



# AnDHN, a Dehydrin Protein From *Ammopiptanthus nanus*, Mitigates the Negative Effects of Drought Stress in Plants

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Dehydrins (DHNs) play crucial roles in a broad spectrum of abiotic stresses in model plants. However, the evolutionary role of DHNs has not been explored, and the function of DHN proteins is largely unknown in *Ammopiptanthus nanus* (*A. nanus*), an ancient and endangered legume species from the deserts of northwestern China. In this study, we isolated a drought-response gene (*c195333\_g1\_i1*) from a drought-induced RNA-seq library of *A. nanus*. Evolutionary bioinformatics showed that *c195333\_g1\_i1* is an ortholog of *Arabidopsis* DHN, and we renamed it AnDHN. Moreover, DHN proteins may define a class of proteins that are evolutionarily conserved in all angiosperms that have experienced a contraction during the evolution of legumes. *Arabidopsis* plants overexpressing *AnDHN* exhibited morpho-physiological changes, such as an increased germination rate, higher relative water content (RWC), higher proline (PRO) content, increased peroxidase (POD) and catalase (CAT) activities, lower contents of malondialdehyde (MDA), H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>, and longer root length. Our results showed that the transgenic lines had improved drought resistance with deep root system architecture, excellent water retention, increased osmotic adjustment, and enhanced reactive oxygen species (ROS) scavenging. Furthermore, the transgenic lines also had enhanced salt and cold tolerance. Our findings demonstrate that *AnDHN* may be a good candidate gene for improving abiotic stress tolerance in crops.

**Key Message:** Using transcriptome analysis in *Ammopiptanthus nanus*, we isolated a drought-responsive gene, *AnDHN*, that plays a key role in enhancing abiotic stress tolerance in plants, with strong functional diversification in legumes.

**Keywords:** *Ammopiptanthus nanus*, drought stress, abiotic stress, legume, AnDHN, ROS

## INTRODUCTION

For plant scientists, dramatically improving crop yields to meet the needs of an ever-increasing human population is an urgent matter at present and will become more pressing in the near future (Eshed and Lippman, 2019; Zhang et al., 2020a). Crop growth and production are strictly limited by various abiotic stress conditions that work individually or together, such as drought, salt, cold,

and heat (Zhu, 2016; Wei et al., 2019). Among them, drought plays a pivotal role in affecting crop yields, greater even than the annual loss in crop yield caused by all other abiotic stresses worldwide (Zhu, 2016; Feng et al., 2020b; Gupta et al., 2020). Thus, a clearer understanding of the mechanisms underlying the control of drought resistance in different crop species is urgently needed.

Unlike animals that are mobile and can escape from adverse environmental conditions, plants have evolved a series of elaborate morpho-physiological and molecular drought tolerance mechanisms that enable them to survive drought stress (Ullah et al., 2017; Gupta et al., 2020). Many recent studies have focused on drought tolerance in plants, and the findings shed light on enhancing drought resistance (Fang and Xiong, 2014; Zhu, 2016; Liu et al., 2019; Xing et al., 2019; Bao et al., 2020; Feng et al., 2020b; Mao et al., 2020; Yang et al., 2020). There are three strategies, drought escape, drought avoidance, and drought tolerance that plants use to adapt to different degrees of water deficiency (Manavalan et al., 2009; Luo, 2010; Ullah et al., 2017; Gupta et al., 2020). Among the three strategies, drought tolerance is the most critical strategy to prevent water loss and involves a series of physiological processes: (1) stomatal closure (reducing water loss), (2) increased root density and root length (increasing absorption of water), and (3) adjusting osmotic conditions at the cellular level by promoting the production of osmolytes, such as proline (PRO) and trehalose (Xie et al., 2006; Comas et al., 2013; Zhang et al., 2020b; Zhou et al., 2020). These key physiological responses and the expression of numerous downstream responsive genes under drought stress are mainly controlled by the hormone abscisic acid (ABA; Ullah et al., 2017; Gupta et al., 2020). In addition, a recent hypothesis proposes that COST1 coordinates with drought tolerance and autophagosome formation in *Arabidopsis* (Bao et al., 2020). Despite previous hypotheses, the functions of proteins encoded by genes involved in drought resistance remain largely unknown in many diverse plant species.

Dehydrins (DHNs) are a group of environmental stress-responsive proteins that belong to Group II of the late embryogenesis abundant (LEA) protein family. Based on the several highly conserved motifs (K-, Y-, S-, and  $\phi$ -segments), DHNs can be divided into five subgroups, such as YnSKn, YnKn, SKn, Kn, and KnS (Rorat, 2006). The K-segment, harboring a lysine-rich repetitive domain (EKKGIMDKIKEKLP), is an exclusive conserved motif present in all DHNs, and this segment may play a pivotal role in protein-lipid interactions (Close, 1996; Koag et al., 2009). The rest three motifs are not essential to characterize the DHN proteins. The Y-segment, a conserved sequence [(T/V)D(E/Q)YGNP] showing partial identity to plant and bacterial chaperones binding sites, is found in the N-terminal region of DHNs (Malik et al., 2017). The S-segment, consisting of four to eight serine residues modifiable by phosphorylation, may regulate protein conformations and ion-binding activities (Yang et al., 2012). The poorly conserved regions, so-called  $\phi$ -segments, are enriched with polar amino acids (Vornam et al., 2011; Graether and Boddington, 2014).

In response to abiotic stresses (e.g., drought, osmotic stress, salinity, and temperature), many DHN genes are upregulated in all vegetative tissues (Close, 1997; Nylander et al., 2001;

Kosová et al., 2007; Peng et al., 2008; Kim and Nam, 2010; Shekhawat et al., 2011; Riyazuddin et al., 2021). In *Arabidopsis*, overexpression of EARLY RESPONSIVE TO DEHYDRATION 10 (ERD10), LOW TEMPERATURE-INDUCED 30 (LTI30), and DHN5 enhance freezing and salt tolerance (Puhakainen et al., 2004; Brini et al., 2007). *OsDHN1* has been shown to play a core role in drought and salt stress (Kumar et al., 2014; Verma et al., 2017). *ShDHN* has been reported to promote resistance against drought and cold stress (Liu et al., 2015), and *MsDHN1* can increase tolerance to Al stress in *Medicago sativa* (Lv et al., 2021). In addition to abiotic stresses, there is evidence that LEA family proteins are involved in antibacterial activity. Overexpression of *LEA2* and *LEA4* derived from *Arabidopsis* in *Escherichia coli* both leads to the inhibition of bacterial growth (Campos et al., 2006). The conserved K-segment peptides are responsible for the antibacterial activities against Gram-positive bacteria (Zhai et al., 2011). While DHN functions remain elusive in other species though a few clues have been found.

In China, *Ammopiptanthus nanus* (*A. nanus*; Leguminosae) is a rare and endangered species. *A. nanus* is an evergreen broadleaf shrub that is only found in severely arid regions of the Xinjiang Uygur Autonomous Region in northwestern China (Liu et al., 2016, 2019). The weather in this region can be extreme, with temperatures varying from  $-29.3^{\circ}\text{C}$  to  $34.7^{\circ}\text{C}$ , annual precipitation of  $<200$  mm, and annual evaporation  $>2,500$  mm (Liu et al., 2016, 2019). Thus, *A. nanus* is an excellent species in which to study the mechanisms underlying drought resistance. Because species in the legume family provide abundant and sustainable food, feed, and industrial materials worldwide, elucidating the abiotic stress resistance mechanisms in *A. nanus* will provide a theoretical basis for enhancing abiotic stress tolerance in other legumes.

In this study, we successfully isolated and characterized a drought response gene (*c195333\_g1\_i1*, NCBI GenBank: AFH89648) from *A. nanus* using rapid amplification of cDNA ends (RACE). The evolutionary history of this gene family in monocots and eudicots is reconstructed, and we renamed it *AnDHN*, the exclusive DHN is isolated from a drought-induced RNA-seq library of *A. nanus*. Moreover, we show that the expression of *AnDHN* is induced by drought stress. Overexpression of *AnDHN* in *Arabidopsis* enhances drought tolerance through increased root length, excellent water retention, and enhanced ROS scavenging. Intriguingly, the promoter of *AnDHN* potentially responds to multiple abiotic stresses and hormones. *AnDHN* is further shown to act as a positive factor against salt and cold stress. Overall, our results will potentially be beneficial to agriculture by introducing genes from resilient legume crops to counter the effects of changing environmental conditions to meet the needs of a growing global human population.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Ammopiptanthus nanus* seeds were collected from Wuqia County ( $39^{\circ}72'N$ ,  $75^{\circ}26'E$ ), Xinjiang Uygur Autonomous Region, China. Seeds were sown in a Murashige and Skoog (MS) solid medium

and germinated in an artificial growth chamber under the following conditions: 16-h light/8-h dark at 25°C. Abiotic stress was applied to 20-day-old seedlings of different periods (0, 6, 12, 18, 24, and 48 h) with 4°C, 20% PEG 8000, and 0.25 M NaCl as our previous study (Liu et al., 2019). In addition, the seedlings were treated with different concentrations of hormones (ABA, 2 μM; IAA, 1 μM; NAA, 2 μM; MeJA, 10 μM, ETH, 50 μM; and GA<sub>3</sub>, 50 μM), according to the previous study (Liu et al., 2019). The different tissues of roots, stem, and leaves were then frozen with liquid nitrogen and conserved at -80°C for RNA isolation.

