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Molecular cloning and characterization of a novel SK3-type dehydrin gene from *Stipa purpurea*

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ABSTRACT

Stipa purpurea, an endemic forage of the grass family in the Tibetan Plateau, is highly resistant to drought. Dehydrins (DHNs) are stress proteins involved in plant protective reactions against environmental stress. In this study, the full-length *DHN* open reading frame (ORF) cloned from *S. purpurea*, named *SpDHN1*, was 816 nucleotides length and encoded a protein of 271 amino acids. Phylogenetic and sequence characterization analysis revealed that the *DHN* gene was an SK3-type DHNs. Subcellular localization analysis indicated that *SpDHN1* was localized in the cytoplasm and the plasma membrane. *SpDHN1* function analysis provided new evidence to support the antioxidation of *SpDHN1* in plant responses drought stress. Ectopic expression of *SpDHN1* in *Arabidopsis thaliana* plants showed more resistance to drought stress than the wild-type, indicating that *SpDHN1* may be a potential candidate gene for genetic improvement of crops to improve stress tolerance.

1. Introduction

Plants have evolved various survival mechanisms to enhance their ability to tolerate drought, including stress signal perception and transduction, and the associated molecular regulatory network. Late embryogenesis abundant (LEA) proteins are the main proteins accumulated under water stress [1]. Dehydrins belong to the group 2 LEA protein family. DHN proteins are divided into five subclasses, including Kn, KnS, YnKn, SKn and YnSKn, which based on the number of conserved K-, S- and Y-segment motifs. All DHN proteins contain at least one highly conserved 15 amino acid motif (EKKGIMDKIKEKLPG) forming an amphiphilic α-helix, located at the C-terminal end of the protein [2]. The Y-segment (DEYGNP) shares high homology with the nucleotide binding site motif of chaperone proteins and is located near the N-terminus of the DHN protein. A characteristic of the S-segment is a block of serines and a protein phosphatase binding site at the end of the sequence. The dehydrins are considered as stress proteins involved in formation of plant protective reactions against dehydration. Recently, Saavedra et al. reported that the expression of a dehydrin protein increased significantly in *Physcomitrella patens* in response to abiotic stress [3]. Improvement of the plant response to abiotic stress would enhance plant adaptation mainly because of the function of DHNs, which can protect cells from dehydration, stabilize the cell membrane, eliminate free radicals and bind metal ions or act as molecular chaperones [4].

Stipa purpurea is an endemic but dominant species in the alpine steppe that are widely distributed in the alpine areas of five provinces in China: Xizang, Qinghai, Gansu, Xinjiang and Sichuan [5]. Its extensive distribution means that it plays a very important role in safeguarding soil and water resources, acting as a windbreak and preventing erosion of sand [6]. In addition, *S. purpurea* contains high levels of crude proteins and fats, making it to be an important natural forage resource of major ecological and economic value. The results of other studies suggest that *S. purpurea* is a perennial grass with high resistance to adverse environmental conditions such as cold, drought and strong winds [7]. Here, we reported the isolation and characterization of a novel SK3-type dehydrin gene from *S. purpurea*, designated *SpDHN1*. As dehydrins play important roles in the response of plants to various abiotic and biotic stresses [4], their genes functions may help us to understand the adaptation of *S. purpurea* to harsh environment on the molecular level. To investigate the function of *SpDHN1*, we

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generated 35S: *SpDHN1-GFP Arabidopsis thaliana* plants in this study and determined the plant's tolerance to drought stress.

2. Materials and methods

2.1. Plant materials

S. purpurea samples were collected from the Qinghai-Xizang Plateau (N 34°57′27″E 98°07′25″), China. These materials were frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation. The *A. thaliana* (ecotype: Col-0) plants used for ectopic expression experiments were grown on 1/2 solid Murashige and Skoog (MS) medium for about 10 days before being transferred to soil. All plants were incubated in a growth chamber at 21 °C and a photoperiod of 16-h light/8-h darkness.

