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ORIGINAL PAPER

SsDHN, a dehydrin protein from *Suaeda salsa*, enhances salt stress tolerance in transgenic tobacco plants

Hui Li[1,2] · Li Zhang[1] · Jingwei Lin[1] · Shuisen Chen[1] · Weiming Gao[1] · Jiayi Zhang[1] · Hui Ma[1] · Ming Zhong[1]

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Abstract

Dehydrins (DHNs) are late embryogenesis-abundant (LEA) proteins, which enhance abiotic stress tolerance in plants. However, little is known regarding the function of DHNs in *Suaeda salsa* L. (*S. salsa*), which can grow in saline soil. Here, we successfully cloned and functionally characterized a dehydrin gene from *S. salsa,* designated *SsDHN*. Sequence alignment analysis showed that SsDHN belongs to SKn-type DHNs and shares relatively high level of homology with *Spinacia oleracea* COR47-like (XP_021846321.1). Quantitative RT-PCR analysis indicated that *SsDHN* expression level increased significantly under salt stress. We also generated *SsDHN*-overexpressing transgenic tobacco lines and analyzed their salt stress response. Seeds of transgenic tobacco plants grown under a range of salt concentrations (100, 200, and 300 mM) showed significantly higher germination rates relative to wild-type seeds. Transgenic plants had longer root length, lower relative electrical conductivity (REC), lower malondialdehyde (MDA) content, higher proline (PRO) content, increased peroxidase (SOD) activity, and less damage to the chloroplast ultrastructure. Our results showed that the transgenic tobacco plant lines had improved salt resistance and osmotic adjustment, enhanced reactive oxygen species scavenging ability, maintenance of the K^+/Na^+ balance, and reduced chloroplast membrane damage. These results suggest that the *SsDHN* gene may be used for improving abiotic stress tolerance in economically important crops.

Keywords Dehydrin · Tobacco · Salt stress · Relative electrical conductivity · Chloroplast membrane damage

Introduction

Plants live in open environments and cannot escape from adverse environmental conditions. As a result, plants are susceptible to numerous abiotic stresses such as high salinity, drought, and extreme temperatures. These stresses, individually or in combination, adversely affect growth, development, and yield, and they may threaten plant survival (Bartwal et al. 2013; Ahanger et al. 2017). To confront various environmental stresses, plants have evolved a series of regulatory pathways to respond and adapt to their environments in a timely manner (Bartels and Sunkar, 2005). Late embryogenesis abundant (LEA) proteins are a large family of hydrophilic proteins that were initially identified in the late stages of seed maturation and were later found in most plants and in different plant tissues. LEA proteins are characterized by different conserved sequence motifs and are rich in alanine, glycine, and serine residues (Close, 2010). There are seven groups of LEA proteins, based on sequence similarity and structural properties (Battaglia et al. 2008). LEA proteins play vital roles in plant growth and abiotic stress response (Jin et al. 2019; Shen et al. 2014). Under abiotic stress conditions, LEA protein expression is upregulated. These proteins play several roles, including the protection of cellular structures (Serrano and Montesinos, 2003), sequestration of ions (Grelet et al. 2005), folding of denatured proteins (Bray, 1993), and protection of cells against membrane damage (Umezawa et al. 2006).

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✉ Hui Ma
mahui@syau.edu.cn

✉ Ming Zhong
mingzhong@syau.edu.cn

[1] Key Laboratory of Agricultural Biotechnology of Liaoning Province, College of Biosciences and Biotechnology, Shenyang Agricultural University, Shenyang, China

[2] Key Laboratory of Protected Horticulture (Ministry of Education), College of Horticulture, Shenyang Agricultural University, Shenyang, China