The wild-type (WT) *Arabidopsis thaliana* (*Arabidopsis*) seeds used for transformation in this study were ecotype “Columbia” (Col-0). The seedlings were grown in a standard growth chamber (60% humidity, and 120 μE m<sup>-2</sup> s<sup>-1</sup> at 22°C) with a 16-h/8-h light/dark cycle.

## RNA and DNA Extraction

For real-time quantitative PCR analysis, three *A. nanus* tissues, such as roots, stems, and leaf materials, were collected for total RNA extraction with a TRIzol reagent (Invitrogen, Carlsbad, CA, United States) as described by Liu et al. (2019). Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, United States) was used for first-strand cDNA synthesis. The genomic DNA was isolated from the young leaf (0.4 cm length) of *A. nanus* with a Plant Genomic DNA kit (Tiangen, Beijing, China). Total RNA and DNA quality was determined using a BioDrop μLITE+ (Harvard Bioscience Shanghai).

## 5', 3' Rapid Amplification of cDNA Ends of AnDHN

Gene-specific primers for *AnDHN* were designed with primer-blast<sup>1</sup> using partial sequence fragment that was obtained from transcriptome sequencing results. The partial cDNA sequence was isolated with a SMART RACE cDNA Amplification Kit (Clontech, San Jose, CA, United States). The cDNA pools for 3' and 5' RACE were generated with the total RNA extracted from leaves of *A. nanus*. The product of reverse transcription was used for outer PCR with the 5' and 3' RACE outer primers (gene-specific primers, GST1 and GST4, respectively), and the first-round products were further used for the inner PCR (gene-specific primers, GST2, GST3, and GST5). The detail sequences of outer and inner primers are described in **Supplementary Table 4**. The final PCR products were purified and cloned into *pGEM-T* vector (Takara, Dalian, China). The positive clones were extracted as recombinant plasmids. More than three independent positive clones were sequenced by Sangon Biotech (Shanghai, China). The full-length cDNA of *AnDHN* was obtained by aligning and assembling to the 5' and 3' sequences with an overlapping fragment using Vector NTI Advance<sup>®</sup> 11.5 software.

## Phylogenetic Analysis

For the phylogenetic tree of DHNs, we obtained multi-species DHN protein sequences from Phytozome 13<sup>2</sup>

(Goodstein et al., 2012) and aligned with Clustal X version v2.1 with default parameters (Larkin et al., 2007). A maximum-likelihood (ML) phylogenetic tree was constructed with IQ-Tree v1.6 using JTT + F + G4 model as suggested by IQ-Tree model test tool (BIC criterion) with 1,000 times of bootstrap replicates, edited and visualized using iTQL<sup>3</sup>. The phylogenetic tree was carried out using a common taxonomy tree application of NCBI<sup>4</sup>, visualized using MEGA5 software (Tamura et al., 2011) and manually optimized for viewing according to recent studies (Puttick et al., 2018).

## Real-Time-qPCR

Real-time-qPCR and data analysis were conducted as described previously by Sun et al. (2020). Gene-specific primers were designed using primer-blast (see text footnote 1) with *AnACTIN* (GenBank: KJ873129.1) and *AtACTIN* as reference. Tissues from five seedlings with the same treatment were pooled for RNA extraction as one biological replicate. Three biological replicates were included for each treatment for one independent experiment, and each sample was amplified in three parallel reactions as technical replicates. PCR was carried out using Real Master Mix (SYBR Green) (Tiangen, Beijing, China) on QuantStudio 7 Flex (Applied Biosystems, Waltham, MA, United States) with 96-well format. The relative expression level was determined according to Sun et al. (2020). The 2<sup>-ΔΔCt</sup> methods were used to calculate the relative expression level of *AnDHN* (Yin et al., 2020).

## Vector Construction and Subcellular Localization

The *AnDHN* coding sequence (CDS) was fused with green fluorescent protein (GFP) driven by *CaMV* 35S promoter. The recombinational fragment was inserted into the *pCAMBIA1302* vector for subcellular localization. After sequenced, the 35S: *AnDHN-GFP* constructions were transformed into *Agrobacterium tumefaciens* GV3101 strain. *Agrobacterium tumefaciens* transformant strains were grown overnight at 28°C in 20 ml yeast extract mannitol broth (YEB) medium plus selective antibiotics, collected by centrifugation, and resuspended in infiltration medium (2 M MgCl<sub>2</sub>, 0.2 M MES, and 100 mM acetyluegenone, PH = 5.7) until the optical density (OD) value was 0.5–1.0. About 5-week-old *Nicotiana benthamiana* leaves were infiltrated with the bacterial cell suspensions and the plasma marker PAD62. GFP fluorescence signals were observed and documented under a confocal laser-scanning microscope (Olympus Fluoview Ver. 2.0c Viewer) after 48 h in the dark. This experiment was repeated for three biologicals, and each assay was performed with three *N. benthamiana* leaves.

## Isolation of the AnDHN Promoter

The *AnDHN* promoter was isolated from *A. nanus* genomic DNA using genome walking as described by Liu et al. (2019). For PCR reaction, the *AnDHN* gene-specific primers were designed according to the sequence of *AnDHN* full-length cDNA.

<sup>1</sup><https://www.ncbi.nlm.nih.gov/tools/primer-blast>

<sup>2</sup><https://phytozome-next.jgi.doe.gov/>

<sup>3</sup><https://itol.embl.de/>

<sup>4</sup><https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi>

Putative functional *cis*-acting elements (CRE) of the *AnDHN* promoter were identified by the PlantCARE database<sup>5</sup>, and the transcription factor-binding sites (TFBSs) were predicted by JASPAR<sup>6</sup> (Wasserman and Sandelin, 2004).

## Arabidopsis Transgenic Lines Isolation

The CDS was driven by the *CaMV* 35S promoter. The recombinational fragment was inserted into the *pCAMBIA3301* vector for genetic transformation. Arabidopsis transformation was performed by the floral dip procedure (Clough and Bent, 1998). The seeds were collected from the infiltrated plants and selected on a half-strength MS medium containing 50 µg/ml of hygromycin. Hygromycin-resistant plants were transferred to soil 10 days after germination. The progeny of three lines with 3:1 segregation was further treated with hygromycin reagents to screen for the homozygous single insertion. Finally, 10 independent overexpressed T<sub>3</sub> lines were obtained. Three representative T<sub>3</sub> independent lines (*AnDHN#1*, *AnDHN#2*, and *AnDHN#3*) were used for subsequent scoring phenotypes.

## Abiotic Stress Tolerance Assays Statistical Analysis

For seeds germination assay, seeds of WT and *AnDHN* transgenic lines were germinated under vernalization conditions (4°C) for 3 days. Then, seeds were on a half-strength MS solid medium containing different concentrations of mannitol (0, 200, 300, and 400 mM) and NaCl (0, 100, 150, and 200 mM). To assess cold tolerance, seeds were sown in a normal half-strength MS solid medium and vernalize treatment for 2 days. Then, the samples were transferred to a low-temperature artificial growth chamber at 4°C for 4 days. And then the seeds were transferred to another growth chamber at 22°C for 5 days. Germination rates were counted for different abiotic stress for three biological replicated assays. For every biological replicate, we tested at least 30 seeds of each line from the same batch three times as one technical replicate.

For the root growth experiment, seeds of WT and *AnDHN* transgenic lines were transferred to a constant temperature incubator at 22°C for 4 days after vernalization. Then, these seeds further on a half-strength MS solid medium containing mannitol (0, 300 mM) and NaCl (0, 150 mM) were grown in a chamber with a condition (16/8 h d/night at 22°C) for 7 days. Meanwhile, another part of the seeds was also sown on a normal half-strength MS solid medium has grown in a chamber with a condition (16/8 h d/night at 4°C) for 7 days. The root length of seedlings of WT and *AnDHN* transgenic lines was collected and measured. Average values were calculated from three biological replicates. And for every biological replicate, we tested six seedlings from the same batch.

Ion leakage (IL) was according to Sakuraba et al. (2014) with minor modifications. The leaves were placed in a tube with 25 ml of double-distilled water for 2 h, and then the initial conductivity (C1) was detected. After heated in boiling water for 15 min and cooled until to room temperature, the

final electrolyte conductivity (C2) was detected. Hence, the relative IL (%) =  $C1/C2 \times 100\%$  was obtained. At least three independent biological replicates were analyzed for each line, and no less than 10 seedlings were collected as one sample for each biological replicate.