2.2. RNA isolation and DHN gene fragment cloning

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) from *S. purpurea* samples. After the total RNA was isolated, DNA-free total RNA (5 μg) was used for first-strand cDNA synthesis in a 20 μl reaction volume containing 2.5 units of avian myeloblastosis virus reverse transcriptase XL (Takara, Tokyo, Japan) and 1 μM oligo (dT) primer. Upstream primer E1 5′ GAACACCC AGTCCTACCAGAGC 3′ and downstream primer E2 5′ CCTTG ATCTTCTCCTTGAGCC 3′ were designed based on the assembled dehydrin (*DHN*) gene fragment (DNA sequences: NCBI SRA: SRR825213). To obtain the full-length *DHN* cDNA from a *S. purpurea* sample, the RACE was performed using SMART RACE cDNA Amplification Kit (BD Biosciences Clontech, USA) and Advantage™ 2 PCR Enzyme Kit (Clontech) in accordance with the manufacturer. The full-length DHN sequence was generated using primers: *DHNF* (5′ ATGGAGGATGAGAGGAACAC 3′) and *DHNR* (5′ TTAACCGCTG GTCTTGTGC 3′).

2.3. Sequence analysis of DHN gene

The full-length *DHN* cDNA sequence was translated using online ExPASy translate tool (http://au.expasy.org/tools/dna.html). The S- and K-segments of DHN protein were identified using ExPASy prosite server (http:// au.expasy.org/prosite/). DNASTAR software was used to carry out the Sequence analyses. MEGA 5.1 was used to perform the phylogenetic reconstruction by the neighbor-joining (NJ) method [8]. Bootstrap values were estimated (with 1000 replicates) to assess the relative support for each branch.

2.4. Generation and screening of transgenic A. thaliana plants

The full-length cDNA of DHN without end codon was inserted into a binary vector pBIN, with a GFP and driven by *Cauliflower mosaic virus* (*CaMV*) 35S promoter, forming a *35S: DHN-GFP* construct. The recombinant plasmid was then introduced into *Agrobacterium tumefaciens* GV3101 and used for wild-type *A. thaliana* transformation via the floral dip method [9]. Transgenic plants (T0) were selected on 1/2 MS medium supplemented with 50 mg L^{-1} kanamycin, and the overexpressing *35S: DHN-GFP* transgenic lines (T1) were verified by PCR using an npt II primers (5′ GAGGCTATTCGGCTATGACT 3′ and 5′ AATCTCGTGATGGCAGGTTG 3′) according to Sun [10]. Fluorescence images were detected using a Laser-Scanning confocal microscope (Olympus Optical Co. Ltd., Tokyo, Japan). GFP was excited with the 488 nm laser line and emission was captured using a 505–530 band-pass filter. For propidium iodide staining, seedlings were incubated with propidium iodide (10 mg L^{-1}) for 5 min and washed two times to remove traces of the treatment agents. Propidium iodide was observed using wavelengths of 600–640 nm.

2.5. Drought stress and photosynthesis capability measurement

For the drought tolerance test, seeds of wild-type and transgenic plants (T3) were clean and surface-sterilized. The seeds were sown on 1/2 MS medium supplemented with 10% polyethylene glycol 6000 (PEG) in the dark at 4 °C for 3 day, and then transferred to growth chambers. Germination rates were measured after 10 days. For seedling growth in PEG, 5-day-old seedlings were transferred on 1/2 MS medium supplemented with 10% PEG. Root lengths were measured after 14 day.

To determine the drought tolerance of transgenic plants in soil, wild-type and transgenic plants were grown on 1/2 MS medium for 10 days. Plants were transferred to soil under a normal watering regime for 3 weeks. Drought tolerance tests were performed by withholding water to the seedlings for 15 days, and then watering was resumed. Plants were allowed to grow for a subsequent 7 days. Chlorophyll fluorescence was measured using Pulse-Amplitude-Modulation (PAM) Chlorophyll Fluorometer (Heinz-Walz-GmbH, Effeltrich, Germany). To measure the maximum quantum yield of PS II, plants were dark-adapted for 30 min. Fv/Fm was recorded during a saturating photon pulse (4000 μmol m^{-2} s^{-1}) using a whole plant.

