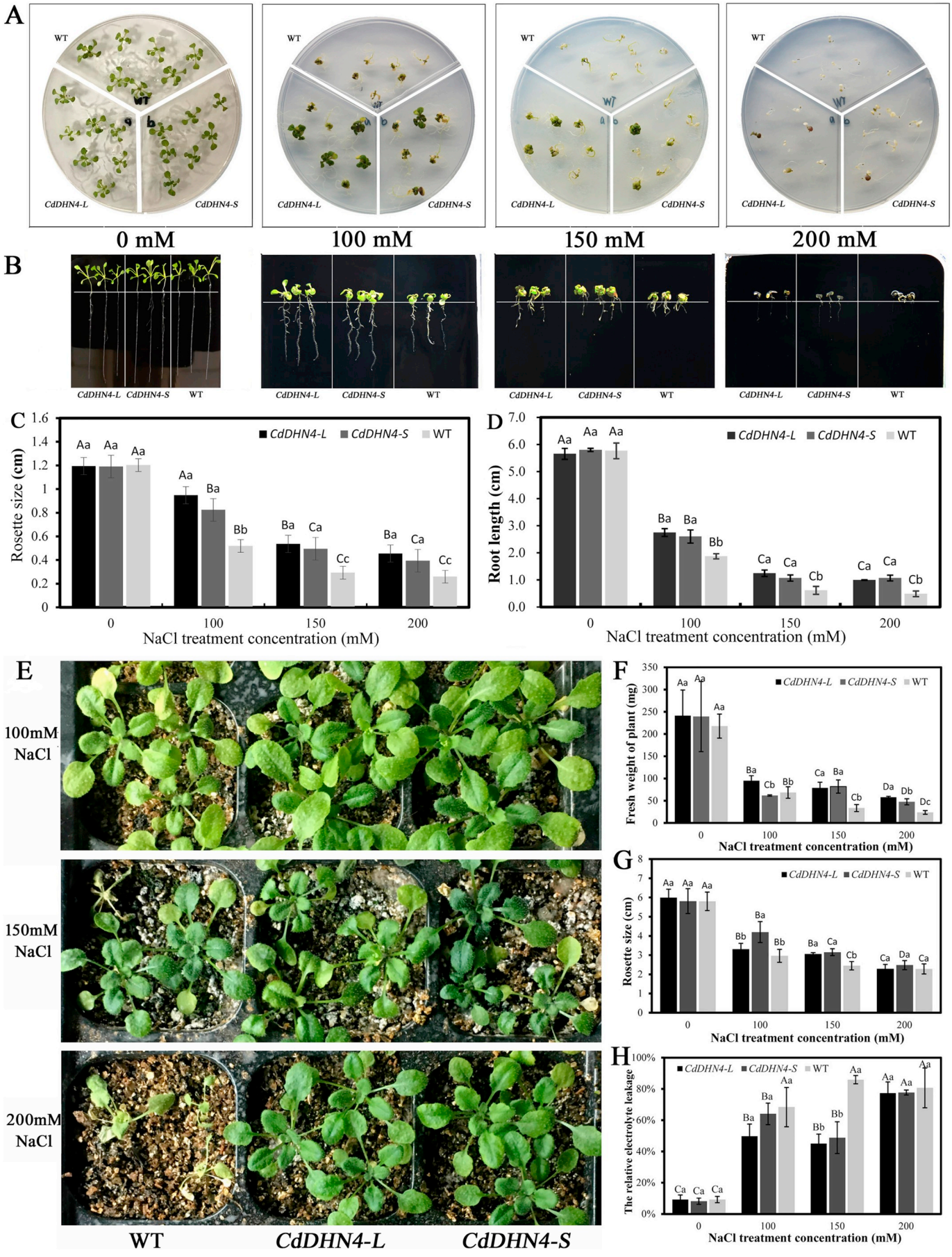

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Fig. 3. Effects of overexpression of *CdDHN4s* on *A. thaliana* growth under salt stress.
(A) Phenotypes of wildtype and *CdDHN4s*-overexpressing *A. thaliana* under normal and salt stress conditions. Five-day-old seedlings were transferred to salt 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) Root length of wildtype and *CdDHN4s*-overexpressing *A. thaliana* under normal and salt 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 100 mM, 150 mM or 200 mM NaCl. Plants were photographed after 15 days. (F) and (G) Fresh weight and rosette size of plants under salt stress. (H) 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).