Relative water content was calculated according to the previous study described in Vysotskaya et al. (2010) with minor modifications. Fresh weight (FW) was measured, and the leaves were in double-distilled water for 4 h in darkness at room temperature. The turgid weight (TW) further was recorded. For dry weight (DW), the leaves were dried at 80°C for 24 h. Relative water content (RWC) equation,  $RWC (\%) = [(FW - DW)/(TW - DW)] \times 100\%$ . Three biological replicates were used for each line, and at least 10 seedlings were collected as one sample detected for each biological replicate.

Malondialdehyde (MDA) content was detected using a maleic dialdehyde assay kit (A003-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Three biological replicates for each sample and at least 10 seedlings were collected as one sample for each biological replicate.

The ROS scavenging enzymes activities of PRO content, catalase (CAT) activity, superoxide dismutase (SOD) activity, peroxidase (POD) activity, and glutathione (GSH) content were detected with kits also produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Three biological replicates for each sample and at least 10 seedlings were collected as one sample as one biological replicate.

For histochemical staining assays, the leaves were collected from 2 to 3-week-old seeding of WT and *AnDHN* transgenic lines treated with mannitol (300 mM), NaCl (150 mM) and 4°C. The histochemical staining assays were conducted with 0.2% nitro-blue tetrazolium (NBT) and 1 mg/ml 3,3'-diaminobenzidine (DAB) solutions with vacuum infiltration for three times. The leaves were kept for 12 h in NBT and DAB solutions. Then, the leaves were decolorized by boiling in 75% ethanol. Images were visualized using stereomicroscopy (SZX16, Olympus).

These experiments were repeated at least three times. All of the statistical analyses were performed using IBM SPSS statistics 19. Significant variation was estimated with Student's *t*-test, \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001. The data were presented as the mean ± SD of three independent experiments.

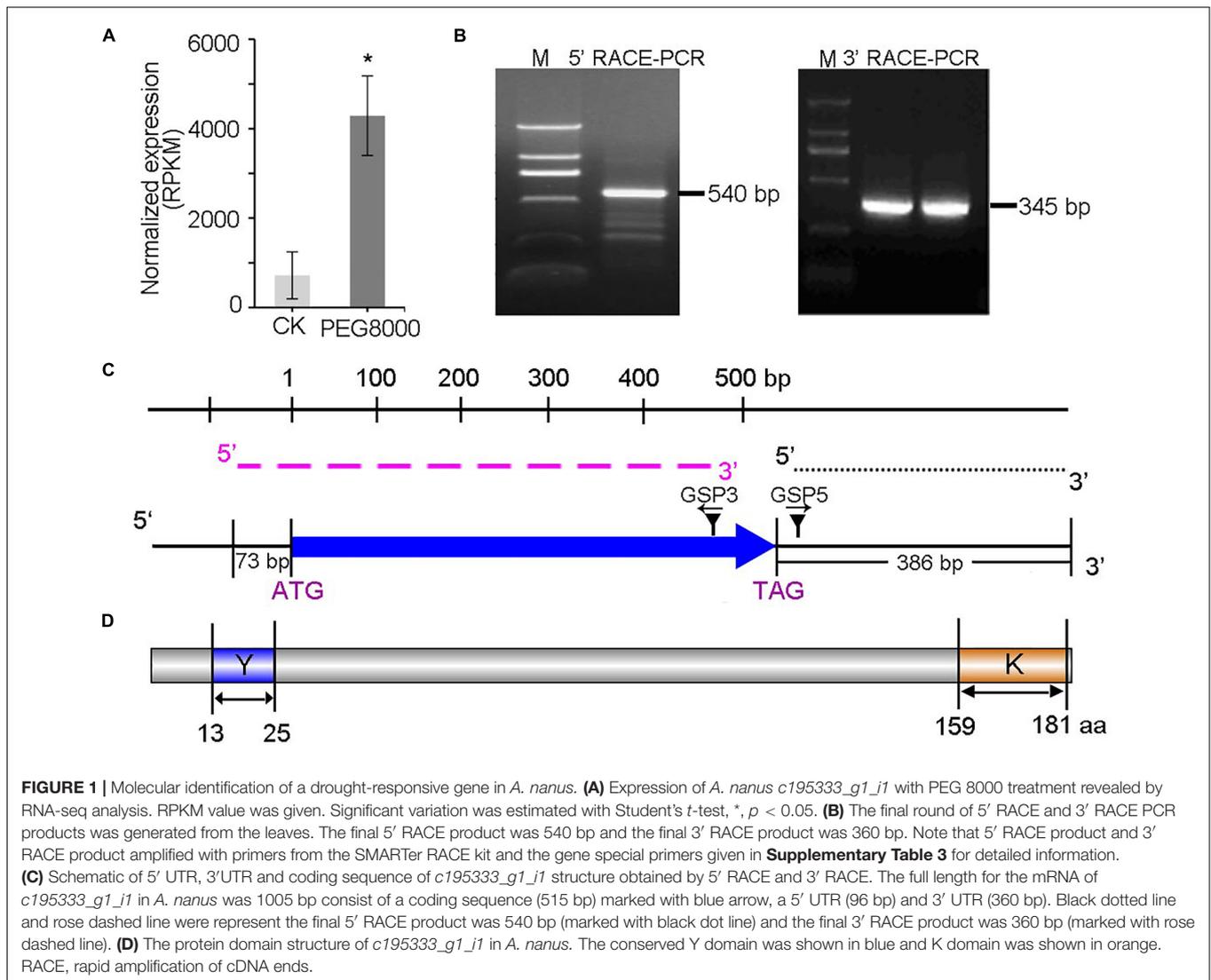
## RESULTS

### A Drought-Responsive Gene in *A. nanus*

To identify the key regulator of drought resistance in *A. nanus*, a significantly upregulated candidate gene (*c195333\_g1\_i1*) (NCBI GenBank: AFH89648) was obtained from an RNA-seq library prepared from drought-induced *A. nanus* seedlings (Figure 1A and Supplementary Table 1). Lacking genomic information, the full-length sequence of this gene was isolated using 5' and 3' RACE. The gene-specific primers were designed from the partial sequence obtained from the RNA-seq data for degenerate RT-PCR (Figure 1C). As a result, the final 5'- and 3'-fragments were amplified through three and two rounds, respectively (Figure 1B and Supplementary Figure 1A), and the final PCR products