2.6. Detection of H_2O_2, O_2^- and malondialdehyde (MDA)

The *in situ* detection of H_2O_2 and O_2^- were performed using the described method with some modifications [11]. H_2O_2 was detected with 1 mg mL^{-1} of diaminobenzidine (DAB), with which leaves were vacuum-infiltrated in 10 mL DAB solution for 2 h and then cleared in boiling ethanol (95%) for 10 min. The amount of O_2^- in the leaves was monitored by 10^{-2} M nitro-blue tetrazolium (NBT) reduction. The leaves were vacuum-infiltrated with 10 mL NBT for 2 h, cleared in boiling ethanol (95%) for 10 min, and stored and examined in 95% ethanol.

The MDA content was determined as previously described [12]. Approximately 0.5 g of fresh leaves were homogenized in 10 mL of 10% trichloroacetic acid (TCA) and centrifuged at 12000*g* for 10 min. Then, 2 mL of 0.6% thiobarbituric acid in 10% TCA were added to an aliquot of 2 mL of the supernatant. The mixture was heated in boiling water for 30 min and then quickly cooled in an ice bath. After centrifugation at 10000*g* for 10 min, the absorbance of the supernatant at 450, 532, and 600 nm was determined.

3. Results and discussion

3.1. Isolation and characterization of the DHN gene in S. purpurea

The full length ORF of *SpDHN1* was 816 bp, encoded 271 amino acid residues, and was named *SpDHN1* (Fig. 1A; GenBank accession: KJ000690). BLASTn analysis showed that it was highly homologous to dehydrin WZY1–2 from *Triticum aestivum* (GI: 532236396), dehydrin 8 from *Hordeum vulgare* (GI: 4105115), dehydrin COR410 from *Brachypodium distachyon* (GI: 357163676), and SK3-type dehydrin from *Oryza sativa* (GI: 152940803) (Fig. 1B). Analysis of the protein sequence using the ExPASy-Compute pI/Mw tool (http://web.expasy.org/compute_pi/) indicated that *SpDHN1* contained 271 amino acids with a calculated isoelectric point of 5.34 and a molecular mass of 29.25 kDa [13]. Further analysis showed that the *SpDHN1* protein contained an S segment (SSSSSSS) and three K segments (RKKGLKEKIKEKLPG) (Fig. 1B).