Dehydrins (DHNs), belonging to the group II LEA proteins, are a group of environmental stress-responsive proteins (Wang et al. 2014). DHNs are named based on their overexpression during seed dehydration stress, which is related to the protective mechanisms against plant dehydration (Jin et al. 2019). Generally, DHNs are hydrophilic proteins that include four conserved motifs (Y-, S-, K-, and φ-segments). Based on the presence of these four conserved motifs, DHNs have been divided into five groups: YnSKn, YnKn, SKn, Kn, and KnS (Serrano and Montesinos, 2003). The K-segment is a lysine-rich sequence motif (EKKGIMDKIKEKLPG) which is prevalent in all DHNs except in maritime pine DHN, and it is thought to be a signature fragment (Grelet et al. 2005; Bray, 1993). The Y segment is a conserved sequence, [V/T]D[E/Q]YGNP, which is found in the N-terminal region of DHNs (Hughes et al. 2013; Malik et al. 2017). The S-segment consists of serine residues (SSSSSSSD) that are modifiable by phosphorylation (Close, 1996; Liu, et al. 2017; Yang et al. 2012). The poorly conserved regions, the so-called φ-segments, are enriched in polar amino acids (Vornam et al. 2011; Graether and Boddington, 2014). DHNs play key roles in plant response to abiotic stress. Overexpression of *DHN5* confers tolerance to freezing and salt stress in *Arabidopsis* (Brini et al. 2007). OsDHN1 has been shown to play a key role in drought and salt stress responses through scavenging of reactive oxygen species in rice (Kumar et al. 2014; Verma et al. 2017). Overexpression of wheat DHN-5 confers tolerance to salt stress in transgenic *Arabidopsis* plants (Saibi et al. 2015). MsDHN1 can increase tolerance to Al stress in *Medicago sativa* (Lv et al. 2021). Overexpression of *HbDHN1* and *HbDHN2* (two DHN genes *of Hevea brasiliensis*) can enhance salt tolerance, drought responses, and osmotic stress resilience in *Arabidopsis* (Cao et al. 2017). SiDHN1 and SiDHN2 can enhance cold tolerance in transgenic tobacco plants, and they exhibit an induced expression pattern under cold stress in *Salvia involucrata* (Qiu et al. 2014; Guo et al. 2017). AnDHN, a dehydrin protein from *Ammopiptanthus nanu*s, mitigates the negative effects of drought stress in plants (Sun et al. 2021). CaDHN3 enhances tolerance to salt and drought stress (Meng et al. 2021). ShDHN has been reported to promote resistance to drought and cold stress (Liu et al. 2015), and *GhDHN_03* and *GhDHN_04* knockdown demonstrated putative roles of DHNs in augmenting osmotolerance and salt tolerance in cotton (Kirungu et al. 2020). Although DHN functions remain elusive in other species, several clues have been found.

Suaeda salsa (L.) Pall. is an annual herb of the Chenopodiaceae family that mostly grows in coastal wetlands, tidal flats, and other saline–alkaline environments. *S. salsa* plays an important role in protecting the marine ecological environment and in purifying wetland sewage because of its natural salt-resistant gene bank and its strong stress resistance (Zhang et al. 2005; Song et al. 2017; Guo et al. 2020a, 2020b). Therefore, studying the molecular mechanisms involved in its salt tolerance would be helpful to further understand the plant response mechanism to salt stress. This understanding is also of great significance for promoting genetic improvement in other plants, enhancing the salt-tolerance of plants, and repairing and improving salt-tolerant soils (Song and Wang, 2015).

DHNs have been reported to be involved in resistance to abiotic stresses and are related to mechanisms protecting against plant dehydration. However, *S. salsa* DHNs remain less explored. In this study, we successfully cloned and characterized the *SsDHN* gene from *S. salsa* using homology-based cloning and RACE (rapid amplification of cDNA end) separation methods. Sequence alignment and evolutionary analyses revealed that SsDHN belongs to SKn-type DHNs and shares relatively high level of sequence homology with *Spinacia oleracea* COR47-like (XP_021846321.1). Moreover, we showed that the expression of *SsDHN* is induced by salt stress. Therefore, we further explored the relationship between *SsDHN* and salt stress using overexpression techniques. The results showed that *SsDHN*-overexpressing tobacco plants exhibited significantly increased tolerance to salt stress. The results of this study reveal that *SsDHN* functions as a positive factor in salt-stress signaling pathways.

Materials and methods

Plant materials and growth conditions

The test material was *S. salsa* obtained from Panjin Red Beach, Dawa County, China.