<sup>5</sup><http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>

<sup>6</sup><http://jaspar2016.genereg.net/>



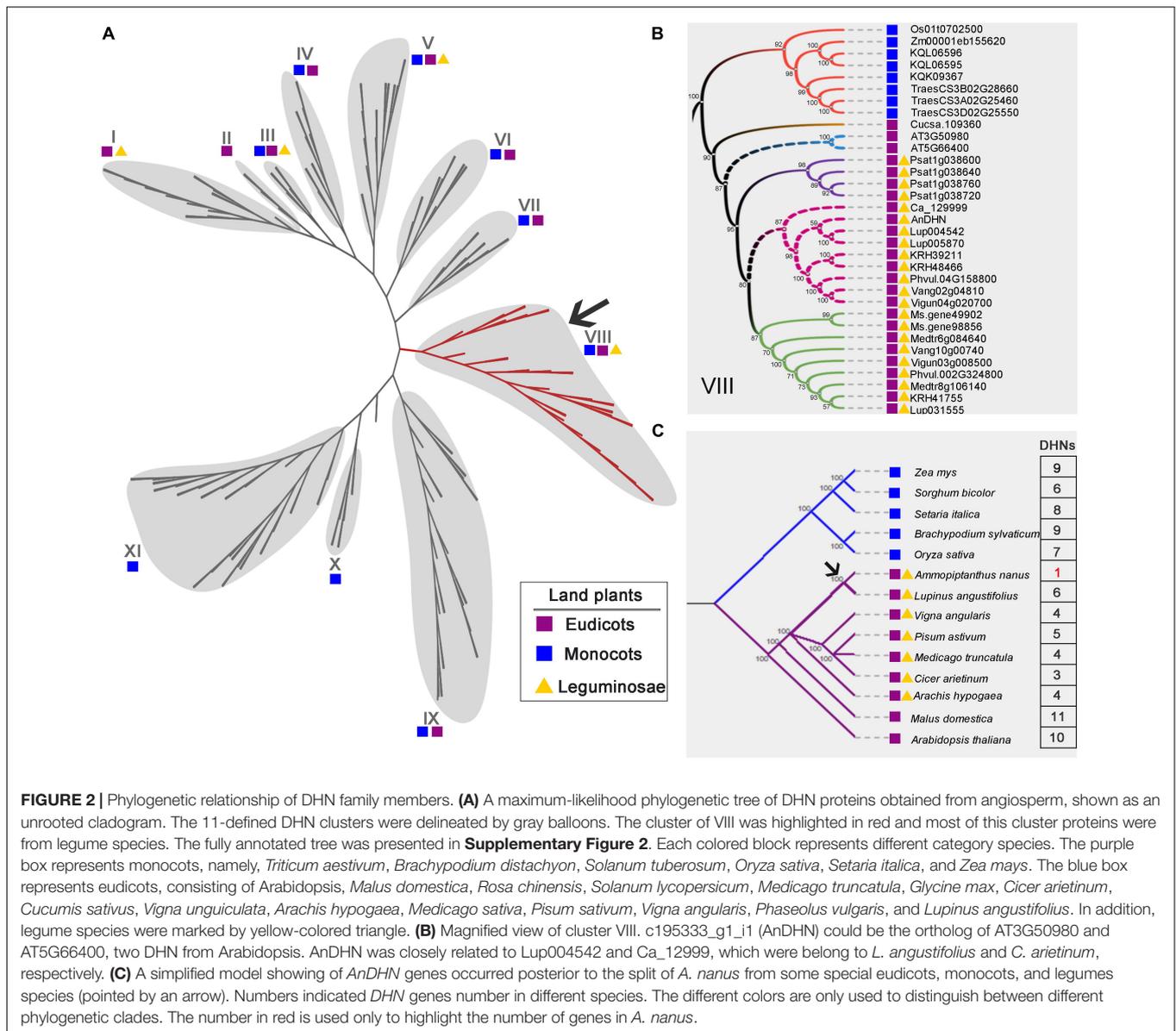
were sequenced. Both the final 5'- and 3'- fragments (540 and 345 bp, respectively) were aligned, and the coding sequence was determined to be 543 bp in length, encoding a putative protein of 181 amino acids (**Figure 1D**). The final obtained 5' UTR and 3' UTR sequences were 73 and 386 bp, respectively. And there is no intron and only one exon exists (**Figure 1C**). The protein had a predicted molecular weight of 18,125.87 MW and a theoretical pI of 5.74. Using the online servers NCBI-CDD and SMART, two conserved motifs, called Y- (DEYGNPV) and K- (KKGIMNKIKEKLPY) segments, were identified (**Figure 1D**), which might play key roles in AnDHN protein. These results demonstrate that *c195333\_g1\_i1* is a new drought-responsive gene from *A. nanus*.

## Evolution of the Dehydrin Proteins

Because of its adaptation to extreme environmental conditions, *A. nanus* is an excellent model species in which to study the drought and cold tolerance in legumes and other species. To further understand the evolutionary history of *c195333\_g1\_i1*,

we constructed a phylogenetic tree. The ML phylogenetic tree was derived from an alignment of 189 DHN sequences from 23 species of both monocots and eudicots (**Figure 2A** and **Supplementary Figure 2**). Based on our results, the gene from *A. nanus* encoding a protein could be orthologous of the proteins encoded by AT3G50980 (XERO1) and AT5G66400 (RAB18) (**Figures 2A,B** and **Supplementary Figure 2**). XERO1 and RAB18 are known as DHN proteins from *Arabidopsis*. Among them, RAB18 is the protein extensively studied, which is widely reported involved in cold tolerance and drought tolerance of plants (Lång and Palva, 1992; Zou et al., 2021).

Previous studies have focused only on the structural types of DHN proteins, with little consideration given to the evolutionary relationships between the DHN family members. Our results show that the DHN family is widely distributed in angiosperms (**Figure 2A**). Among these, all of the DHN family members from legume species were clustered in subfamilies I, III, V, and VIII, with most found in subfamily VIII (**Figures 2A,B**). We identified AnDHN in *A. nanus* as being a protein closely related

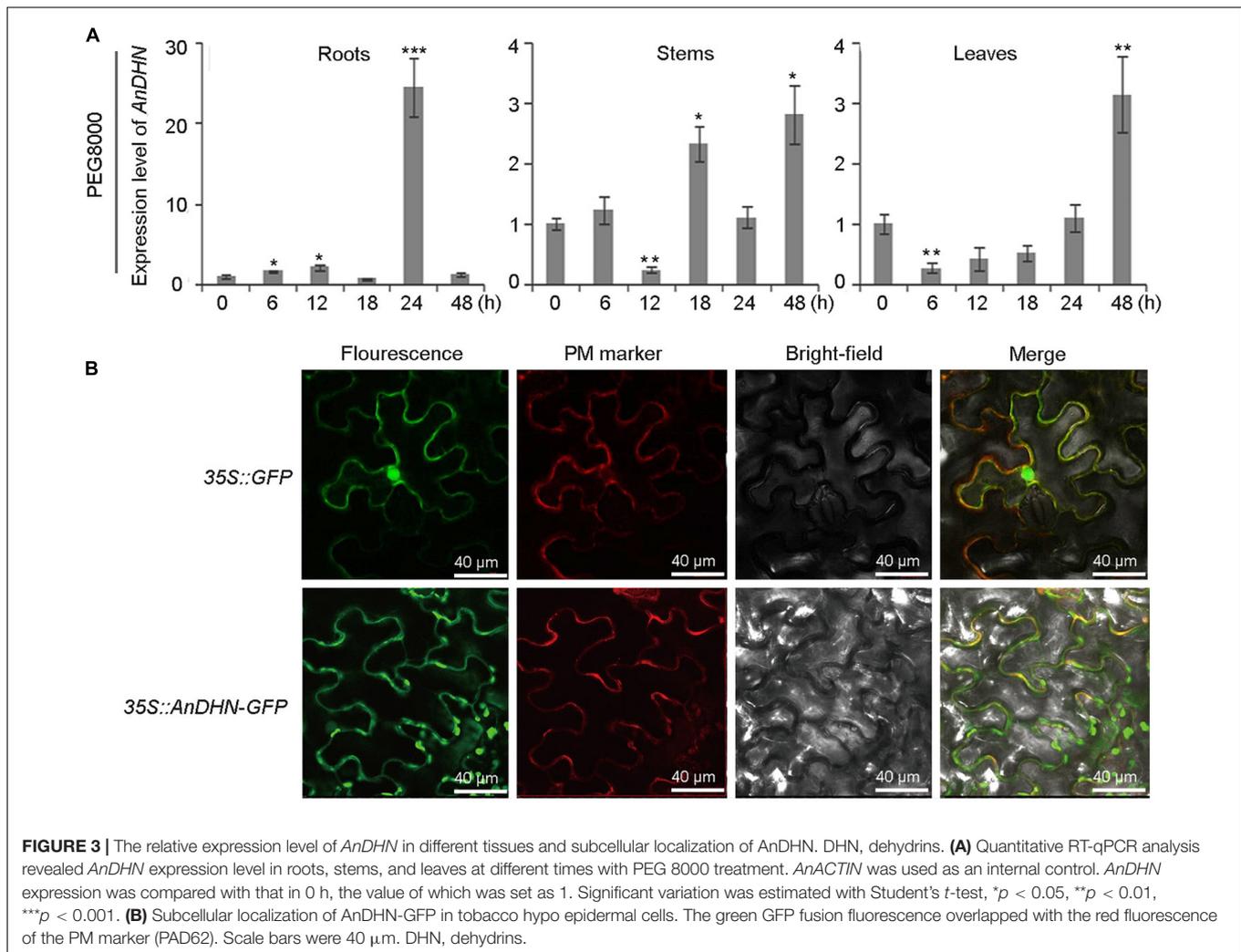


to Lup004542 and Ca\_12999 in legumes (**Figure 2B**). A multiple sequence alignment showed that AnDHN harbors conserved K- and Y-segment domains similar to AT3G50980, AT5G66400, Lup004542, and Ca\_12999 (**Supplementary Figure 3**), consistent with our bioinformatic prediction (**Figure 1D**). The sequences of predicted DHN proteins from *Zea mays*, *Sorghum bicolor*, *Setaria italica*, *Brachypodium sylvaticum*, *Oryza sativa*, *A. nanus*, *Lupinus angustifolius*, *Vigna angularis*, *Pisum sativum*, *Medicago truncatula*, *Cicer arietinum*, *Arachis hypogaea*, *Malus domestica*, and *Arabidopsis* were further used in phylogenetic reconstruction (**Figure 2C**). Interestingly, all of the monocot species had more than six DHN proteins, and the genomes of the eudicot species *Arabidopsis* and apple contain at least 10 DHN proteins of different coding for each. However, the number of *DHN* genes in legume species ranged from three to six, except for *A. nanus*, which lacks genomic information. Our findings suggest that

DHNs are evolutionarily conserved in multiple species, which is consistent with previously reported (Close, 1997; Svensson et al., 2002; Rorat et al., 2006).