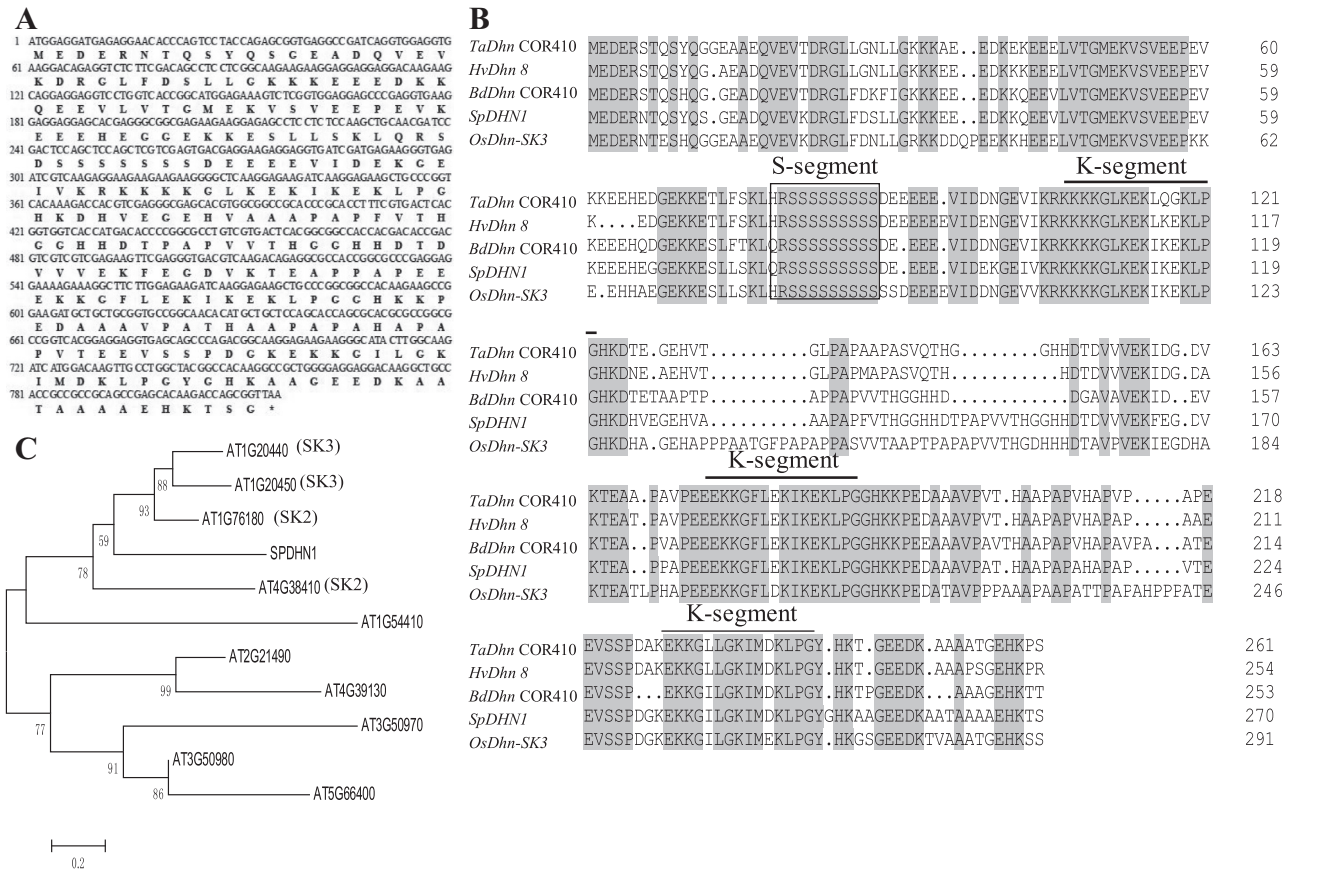

Fig. 1. Sequence analysis of *SpDHN1* and phylogenetic relationships between *SpDHN1* protein and the 10 dehydrin proteins from *A. thaliana*. (A) Nucleotide and deduced protein sequence of *SpDHN1*. (B) Multiple sequence alignment of *SpDHN1* with its homologous sequences. The amino acid sequences of K-segments are highlighted with ledgement and S-segments with box. (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.

In addition, a phylogenetic tree including the *SpDHN1* protein and the ten DHN proteins identified in the *A. thaliana* genome was created by Neighbor joining with the MEGA 5.1. Based on our phylogenetic results, the *SpDHN1* was clustered with the *A. thaliana* SKn-type DHNs, including At1g20440, At1g20450, At1g76180 and At4g38410, which were involved in drought and cold stress tolerance (Fig. 1C) [14]. Genes with the same functions are often closely related and this has been confirmed in previous reports [15–17]. K-segment of DHN protein could form amphipathic α-helices, and the amphipathic α-helices could interact with partly dehydrated surfaces of various other proteins and also with surfaces of biomembranes [18]. Several K-segments in one DHN protein can form bundles to enhance their amphipathic character in protein–protein or protein–biomembrane interactions. These interactions were the basis of dehydrin protective functions [19]. Under drought conditions, protective enzymes activities have been reported for several SKn-type DHNs, such as ERD10/EDD14 (At1g20450/At1g76180) from *A. thaliana* [20], dehydrin COR410 from maize [18]. Moreover, DHNs can also keep the original cell volume and prevent cellular collapse due to their unfolded state, higher accumulation and capability to bind water under drought stress. In addition, S-fragment of several DHNs contains phosphorylation sites. For example, a differential phosphorylation pattern of DHN-5 was observed in wheat response to drought and salt stress [21]. These results suggested that *SpDHN1* was a putative member of SK3-type DHN family in *S. purpurea* and possibly contributed to drought stress tolerance.

3.2. Identification of ectopic-expressing 35S:DHN-GFP transgenic plants

To elucidate the role of *SpDHN1* gene in the growth and development of *A. thaliana*, A fusion of *SpDHN1* to GFP under the control of the CaMV35S promoter was introduced into a binary vector (pBIN) (Fig. 2A). Transgenic plants were generated by introducing the *35S: SpDHN1-GFP* into the wild-type *A. thaliana* plants. Transgenic plants were verified by detecting *npt II* gene in all of the kanamycin resistant transgenic lines rather than the wild-type *A. thaliana* using specific PCR primers (data not shown). RT-PCR analysis was used to confirm the expression of *SpDHN1*, whereas no signal was detected in the wild-type plants (Fig. 2B). Two homozygous lines (SP-2, SP-3) with *SpDHN1* expressed were selected for further analysis.