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 significant 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

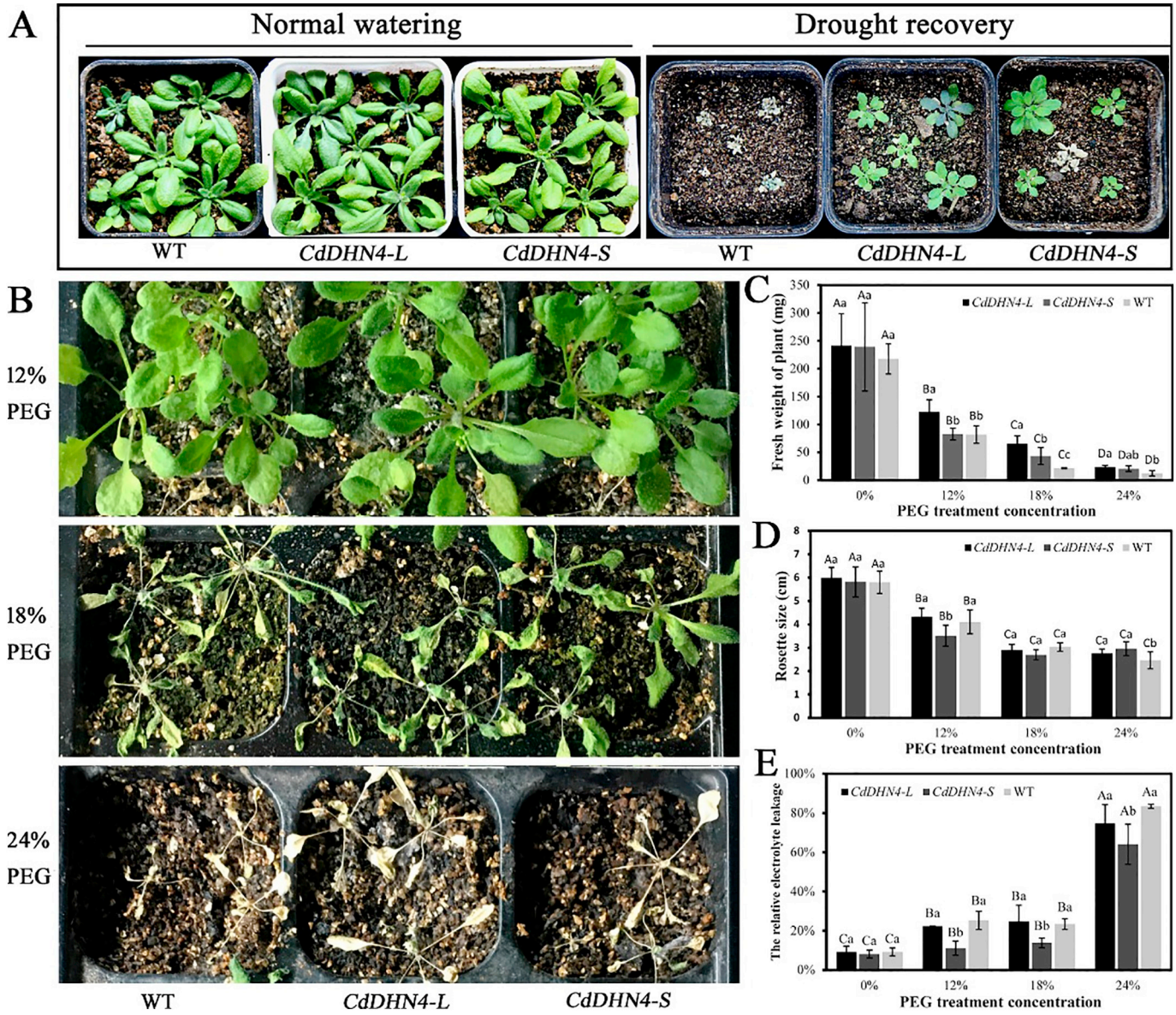

Fig. 4. Effects of drought stress on *A. thaliana* wildtype and *CdDHN4s* transgenic plants.
(A) Phenotypic comparison of 2-week-old wildtype, *CdDHN4-L* and *CdDHN4-S A. thaliana* grown in a soil and vermiculite mixture (1:4) under normal watering and recovery from 3 weeks of drought stress. (B) Phenotypic 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).