### Expression Pattern of AnDHN in Response to Drought Stress and Subcellular Localization of AnDHN

We next conducted a comprehensive set of experiments to functionally analyze AnDHN for its potential roles in simulated drought stress. The temporal expression patterns of *AnDHN* were analyzed in roots, stems, and leaves from 0 to 48 h of PEG 8000 treatment. Our results further confirmed that *AnDHN* is a drought-responsive gene (**Figure 3A**). Our data show that *AnDHN* expression in roots was significantly upregulated after 6 h of drought treatment and that expression peaked

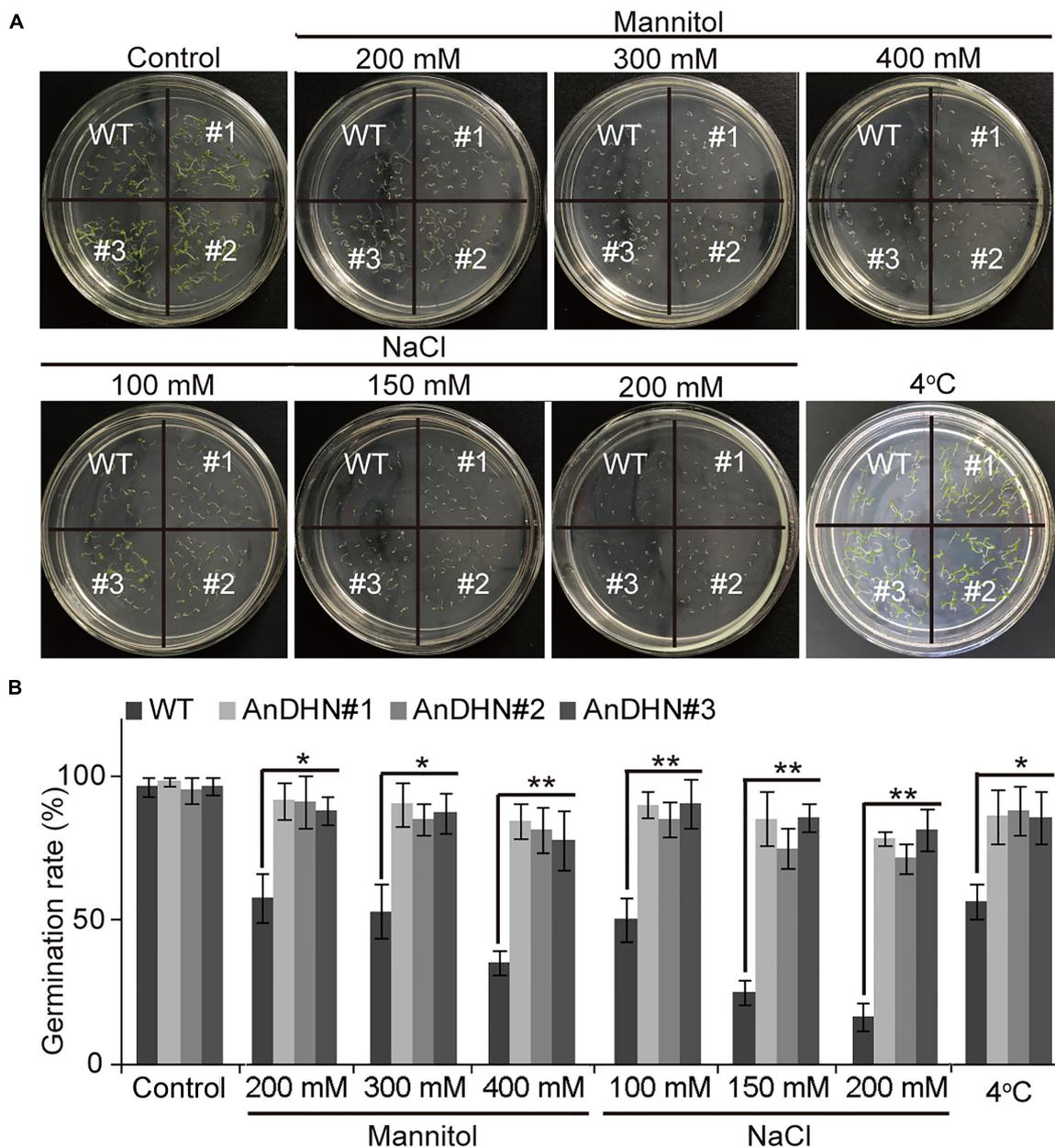


at 24 h of PEG 8000 treatment. Unlike roots, *AnDHN* expression was increased by 2.8-fold and 3.1-fold at 48 h compared to the 0 h control treatment in stems and leaves, respectively (Figure 3A). To determine why *AnDHN* expression responds to drought stress, we obtained the *AnDHN* promoter region, which was 830 bp in length, by genomic walking (Supplementary Table 2). Several types of drought-induced *cis*-acting elements were identified from the PlantCARE and JASPAR databases as expected (Supplementary Figure 4 and Supplementary Table 3). Unexpectedly, we also identified numerous cold and salt-induced elements in the promoter of *AnDHN* (Supplementary Figure 4 and Supplementary Table 3). To test our predictions, the expression pattern of *AnDHN* was analyzed after following cold and salt stress treatments. *AnDHN* expression was markedly upregulated at 12 h in the roots, 24 h in stems, and within 24 h in leaves exposed to salt stress (Supplementary Figure 5). In response to cold (4°C) treatments, *AnDHN* expression was dramatically different from the 0-h control at most times during the 48-h experiment in the roots, stems, and leaves (Supplementary Figure 5).

To determine the subcellular localization of the AnDHN protein, the AnDHN-GFP fusion was transiently expressed in *N. benthamiana* leaf epidermal cells. The result showed that GFP fluorescence overlaps with the plasma membrane (PM) marker, indicating the AnDHN protein is localized at the PM (Figure 3B).

### Overexpression of *AnDHN* Enhanced Drought Tolerance by Increased Germination Rate and Deep Root System Architecture

To further verify whether *AnDHN* is related to drought stress, *AnDHN* was driven by the 35S promoter, and 10 overexpressions of *AnDHN* transgenic lines were generated in *Arabidopsis*. Among these lines, we chose three representative independent lines for further analysis (abbreviated as *AnDHN*#1, *AnDHN*#2, and *AnDHN*#3), and *AnDHN* had a relatively high expression level in these lines (Supplementary Figure 6). Germination rates in the transgenic plants showed no differences from the WT plants under normal conditions (Figures 4A,B). However, we observed higher germination rates in seeds from transgenic



**FIGURE 4 |** The germination rate of *AnDHN* overexpression transgenic lines. **(A)** Germination rate of the WT seedling and *AnDHN*-OX seedlings on half-strength MS medium treatment with or without 200, 300, and 400 mM mannitol; 100, 150, and 200 mM NaCl; 4°C. The average was calculated for three biological replicates and each line of seeds was pooled from more than 30. **(B)** The germination rate was calculated by ANOVA. Values were means  $\pm$  SD. Significant differences based on Student's *t*-test \* $p < 0.05$ , \*\* $p < 0.01$ . DHN, dehydrins.

lines compared to seeds from WT plants at three different concentrations of mannitol (Figures 4A,B). As expected, the germination rate of WT seeds was decreased in the presence of mannitol compared to that of WT seeds without mannitol (Figures 4A,B). This may indicate that the germination rate was related to drought stress.

Under drought stress, a deep root system architecture is beneficial and allows plants to adapt to adverse living conditions (Wei et al., 2020; Yang et al., 2020; Zhou et al., 2020).

To confirm this, we compared the root length of WT and overexpressed plants under normal conditions and 300 mM mannitol treatment. As the result, an insignificant difference can be visualized between WT plants and overexpressing plants grown under normal conditions except for *AnDHN#1*, and there is a significant difference between the WT plants and *AnDHN#1* (Figures 5A,B). However, we found that all three transgenic lines displayed markedly increased root length when grown on a medium containing 300 mM mannitol, especially for *AnDHN#1*

(Figures 5C,D). Thus, our data suggest that overexpression of *AnDHN* enhances drought tolerance by increasing the germination rate and the deep root system architecture.

### **AnDHN-OX Plants Enhanced Drought Resistance With Excellent Water Retention, Increased Osmotic Adjustment, and Enhanced Reactive Oxygen Species Scavenging**

Under drought stress, plants that are capable of physiological change will be able to adapt to changeable environments. We therefore investigated two physiological indexes, IL and relative water content (RWC), to investigate water retention in the plants. We found a slight decline in electrical conductivity under drought stress (Figure 6A). However, all three independent *AnDHN-OX* transgenic lines retained much more water than did the WT plants under drought stress (Figure 6B). Our results suggest that overexpression of *AnDHN* in *Arabidopsis* can enhance drought tolerance by increasing water retention.