GFP, an ideal visual marker, has been widely used to select transgenic plants and protein subcellular localization [22]. Fluorescence microscopy of the roots showed that *SpDHN1*-GFP fusion protein was localized in the cytoplasm and the plasma membrane (Fig. 2C). Localization work about other dehydrins has established that they can be present in the membrane, nucleus or cytoplasm of cells [23,24]. The localization of these membrane proteins suggest that they are involved in the protection of the plasma membrane against freezing or dehydration stress through preventing the destabilization of the plasma membrane [24]. Localized in the cytoplasm has been reported on potato DHN24 (SK3) subjected to drought and cold [25,26]. Meanwhile, their amounts substantially increase under the cell dehydration conditions that occur

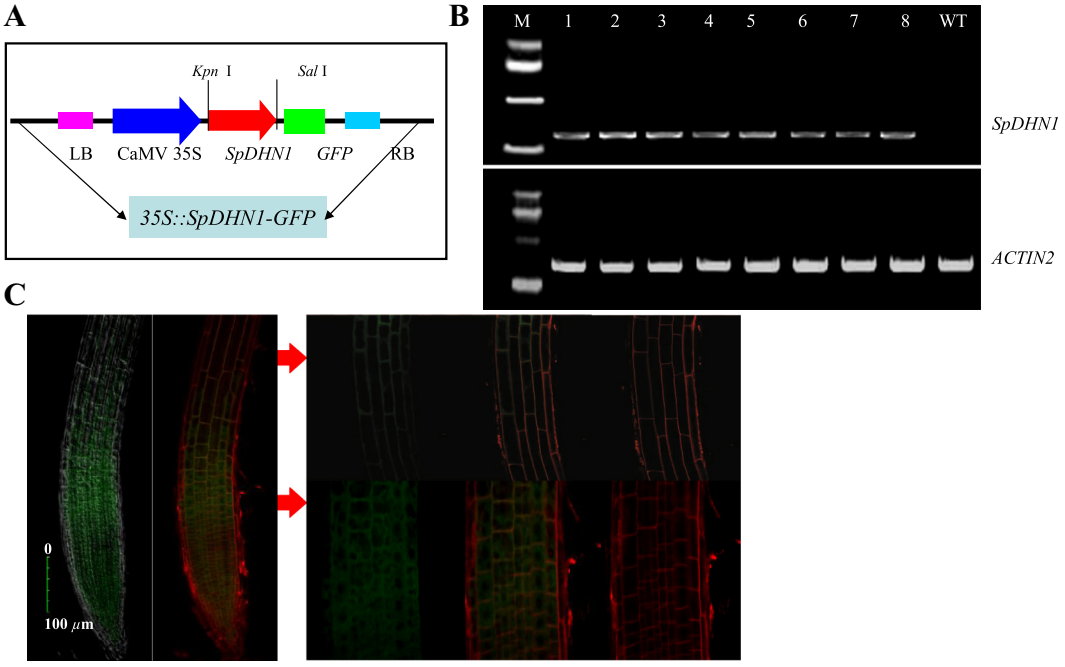

Fig. 2. Construction of the *35S::SpDHN1*-GFP expression vectors and Subcellular localization of *35S:SpDHN1*-GFP fusion protein (A) Schematic diagram of the plant expression vectors. The vector used for the introduction of *SpDHN1* cDNA sense orientation into *A. thaliana*. The sense *SpDHN1* cDNA insert is flanked by the cauliflower mosaic virus 35S promoter, *Kpn* I and *Sal* I sites for recombination. LB left border, RB right border. (B) The RT-PCR analysis of the transgenic plants and the wild-type plants. 1–7, transgenic *A. thaliana* plants; 8, positive control; WT, wild-type plants. M, DNA molecular marker. (C) Confocal laser scanning microscopy images of GFP fluorescence (green) and propidium iodide (PI) fluorescence (red) in the transgenic *A. thaliana* root (left). The local enlarged images of GFP fluorescence (right). Bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

during seed maturation or in vegetative tissues subjected to environmental stress such as low temperature and drought [24,25].