Plants showing good growth were selected and sampled after treatment with 300 mM NaCl. Some of the samples were used for RNA extraction, and the remaining samples were frozen in liquid nitrogen and stored at − 70 °C for future use.

The wild-type (WT) *Nicotiana tabacum* L. seeds used for transformation in this study were "NC89". The seedlings were grown in a standard growth chamber (2000 Lx, 23 ± 1 °C) with a 16-h/8-h light/dark cycle.

Homology cloning and 3′ rapid amplification of cDNA of SsDHN

Related sequence information for *DHN* was extensively searched in the EST and GenBank databases. Degenerate primers DHNF1, DHNF2, DHNR1, DHNR2, and DHNR3 were designed using Primer 5.0, combined with BLASTn, for sequence comparison analysis. Oligo (dT) was used as a joint primer, and the cDNA obtained by reverse transcription was used as a template to amplify the conserved

region fragment. A pair of 3′ RACE specific primers, namely DHN3-1 and DHN3-2, were designed according to the conserved region of the cloned *SsDHN* gene. Using total RNA as a template and CDS (Coding Sequence) as the primer, first-strand cDNA was synthesized using M-MuLV Reverse Transcriptase (Promega). Next, nested PCR amplification was performed with DHNR3-1 and DHNR3-2 as upstream primers and PCR-G as downstream primers with the above cDNA to obtain a single 3′ segment. The two obtained fragments were spliced using DNAMAN software to obtain the complete sequence. Specific primers, namely DHNF and DHNR, were designed according to the complete sequence, and the full-length coding region of the *DHN* gene was amplified by PCR using cDNA of *S. salsa* as a template. Detailed sequences of the outer and inner primers are listed in Supplementary Table S1.

SsDHN sequence analysis

The CDS of *SsDHN* cDNA sequence was translated using the online ORF finder translator. The S- and K-segments of SsDHN were characterized using the ExPASy prosite server. A phylogenetic tree was constructed using MEGA6 software employing the neighbor-joining method (Tamura et al. 2013). The SsDHN protein sequence (NCBI accession no.: AGC55011.1) was aligned with known DHNs using ClustalX software. The amino acid sequences of different DHNs used were as follows: *Chenopodium quinoa* (CqDHN; NCBI accession no.: XP_021756500.1; XP_021732246.1), *Atriplex canescens* (AcDHN; NCBI accession no.: AFC98463.1), *Atriplex halimus* (AhDHN; NCBI accession no.: AGZ86543.1), *Spinacia oleracea* (SoDHN; NCBI accession no.: XP_021846321.1), *Suaeda glauca* (SgDHN; NCBI accession no.: AEA29617.1), *Capsella bursa-pastoris* (CbDHN; NCBI accession no.: ABV56004.1), *Arabidopsis thaliana* (AtDHN; NCBI accession no.: CAA62449.1), *Momordica charantia* (McDHN; NCBI accession no.: XP_022152554.1), *Manihot esculenta* (MeDHN; NCBI accession no.:XP_021614140.1), *Coffea canephora* (CcDHN; NCBI accession no.: ABC68275.1).

Expression analysis by quantitative real-time PCR

The four to five true leaves of *Suaeda salsa* seedlings were transferred from the greenhouse to flower-pots, and placed in a light culture room at a temperature of 22 ± 2 °C and a relative humidity of 75% for cultivation. After culturing for 20 d, *Suaeda salsa* seedlings were treated with an aqueous solution containing 300 mM NaCl for 0, 6, 12, 24, and 48 h, respectively. Tobacco roots and leaves treated with NaCl were used for total RNA extraction (TransGen, Beijing, China) and cDNA synthesis (TransGen). The qRT-PCR analysis was performed using an QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). The *NtActin* gene of tobacco was used as an internal control. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Three biological and three technical replicates were performed for each gene. The primers used in this study are listed in Supplementary Table S1.