Proline is a proteinogenic amino acid that contributes to osmotic adjustment in drought tolerance. Our assays confirmed that, when treated with 300 mM mannitol, proline accumulated to higher levels in the *AnDHN-OX* transgenic plants than in WT plants, indicating that *AnDHN-OX* plants have increased drought tolerance (Figure 6C). We next measured the MDA contents, because MDA is an important indicator of damage of cellular membranes (lipid peroxidation) caused by abiotic stresses. The MDA contents in *AnDHN-OX* lines were significantly lower than in WT plants under drought treatment (Figure 6D) showing that increased expression of *AnDHN* relieves damage to the cellular membranes.

Excess ROS ( $H_2O_2$  and  $O_2^-$ ) production can damage cellular membranes (Feng et al., 2020a), and the ROS levels are strictly controlled by antioxidant enzymes. Therefore, we also determined the activities of POD, CAT, SOD, and GSH that are responsible for ROS scavenging. As shown in Figures 6E–H, the activities of these indicator enzymes, except for SOD, were increased in the transgenic lines in response to drought stress (Figures 6E–H). Nitro blue tetrazolium (NBT) and DAB staining were performed to detect  $H_2O_2$  and  $O_2^-$ , respectively. We observed only slight staining in the leaves of the WT and *AnDHN-OX* plants grown under normal conditions, with no visible differences between them (Figures 7A,B). Under drought stress, however, weaker staining was found in the leaves of the *AnDHN-OX* lines compared with the WT plants (Figure 7). These results indicate that *AnDHN-OX* plants had increased ROS scavenging capability in response to drought stress.

### **AnDHN Simultaneously Enhanced Plant Tolerance to Salt and Cold Stress**

In our previous study, we found that *AnDHN* expression could also be induced by both salt and cold stress (Supplementary Figure 5). Moreover, the promoter of *AnDHN* contains salt and cold stress-induced *cis*-acting elements (Supplementary Figure 4 and Supplementary Table 3). We next performed experiments to determine whether *AnDHN* affects salt and

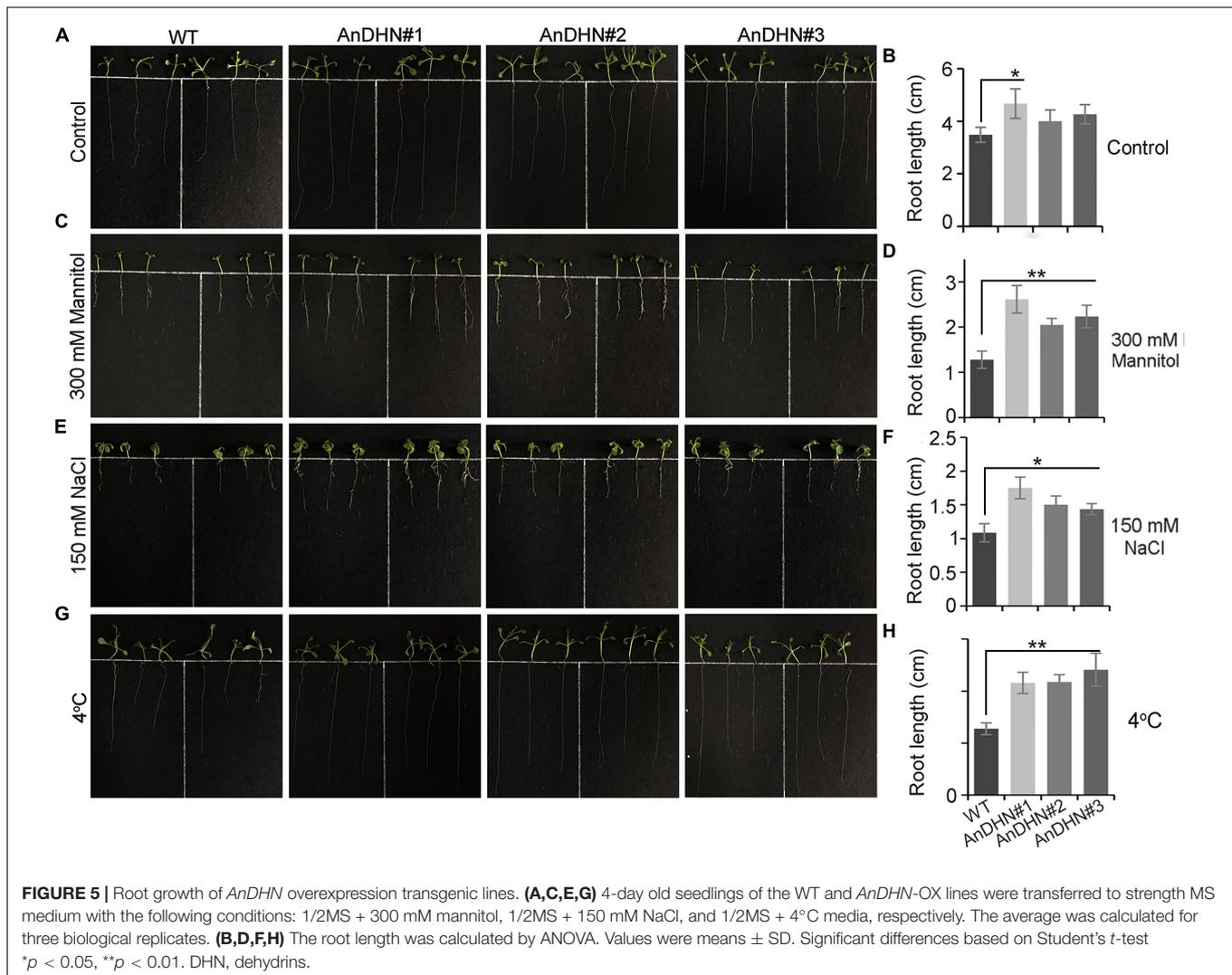
cold stress by assaying seed germination rate, root length, IL content, RWC, and the activities of crucial antioxidant enzymes. As expected, our results indeed confirmed our hypothesis that *AnDHN* simultaneously improves plant tolerance to both salt and cold stress. The *AnDHN-OX* lines had relatively higher germination rates and longer root lengths in comparison to the WT under salt and cold stress conditions (Figures 4, 5). Furthermore, the RWC and proline contents and POD and GSH activities were significantly higher in *AnDHN-OX* line plants under salt stress (Figures 6B,C,E–H). The concentrations of IL, proline, and the POD, CAT, and SOD activities in the *AnDHN-OX* lines were higher than in the controls in response to cold stress (Figures 6A,C,E–G). In addition, there was less DAB and NBT staining in the overexpression lines relative to that in the WT plants (Figure 7). These results show that *AnDHN* also has the potential to enhance salt and cold tolerance in plants.

## **DISCUSSION**

### **AnDHN Improves Drought Tolerance via Abscisic Acid**

Although DHNs are predicted to be stress-related proteins (Nylander et al., 2001; Kosová et al., 2007; Peng et al., 2008; Kim and Nam, 2010; Shekhawat et al., 2011), the study of DHN functions in many species is still in its infancy. Previous studies have been focused on analyses of the putative promoters of *DHN* genes (Zhu et al., 2014; Liu et al., 2015; Aguayo et al., 2016). All of these studies showed that the expression of *DHN* genes is upregulated in response to multiple hormones and abiotic stresses (Zhu et al., 2014; Liu et al., 2015; Aguayo et al., 2016). Our results show that *AnDHN* positively improves drought tolerance through morphological and physiological changes in *A. nanus*.

Abscisic acid can improve stress tolerance in plants by allowing them to adapt to changeable environments (Zhu, 2016). Previous studies have shown that ABA regulates the expression of many downstream genes in response to drought stress with morphological and physiological changes, such as deep root system architecture, stomatal closure, and enhanced ROS scavenging (Ullah et al., 2017; Xing et al., 2019; Gupta et al., 2020). Also, ABA-dependent signaling pathways play a critical role in the response to drought stress (Ullah et al., 2017). Our data demonstrate that *AnDHN* is a membrane protein (Figure 3B), suggesting that *AnDHN* could be an upstream gene involved in drought tolerance. Moreover, previous studies in rice showed that overexpression of *OsDhn-Rab16D* modulated the expression patterns of ABA signaling genes to alter endogenous ABA concentrations (Tiawari et al., 2019). Thus, we can also hypothesize that *AnDHN* indirectly changes the expression of ABA biosynthesis genes or ABA signaling genes to alter physiological responses and the expression of numerous downstream tolerance genes under drought stress in *A. nanus*. To test this hypothesis, we also detected the expression of ABA biosynthetic and catabolic genes in the WT plants and *AnDHN* overexpressed lines. The results showed that the expression levels of *AtNECD3* and *AtNECD5* (two ABA biosynthetic genes) were significantly upregulated, while the expression levels of



*AtCYP707A1* and *AtCYP707A3* (two ABA catabolic genes) were downregulated (**Supplementary Figure 7**). Interestingly, our results also show that *AnDHN* expression is induced by ABA (**Supplementary Figures 4, 8** and **Supplementary Table 3**), indicating that there might be an AnDHN-ABA loop to improve drought tolerance in *A. nanus*.