3.3. Improved tolerance of transgenic plants to drought-stress treatments

Drought stress is an essential restriction factor of affecting plant growth and development. The identification and study of plant drought tolerance genes is one of the basic research approaches at the molecular level to reveal the mechanism of plant drought stress responses. DHNs are induced in vegetative tissues following salinity, dehydration, cold and freezing stress and play a fundamental role in plant response and adaptation to abiotic stresses [18]. To determine the possible role of the *SpDHN1* gene under stress conditions, the response of *SpDHN1*-overexpressing *A. thaliana* to drought stress was analyzed using transgenic plants.

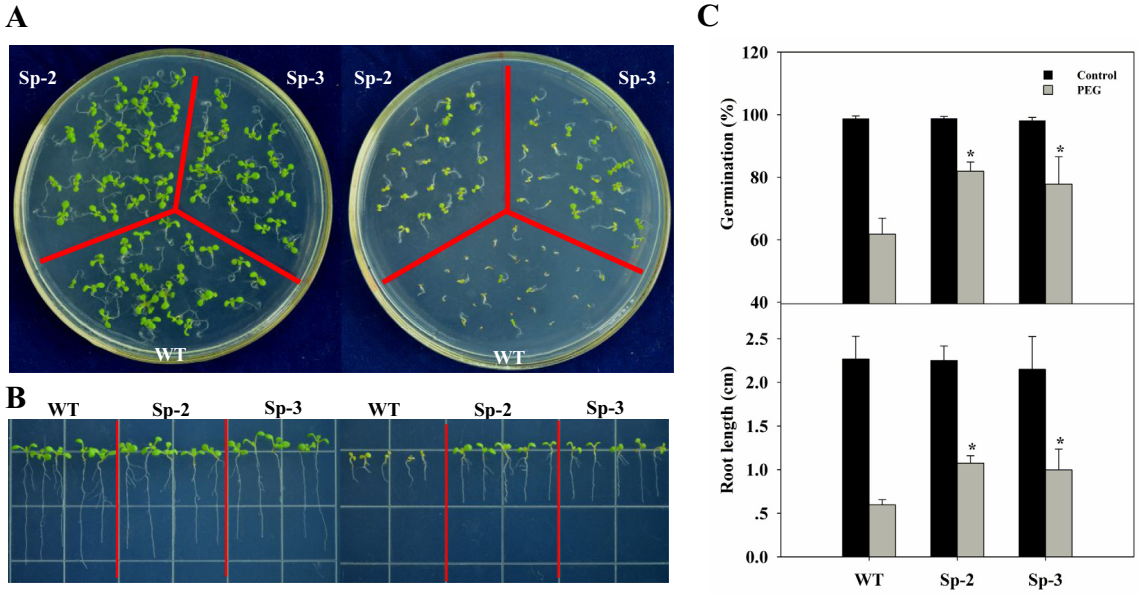

Fig. 3. Drought stress response of transgenic and wild-type seedlings. (A) Phenotype of *A. thaliana* under normal conditions and drought stress. (B) Representative images of wide type and *SpDHN1*-overexpressed *A. thaliana* roots grown on 1/2 MS medium containing 10% PEG. The photograph was taken on 14 day after germination (DAG). (C) Germination of *SpDHN1*-overexpressed and wild-type seeds under normal conditions and drought stress in 10 days. The root length was measured on MS medium containing 10% PEG on 14 DAG. Data mean values ± SE obtained from three independent experiments. *Indicates significant difference among materials at the 0.05 level.

As shown in Fig. 3, the seed germination rate of transgenic plants was comparable to that of wild-type plants without PEG treatment. However, following a lower dosage 10% PEG treatment, transgenic plants exhibited higher germination rates than wild-type after 10 d of treatment. We also compared the root length on 1/2 MS medium under drought stress condition. The primary root length of the transgenic *A. thaliana* plants was dramatically different from the wild-type plants (Fig. 3B, C). The primary root length of the transgenic lines was longer than that of the wild-type plants, and the shoot of *SpDHN1*-overexpressing transgenic plants had more vigorous shoots and lateral roots than wild-type. These results indicated that *SpDHN1*-overexpression rendered plants more resistance to drought during early development. Similar results have been reported that overexpression of *ZmDHN1* (SKn-type) from maize can increase tobacco resistance to drought and low temperature [27]. Recently, Xiong et al. reported that AY587109 (SKn-type) also performed the same function in rice under low temperature and drought conditions [28].