Four to five suaeda seedlings with true leaves were transferred from greenhouse to pots and placed in light culture chamber at 22 ± 2 °C and relative humidity 75%. After 20 days of culture, suaeda seedlings were treated with salt stress. The time from the last watering to the salt treatment was 7 d, and then the seedlings were treated with an aqueous solution containing 300 mM NaCl for 0, 6, 12, 24 and 48 h, respectively. Each treatment was repeated for three times. The leaves and roots of the three repeated samples were taken and quickly put into liquid nitrogen, and then stored in a refrigerator at − 80 °C.

Tobacco transformation

The *SsDHN* cDNA CDS was driven by the CaMV *35S* promoter. A 700-bp fragment was inserted into the PBI121 vector for genetic transformation. The recombinant construct was introduced into Agrobacterium strain GV3101 and transformed into tobacco "NC89" using a leaf explant transformation method as described (Sunilkumar et al. 1999). Finally, six independent overexpressing T3 lines were obtained. Three representative T3 independent cell lines (SsDHN-1, SsDHN-2, and SsDHN-3) were selected for further experiments.

Southern blot hybridization

Genomic DNA was isolated from tobacco leaves using a Plant Genomic DNA Kit (Beijing Tiangen, China). Next, 10 μg of DNA from each sample was digested with HindIII restriction enzyme, electrophoresed on a 1% agarose gel, and transferred to a Hybond N + membrane (Amersham). DIG (digoxigenin)-labeled DNA was used as a probe for DNA hybridization. Color rendering after molecular hybridization was performed using an enzyme-linked immunosorbent assay (ELISA) and the chemiluminescence substrate CSPD. In this experiment, the Agrobacterium plasmid transformed into the PBI121-DHN recombinant vector was used as the positive control, and the WT tobacco was used as the negative control.

Protein extraction and western blot analysis

Leaves of tobacco plants were used to obtain crude protein extracts through extraction buffer [50 mM Tris–HCl (pH 8), 10 mM NaCl, 1% SDS, 5% β-mercaptoethanol, 1 mM

leupeptin, 1.5 mM pepstatin A, 1.5 mM aprotinin, 0.1 mM PMSF], and centrifuged at 4 °C for 15 min at maximum speed by microcentrifugation. Proteins were precipitated with trichloroacetic acid (TCA) (10%, v/v), washed with 0.1 M acetic acid/methanol 3 times, dried, and suspended in an appropriate buffer for gel electrophoresis. Fifty micrograms of protein were isolated from each sample using one-dimensional SDS polyacrylamide gel electrophorese (SDS-PAGE) and electroblotted onto nitrocellulose membranes according to the manufacturer's instructions (Bio-Rad). The nitrocellulose membrane was blocked with 0.5% Tween-20 and 10% milk powder in PBS. The blots were probed with anti-DHN polyclonal antibody (Agrisera, Wuhan, China) and anti-rabbit antibody (ZSGB-BIO, Beijing, China) and incubated overnight at 4 °C. The blots were washed three times in 1 × PBS buffer and immunoreactive bands detected using the BCIP/NBT Chromogenic Kit (Tiangen, Beijing, China).

Abiotic stress tolerance assay

For seed germination and root growth assays, seeds of WT and *SsDHN* transgenic lines were grown on MS (Murashige and Skoog) solid medium containing different concentrations of NaCl (0, 100, 200, and 300 mM) at 22 ± 2 °C for 20 days. Germination rate and root growth were calculated using three biological replicates. For tobacco seedling recovery assays, tobacco seedlings grown in MS medium with 200 mM NaCl for 30 d were transferred to normal MS medium.

WT and transgenic tobacco seedlings (4 × 2 cm) were treated with different concentrations of NaCl (0, 100, 200, and 300 mM) for 3 d. Fresh weight (0.5 g) tobacco leaves were washed with deionized water and wiped dry. 10 ml of deionized water was added; the test tube plug was covered and placed on the bed at 40–50 rpm and shaken slowly for 2 h at 25 °C. Taking the conductivity of deionized water as a blank control (C1), the conductivity of the solution was R1 after shaking well. Then, the solution was boiled in boiling water for 15 min and cooled to room temperature and conductivity was measured as R2 after shaking well. The conductivity of deionized water after boiling was used as a blank control (C2), and the relative electrical conductivity was calculated using the following equation: Relative electrical conductivity (%) = [(R1 − C1)/(R2 − C2)] × 100. At least ten seedlings were collected as one sample for each biological replicate.