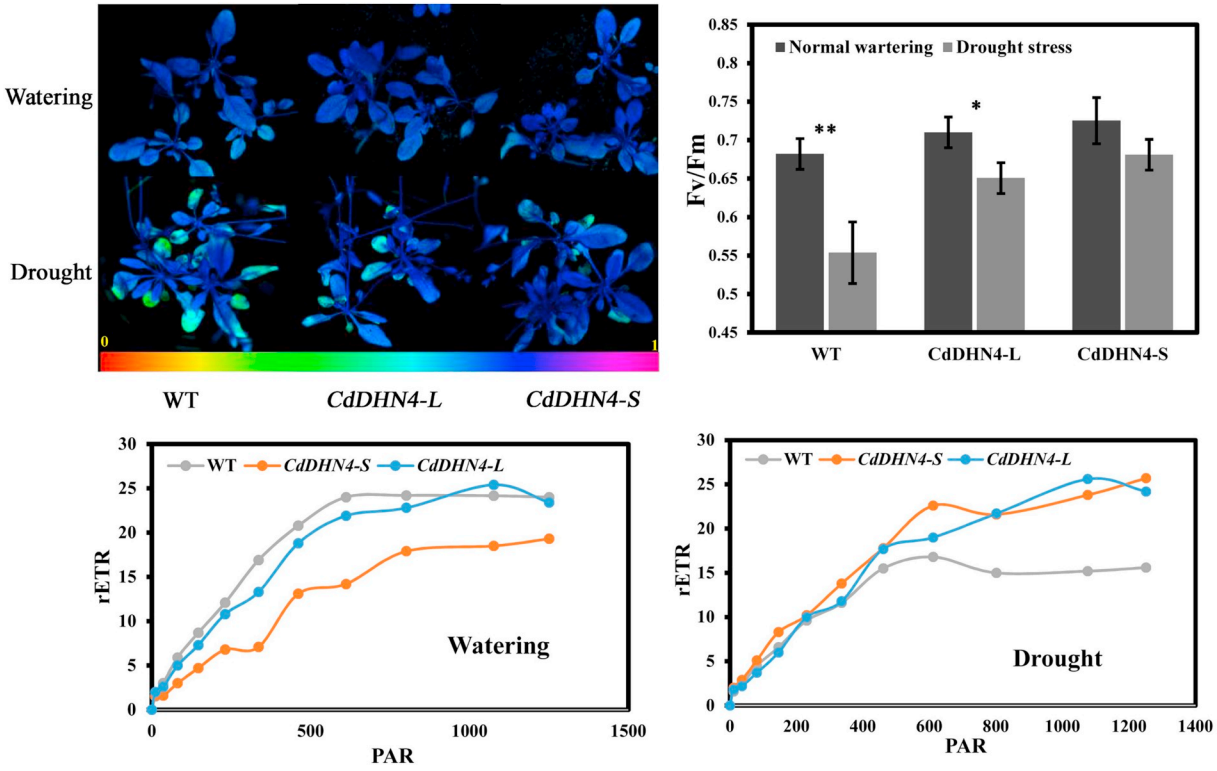

Fig. 5. Photosynthetic capability of wildtype and transgenic *A. thaliana* under drought stress.
(A) and (B) Photosynthetic capabilities were recorded by Fv/Fm imaging using a PAM chlorophyll fluorometer. The pseudocolor code ranges from 0 (red) to 1.0 (purple), the asterisk represents significant differences of each genotypic sample grown under normal watering and drought stress conditions, and significant levels are indicated by * $P < 0.05$ or ** $P < 0.01$. (C) and (D) Rapid light-response curve of ETR parameters of plants under normal watering and drought stress conditions. Measurements were performed at the following light intensities: 0, 11, 36, 81, 146, 231, 336, 461, 611, 801, 1076, and 1251 μmol·m^{-2}·s^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 accounts 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 *CdDHN4s* 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 H_2O_2 content of both *CdDHN4s* plants was significantly lower than in WT under 400 mM mannitol treatment. Furthermore, the *CdDHN4-S* transgenic plants exhibited higher $O_2^{\cdot -}$ inhibition activity than WT plants under salt or drought treatment. All data indicated that *CdDHN4* enables plants to scavenge ROS, such as H_2O_2 and $O_2\cdot^-$.

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 H_2O_2 and $O_2\cdot^-$ 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-S* has higher ROS-scavenging capacity than *CdDHN4-L*.

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 vitro* 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).