In order to further determine *SpDHN1* function, drought tolerance tests were performed using wild-type and transgenic plants in soil. Water was withheld from the 3-week-old wild-type and transgenic plants for 15 days. Transgenic plants showed higher survival rates than the wild-type (Fig. 4A, C). In particular, transgenic plants exhibited relatively higher drought tolerance (Fig. 4A). When they were re-watered after drought treatment, transgenic plants recovered faster and showed better survival than wild-type plants (Fig. 4A, C). We then compared the maximum photochemical efficiencies of PS II in the dark-adapted state (Fv/Fm), which reflected the plants' photosynthetic capabilities. The Fv/Fm of *SpDHN1*-overexpressing plants was higher than that of wild-type under drought conditions (Fig. 4A, B). The *SpDHN1*-overexpressing plants exhibited excellent photosynthetic capability and increased drought tolerance under conditions of drought. A previous study has reported for maize, where drought stress resulted in an increase in the expression of various proteins, including *ZmDHN2*, leading to improved drought stress resistance

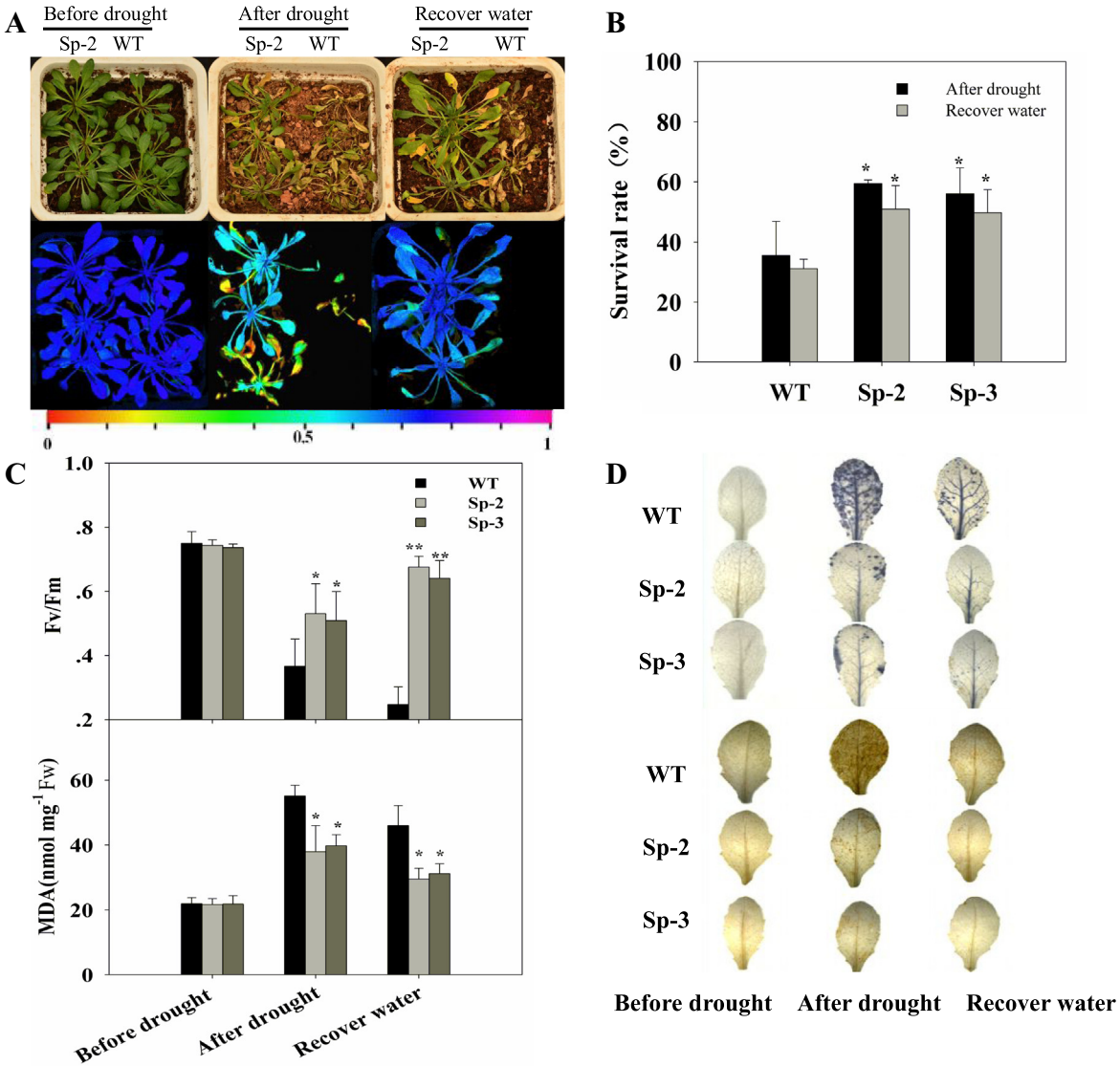

Fig. 4. Effect of drought stress on the growth of transgenic *A. thaliana* seedlings. (A) Leaf phenotypes of wild-type and transgenic *A. thaliana* derived from different treatment. At the indicated times, images were captured (top), and photosynthetic capabilities were recorded by Fv/Fm imaging using a PAM chlorophyll fluorometer (bottom). The pseudocolor code depicted at the right of the images ranges from 0 (red) to 1.0 (purple). (B) Survival rate of wild-type and transgenic *A. thaliana* seedlings. (C) The effects of drought stress on photosynthetic Fv/Fm ratio and leaf MDA content of wild-type and transgenic *A. thaliana* seedlings. Data are mean values ± SE obtained from three independent experiments. *Indicates significant difference among materials at the 0.05 level; **indicates significant difference at the 0.01 level. (D) Effects of drought on H_2O_2 (down) and O_2^- (up) in transgenic *A. thaliana* and wild-type seedling leaves. H_2O_2 and O_2^- were measured by reported method. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and maintenance of photosynthetic activity and metabolism under drought stress [29].