MDA levels were measured using a maleic dialdehyde assay kit (A003-3, A003-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). WT and transgenic tobacco seedlings (4 × 2 cm) were treated with different concentrations of NaCl (0, 100, 200, and 300 mM) for 3 d. Three biological replicates for each sample and at least ten seedlings were collected as one sample for each biological replicate.

The ROS scavenging enzyme activities of PRO and SOD were detected using kits produced by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). WT and transgenic tobacco seedlings (4 × 2 cm) were treated with different concentrations of NaCl (0, 100, 200, and 300 mM) for 3 d. Three biological replicates for each sample and at least ten seedlings were collected as one sample for each biological replicate.

For K^+ and Na^+ content assays, WT and transgenic tobacco seedlings (4 × 2 cm) were treated with different concentrations of NaCl (0, 100, 200, and 300 mM). Each sample was tested three times for each salt concentration, and the treatment time was three days. Tobacco plants were removed from the salt water and rinsed with distilled water. The above ground and underground parts were separated, weighed (into 0.5 g parts), and dried in the oven until constant weight. Then, we added 1 ml concentrated sulfuric acid and 1 ml 30% hydrogen peroxide and put the triangular bottles into boiling water for 1 h. Deionized water (50 ml) was added; a filter paper was spread on the funnel and the solution was slowly poured into the funnel at a constant volume for filtering. An atomic absorption instrument was used to determine its concentration. After all the samples were measured, a standard curve was constructed according to the concentration of the reference standard liquid. The K^+ content = (concentration × 50 ml × 39)/0.5 g, and the Na^+ content = (concentration × 50 ml × 23)/0.5 g. Three biological replicates for each sample and at least 10 seedlings were collected as one sample for each biological replicate.

WT and transgenic tobacco were cultured in medium with NaCl concentrations of 0, 100, and 200 mM for 60 days. Leaf samples (1 × 1 cm) were cut from the same parts of the two materials along the main veins of the leaves. The material was quickly placed in a pre-cooled 3% glutaraldehyde fixation solution and stored in a refrigerator at 4 °C. Phosphate buffer (0.1 mol/l, pH 7.2) was used to wash 2–5 times for 30 min each time. The cells were then immobilized with a 1% osmium acid solution for 2–4 h. Phosphate acid buffer (0.1 mol/l, pH 7.2) was used to wash 2–5 times (for 30 min each time) on an oscillating platform. Ethanol was used for dehydration: 30% ethanol → 50% ethanol → 70% ethanol → 80% ethanol → 90% ethanol (15 min for each) → 100% ethanol → 100% ethanol (30 min for each) → epoxy propane (twice for 30 min). Acetone and incomplete resin (3:1) were used overnight. Tobacco leaves were then placed into an embedding plate for 2 h, polymerized in an oven at 60 °C for 12 to 24 h, and double-dyed with uranyl acetate and lead citrate for 20 min. Finally, the cells were observed and photographed under a transmission electron microscope (LSM 510, ZEISS).

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics software. All experiments were repeated at least three times, and the data are presented as means ± SD. Statistically significant variation was determined using Student's *t*-test, and $*p < 0.05$ was considered as significant.

Results

SsDHN encodes an SKn-type DHN

SsDHN was obtained using homology cloning and 3′-RACE (Supplementary Figure S1). The full-length *SsDHN* cDNA was 847 bp, including a 61-bp 5′-untranslated region (UTR), a 169-bp 3′-UTR region and a 678-bp ORF (Supplementary Figure S2A). Analysis of the protein sequence using ExPASy ProtParam revealed the isoelectric point to be 5.22. The molecular weight was 32.377 kDa, and the overall hydrophilicity average (GRAVY) was 0.804, indicating that SsDHN is a hydrophobic protein.