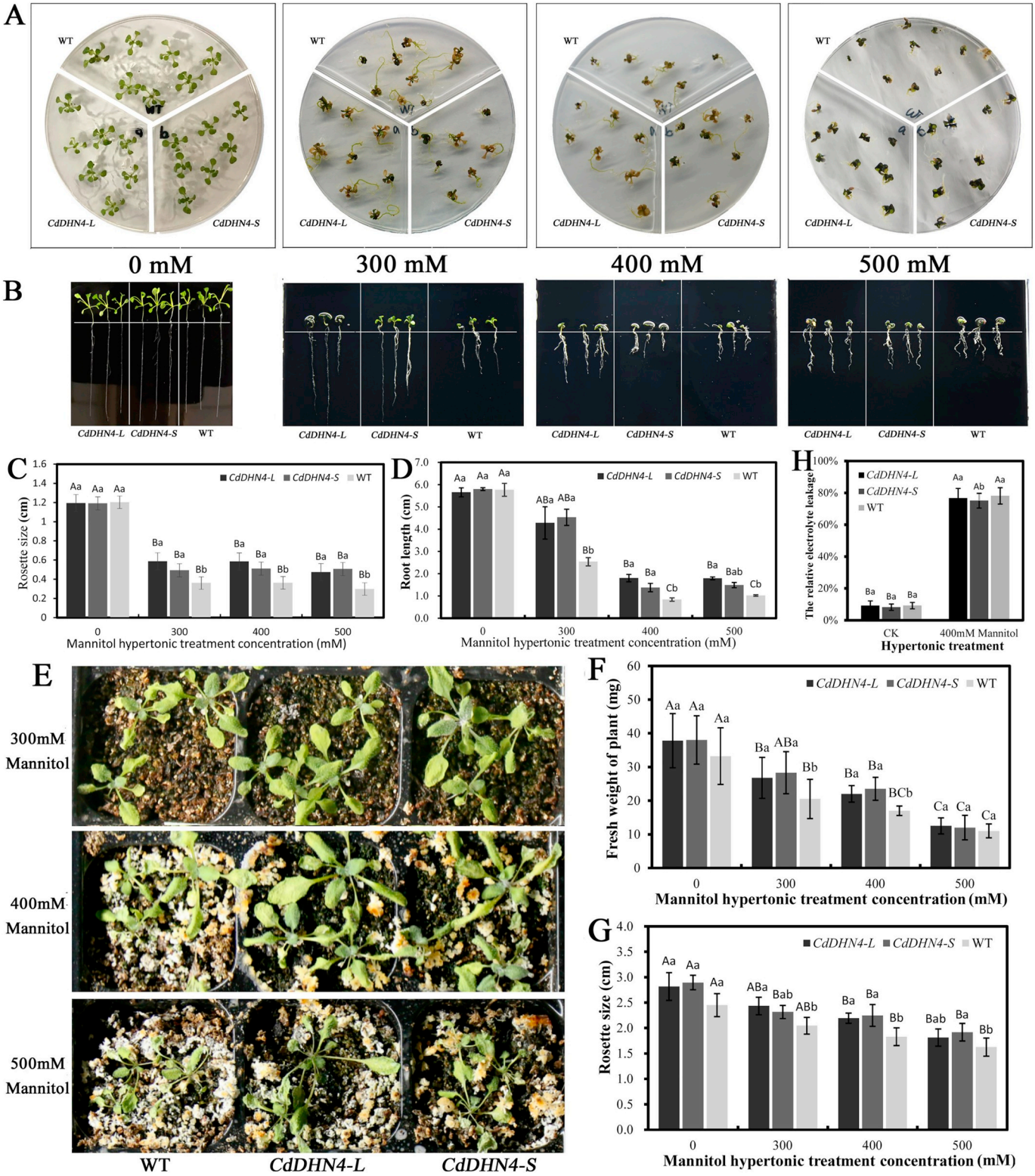

Fig. 6. Effects of overexpression of *CdDHN4s* on *Arabidopsis* growth under osmotic stress.
(A) Phenotypes of wildtype and *CdDHN4s*-overexpressing *A. thaliana* under normal and osmotic stress conditions; five-day-old seedlings were transferred to high osmotic media and grown for 15 days. (B) Root 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).