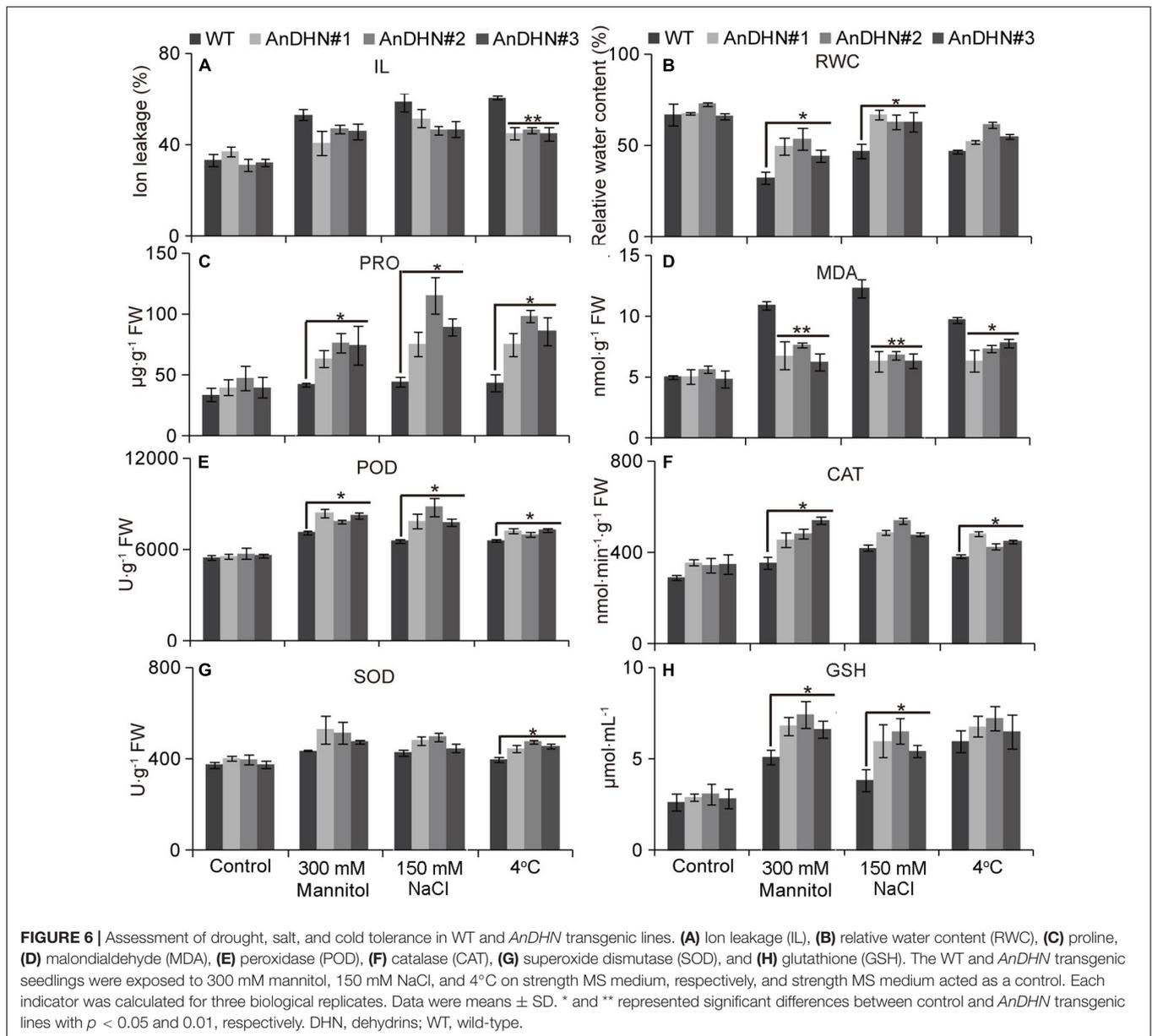
## Subcellular Localization of Dehydrin Proteins

Previous studies have shown that DHN proteins are localized in the cytosol, nucleus, mitochondria, vacuole, and vicinity of the PM (Houde et al., 1995; Danyluk et al., 1998; Rorat et al., 2004). The localization of the DHNs in cells may depend on the existence or deficiency of the Y-, S-, and K- segments (Graether and Boddington, 2014). YnKn, YSK, and Kn types of DHN proteins have been found to localize to the nucleus and cytoplasm (Wisniewski et al., 1999; Lin et al., 2012). SKn type of DHNs is found to localize in the vicinity of the PM (Danyluk et al., 1998; Hara et al., 2003). Y-segment has shown no connection with the

localization of DHN proteins. In our research, we found that the AnDHN protein was localized at the PM (**Figure 3B**). According to the role in this study and the previous reports (Hara et al., 2003; Yang et al., 2014), we guessed that it could play a key role in protecting lipid membranes against peroxidation under drought and freezing stress.

## Dehydrins Are Evolutionarily Conserved in the Abiotic Stress Response in Angiosperms

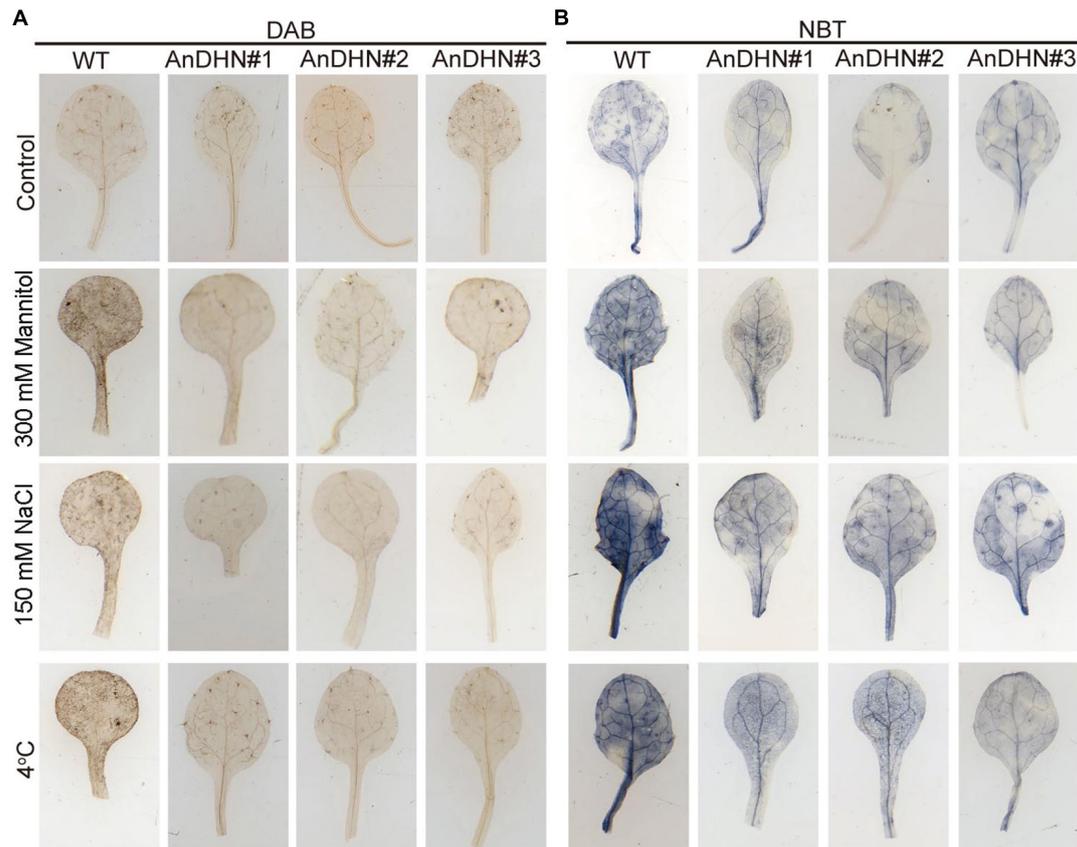
Dehydrin proteins are reported to be involved in responses to various abiotic stresses in plants (Hundertmark and Hincha, 2008; Xu et al., 2014; Zhu et al., 2014). In our study, we also showed that overexpression of *AnDHN* in *Arabidopsis* mediates the effects of salt and cold stress (**Figures 4–7** and **Supplementary Figure 5**), in addition to its effect on drought stress. Some hormones play critical roles in abiotic stress tolerance, and various genes that are involved in regulating abiotic stress tolerance are induced by multiple hormones



(Zhu et al., 2014; Liu et al., 2019; Xing et al., 2019). Changes in *AnDHN* expression following treatment with ABA, Indole-3-Acetic acid (IAA), 1-Naphthyl acetic acid (NAA), Jasmine (keto) acid methyl ester (MeJA), ethylene (ETH), and GA<sub>3</sub> are shown in **Supplementary Figure 8**, consistent with previous results in *A. nanus*, *Cucumis melo* var. *makuwa* Makino, and *Triticum aestivum* (Zhu et al., 2014; Liu et al., 2019; Xing et al., 2019). This suggests that *AnDHN* may play an active role in abiotic stress responses in *A. nanus*. *Cis*-acting elements in promoters that affect gene expression in response to abiotic stress have been widely studied (Yamaguchi-Shinozaki and Shinozaki, 2005; Zhu et al., 2014; Liu et al., 2019). We identified many hormone-inducible elements in the *AnDHN* promoter that respond to MeJA, IAA, and ABA in addition to drought, cold, and salt (**Supplementary Figure 4** and **Supplementary Table 3**).