MDA, as an end product of lipid preoxidation, was induced by drought stress in rice leaves [30], maize leaves [31], and alfalfa roots and shoots [32]. In the present study, the MDA content was also increased by the drought treatments, but the degree of transgenic plants were lower than wild-type (Fig. 4C). Lipid peroxidation in plant membranes is mainly caused by reactive oxygen species (ROS) [33]. Consistent with localization of *SpDHN1* in plasma membrane results, *SpDHN1* protein seemed able to disposal of oxidative damage to plasma membrane by preventing lipid peroxidation. It has been reported that SK3-type DHNs CuCOR19 from *Citrus unshiu* acted as antioxidants and protected plasma membrane from being oxidized by ROS [34]. Similar results were obtained by measuring the *in situ* generation of H_2O_2 and O_2^- using DAB and NBT staining. Drought accelerated the production of H_2O_2 and O_2^- in wild-type plants, and sustained substantial ROS production even after re-water. However, such exposure did not induce ROS over-accumulation in transgenic plants (Fig. 4D). The results showed that leaves of transgenic plants accumulated much less ROS than those of wild-type. DHNs serve as a key antioxidant role because they contain relatively large amounts of H, R and other reactive amino acid residues on their surface, which can interact with ROS species to scavenge ROS [18]. These results show that *SpDHN1* displays important antioxidant functions due to its ability of scavenging ROS, and overexpression of *SpDHN1* enhances transgenic plants tolerance to drought stress.

4. Conclusion

A dehydrin gene (*SpDHN1*) was first cloned from *S. purpurea* leaves. The full-length ORF of *SpDHN1* was 816 bp, encoding 271 amino acids with a predicted molecular weight (MW) of 29.25 kDa and an isoelectric point (PI) of 5.34. *SpDHN1* had the typical structure of a dehydrin protein, namely three K fragments and an S fragment (SK3-type DHN gene). Further, the *SpDHN1* proteins executed their functions in the cytoplasm and the plasma membrane. Finally, *SpDHN1* function analysis indicated that the *SpDHN1*, as an extremely efficient ROS scavenger, played an important role in drought stress tolerance. This work provided a theoretical and experimental basis for further research and use of the gene in genetic manipulation in other crops.

Acknowledgments

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