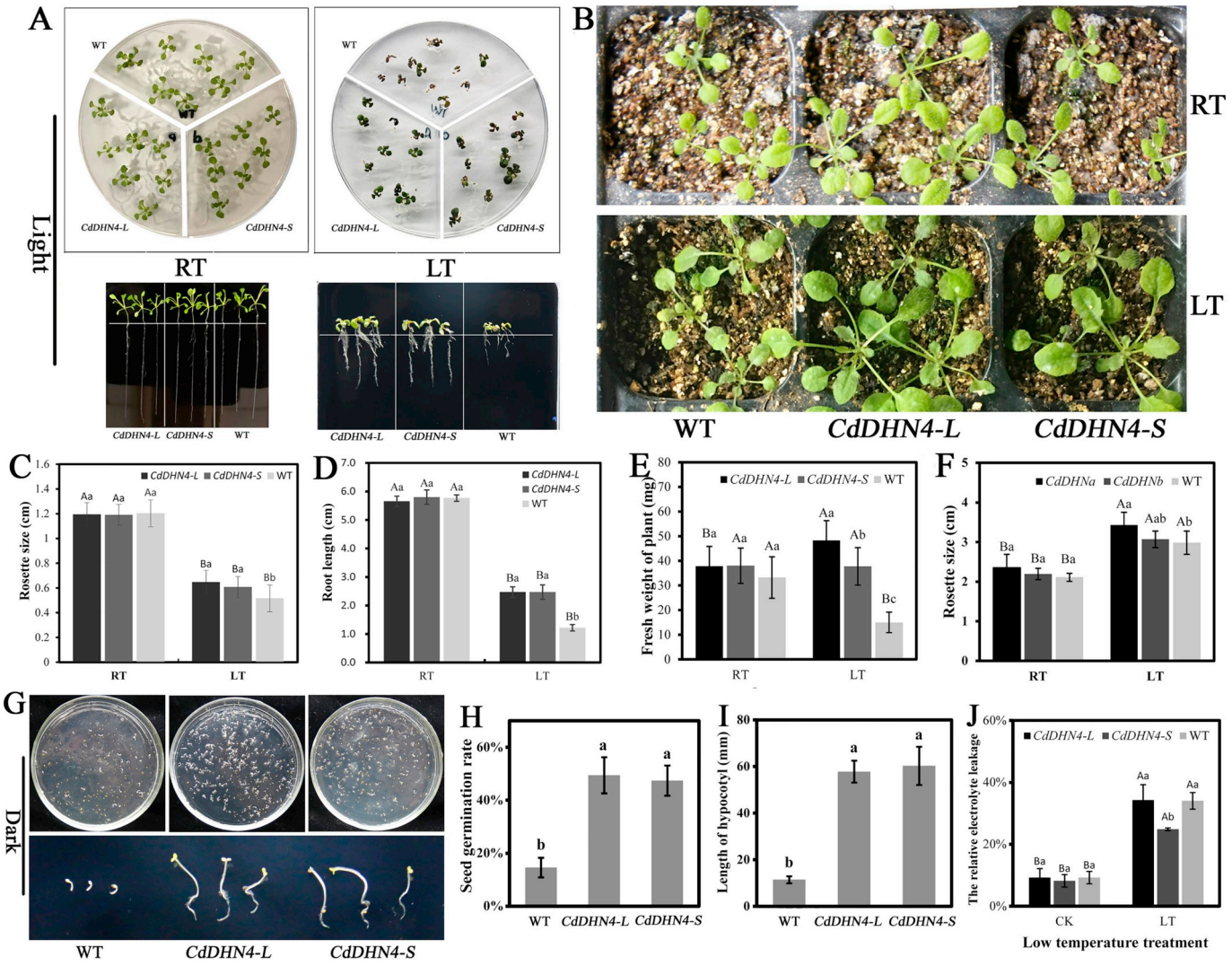

Fig. 7. Effects of overexpression of *CdDHN4s* on *Arabidopsis* growth under low temperature stress.
(A) Phenotypes of wildtype and *CdDHN4s*-overexpressing *A. thaliana* grown at room temperature (22 ˚C) and low temperature (8 ˚C day/0 ˚C night) for 3 weeks. (B) Phenotypic comparison of 2-week-old wildtype, *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).

5. Conclusions

This study demonstrated that the *CdDHN4* gene in the drought-tolerant hybrid bermudagrass genotype 'Tifway' producers two transcripts: *CdDHN4-S* and *CdDHN4-L*. Both CdDHN4-S and CdDHN4-L can be classified as YSK_2-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*.