Moreover, the promoter harbored a lot of TFBSs of abiotic stress and hormones, simultaneously (**Supplementary Table 3**). Thus, our results imply that *AnDHN* expression depends upon the presence of these promoter elements to respond to abiotic stresses.

To the best of our knowledge, the DHN family has multiple members in most species; for example, there are four and 10 *DHN* genes in *Eucalyptus globulus* and *Arabidopsis*, respectively (Fernández et al., 2012; Aguayo et al., 2016). Phylogenetic analysis of DHN proteins from dicots and monocots showed that there are at least three members in each of the species examined, although only a single gene was identified in *A. nanus* (**Figure 2** and **Supplementary Figure 2**). Furthermore, *AnDHN* was isolated from a drought-induced RNA-seq library of *A. nanus*. Hence, we speculate that there may be more than one *DHN* gene in



**FIGURE 7** | Reactive oxygen species (ROS) accumulation in WT and *AnDHN* transgenic lines under drought, salt, and cold stress. **(A,B)** DAB and NBT staining for detecting  $H_2O_2$  and  $O_2^-$  levels in leaves of WT and *AnDHN* transgenic lines treatment with 300 mM mannitol, 150 mM NaCl, and 4°C, respectively. Experiments are repeated three times with similar results and one representative result was shown. DHN, dehydrins.

*A. nanus*, which would be consistent with other species. DHN acts as the pivotal regulator in the response to various abiotic stresses in *Eriobotrya japonica*, *Eucalyptus globulus*, *Oryza sativa*, and *Arabidopsis* (Fernández et al., 2012; Kumar et al., 2014; Xu et al., 2014; Aguayo et al., 2016). We further demonstrated that AnDHN positively regulates drought stress tolerance and tolerance to salt and cold (Figures 3–7). Phylogenetic analysis and sequence alignment revealed that DHN proteins are highly conserved in angiosperms (Figure 2 and Supplementary Figure 3). Taken together, these results indicate that DHNs appear to define a class of proteins that regulate abiotic stress tolerance in all angiosperms.

Apart from the function of stress, LEA family proteins may also play key roles in the growth and development of plants. Unfortunately, research on LEA function is limited by their proteins with structural flexibility and lacking similarity with other known proteins. It will be a hotspot to explore the association between their structural types with different levels of water deficiency based on the previous studies of LEA proteins (Battaglia and Covarrubias, 2013). Moreover, numerous LEAs are detected in developmental root hairs, suggesting that they have the potential for water absorption and nitrogen fixation in symbiotic association rhizobium-legumes

(Battaglia and Covarrubias, 2013). The research on the function of LEA proteins is not enough except for the stress response, and it will be worthwhile to investigate in the future.

### Further Perspectives of Drought Stress

*Ammopiptanthus nanus* is a non-model plant that grows in arid desert habitats in central Asia. Plants can experience conditions, such as very low rainfall, extremely high evaporation, and abnormally high and low temperatures (Liu et al., 2016, 2019). Because it is able to survive in such extreme environments, *A. nanus* has great research potential for elucidating drought and cold tolerance mechanisms that could be applied to other species. Our findings shed light on the control of drought resistance by DHN in *A. nanus*. However, the mechanisms underlying drought resistance are still largely unknown in *A. nanus* and also in other legume species.

In the future, reduced crop yields and quality loss due to drought stress will be more serious (Ullah et al., 2017). Hence the drought tolerance mechanism remaining needs to gain better exploitation. (1) From both model and non-model species, more genes responsible for drought tolerance need to be identified and characterized by genetic variation, transcriptomic, gene editing, and so on. Abiotic stress is a complicated trait. As drought often

couples with others stress, pleiotropic genes are excellent. (2) Stomatal closure often with carbohydrate synthesis is reduced, how to coordinate with growth and drought stress is important. (3) Research studies of plant responses to drought are mostly studied from plants grown in the laboratory greenhouse, not natural. We need to accelerate the application of our results into natural water-deficit conditions for agriculture.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

LC conceptualized the project, had overall responsibility for this project, such as project ideas, guidance on experimental design, data analysis, manuscript writing, and revision, and took part in the project administration and funding acquisition. YS, LL, and SS carried out the laboratory work and data analysis. YS wrote the first draft of the manuscript with the help of XZ. MI helped to review and edit the manuscript. LZ has been involved in critically revising the manuscript for important intellectual content. All authors have read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.788938/full#supplementary-material>

**Supplementary Figure 1 |** The transcript of *AnDHN*. DHN, dehydrins. **(A)** Detection of the 5' and 3'-terminus of the transcript of *AnDHN* by 5' and 3'-RACE. The gene special primers were given in **Supplementary Table 4** for detailed information. **(B)** The complete transcript of *c195333\_g1\_i1* was obtained by 5'

and 3' RACE. The start and stop codons were labeled in red. DHN, dehydrins; RACE, rapid amplification of cDNA ends. The bold sequence represents the coding sequence of *AnDHN*.

**Supplementary Figure 2 |** A maximum-likelihood phylogenetic tree of DHNs. The generated dataset consists of 23 species and 189 DHN proteins. DHN, dehydrins.

**Supplementary Figure 3 |** Amino acid sequence alignments with closely related DHNs. DHNs from *A. nanus* and closely related species, namely, *Arabidopsis*, *C. arietinum*, *L. angustifolius*, *G. max*, *P. vulgaris*, *V. angularis*, and *V. unguiculata*. The Y-domain and K-domain were underlined in blue and brown lines, respectively. DHN, dehydrins. The black and gray highlights represent the conservative interval of the protein of *AnDHN*. The black highlight represents the sequence identified more than 75% and the gray highlight represents the sequence identified more than 50%.

**Supplementary Figure 4 |** Predicted *cis*-regulatory-elements (CREs) in the 830 bp promoter regions of *AnDHN*. DHN, dehydrins.

**Supplementary Figure 5 | (A–C)** Quantitative RT-qPCR analysis revealed *AnDHN* expression level in roots, stems and leaves in different times under 150 mM NaCl treatments. **(D–F)** Quantitative RT-qPCR analysis revealed *AnDHN* expression level in roots, stems and leaves in different times under 4°C treatments. *AnACTIN* was used as an internal control. *AnDHN* expression was compared with that in 0 h, the value of which was set as 1. Significant variation was estimated with Student's *t*-test \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ .

**Supplementary Figure 6 |** Expression of *AnDHN* in the overexpression lines. RNA was isolated from 10-day-old seedlings. *AtACTIN* gene was used as an internal control. Significant variation was estimated with Student's *t*-test of three independent biological replicates. \*\*\*,  $p < 0.001$ .

**Supplementary Figure 7 |** The relative expression level of ABA biosynthetic and catabolic genes of WT and *AnDHN* overexpression lines. RNA was isolated from 10-day-old seedlings. *AtACTIN* gene was used as an internal control. Significant variation was estimated with the Student's *t*-test of three independent biological replicates. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . WT, wild-type; DHN, dehydrins.

**Supplementary Figure 8 |** Quantitative RT-qPCR analysis revealed *AnDHN* expression level in leaves in different times. **(A–F)** Were used ABA, NAA, IAA, MeJA, ETH, GA<sub>3</sub> treatment, respectively. *AnACTIN* was used as an internal control. *AnDHN* expression was compared with that in 0 h, the value of which was set as 1. Significant variation was estimated with Student's *t*-test, \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ .

**Supplementary Table 1 |** Expression level of *c195333\_g1\_i1* in *A. nanus* under drought treatment (RPKM).

**Supplementary Table 2 |** List of the TFBSs and *cis*-acting elements in the 830 bp promoter of *AnDHN*. TFBS, transcription factor-binding site; DHN, dehydrins.

**Supplementary Table 3 |** The 830 bp promoter sequence of *AnDHN*. DHN, dehydrins.

**Supplementary Table 4 |** Primers were used in this study.

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