Funding sources

This work was supported by the National Project on Sci-Tec Foundation Resources Survey (No. 2017FY100600) and the National Natural Science Foundation of China (Nos. 31272198, 31872419, 31670693 and 31872408).

CRediT authorship contribution statement

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

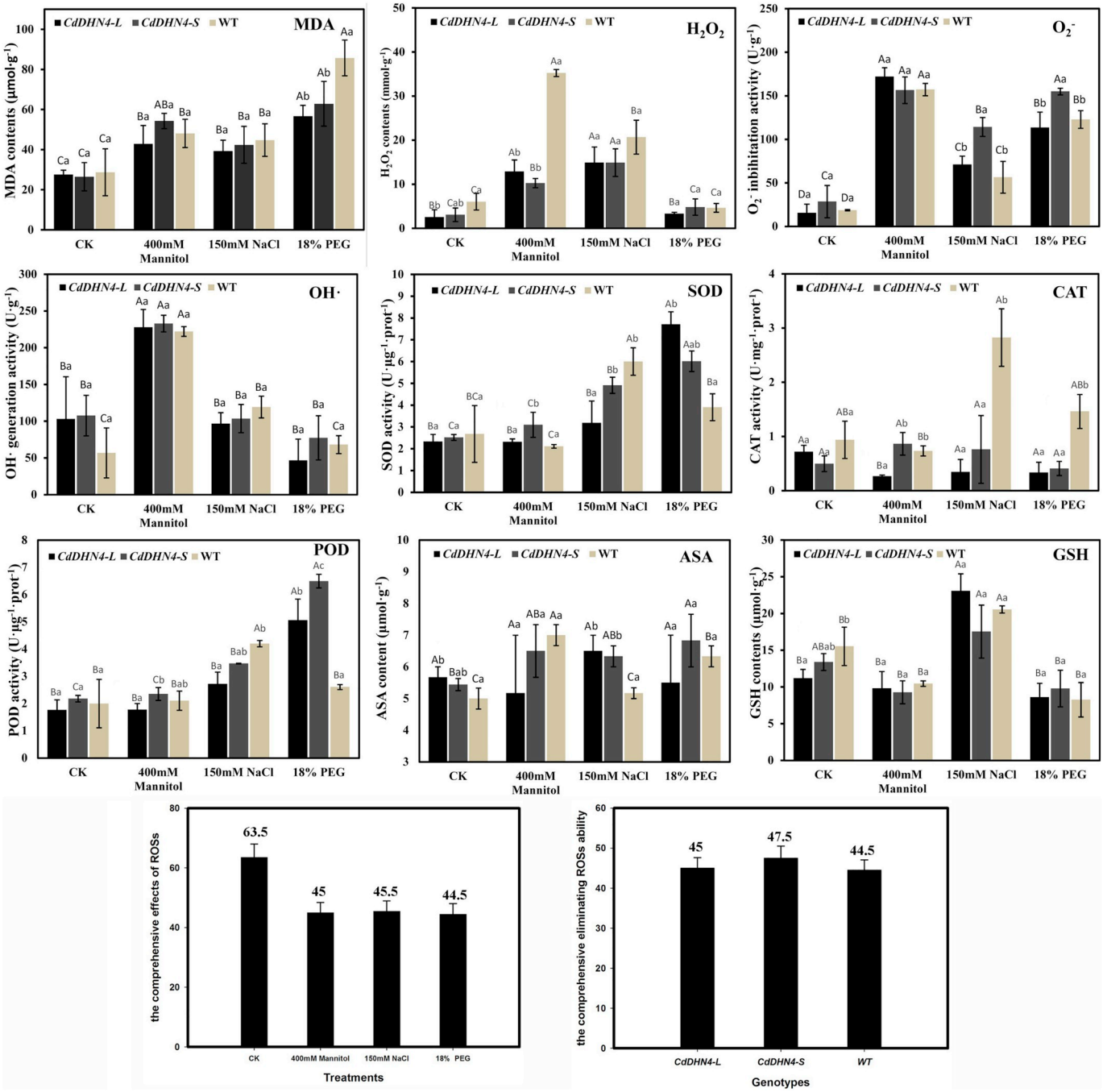

Fig. 8. Quantitative analyses of MDA, ROS, and the antioxidant system of 3-week-old *A. thaliana* wildtype and *CdDHN4s* transgenic plants under different stress conditions, comprehensive analysis of the ROS-eliminating abilities of the three genotypes, and the comprehensive effects of ROS under different 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 in 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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