The amino acid sequence was deduced from the cDNA-coding region of *SsDHN* and compared with the amino acid sequence encoded by the *DHN* genes of other plants. The results showed that there was a sequence of SSSSSSSDEEGEEGDDEEKKK rich in serine and lysine (i.e., S and K residues) in the 130–160 amino acid region, and two K fragments of KIKEKLPG in the C-terminal 226–240 amino acids, suggesting that the SsDHN protein has two characteristic domains of DHN proteins which belongs to members of the SKn family. In addition to this homology, there were certain differences involving DHN of *Suaeda salsa* and other species, which represents a manifestation of species diversity at the genetic level (Supplementary Figure S2B).

To further study the evolutionary relationship between SsDHN protein and DHNs from other plant species, *Suaeda salsa*, *Momordica charantia*, *Manihot esculenta*, *Arabidopsis thaliana*, *Coffea arabica*, *Spinacia oleracea, Chenopodium quinoa*, *Atriplex canesce*ns, *Atriplex halimus*, *Capsella bursa-pastoris* and *Suaeda glauca* sequences were analyzed using ClustalX software and MEGA6.0. We observed that the amino acid coding sequence of *SsDHN* has a distant evolutionary relationship with *M. charantia* and a close evolutionary relationship with *S. oleracea*, which is the same as the result of sequence homology described above. These results indicated that the SsDHN protein could be grouped as an SKn type (Fig. 1).

Expression pattern of *SsDHN* in response to salt stress

Next, we conducted a comprehensive set of experiments to functionally analyze SsDHN for its potential role in simulated salt stress. The temporal expression patterns of *SsDHN* were analyzed in the roots and leaves after 0–48 h of 300 mM NaCl treatment. Our data showed that the expression level of *SsDHN* first increased and then decreased, and it reached its highest level at 12 h. With increasing treatment time, the expression level of *SsDHN* was higher than that under control growth conditions in leaves (Fig. 2A). Similarly, the expression level of *SsDHN*

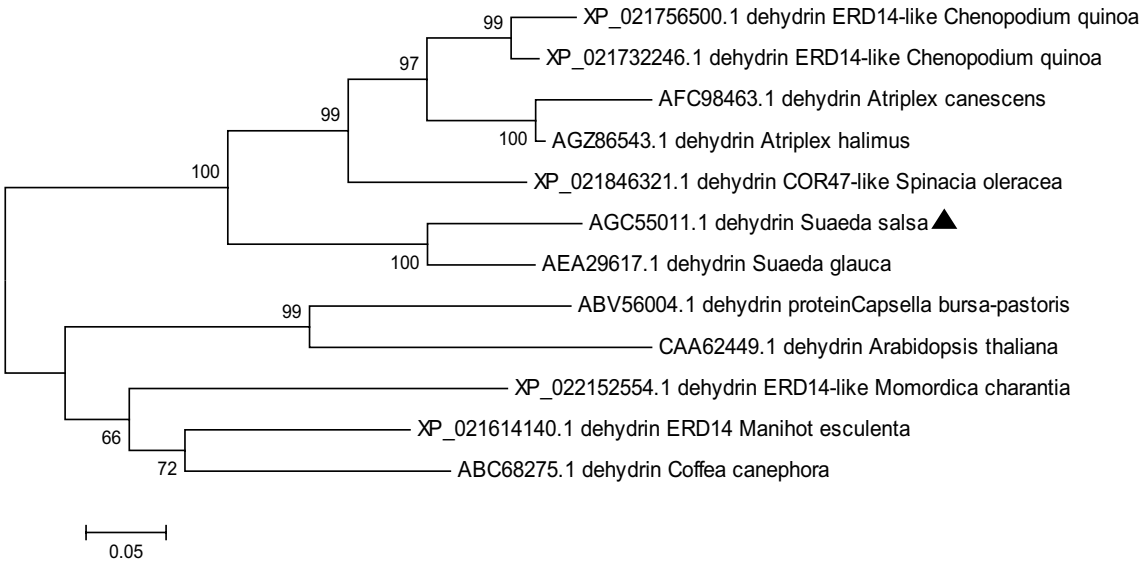

Fig. 1 Phylogenetic relationships between SsDHN protein and DHNs from other plant species. The molecular phylogeny was constructed from a complete protein sequence alignment of DHNs using the neighbor-joining method. The symbol "▲" shows the position of SsDHN in the phylogenetic tree

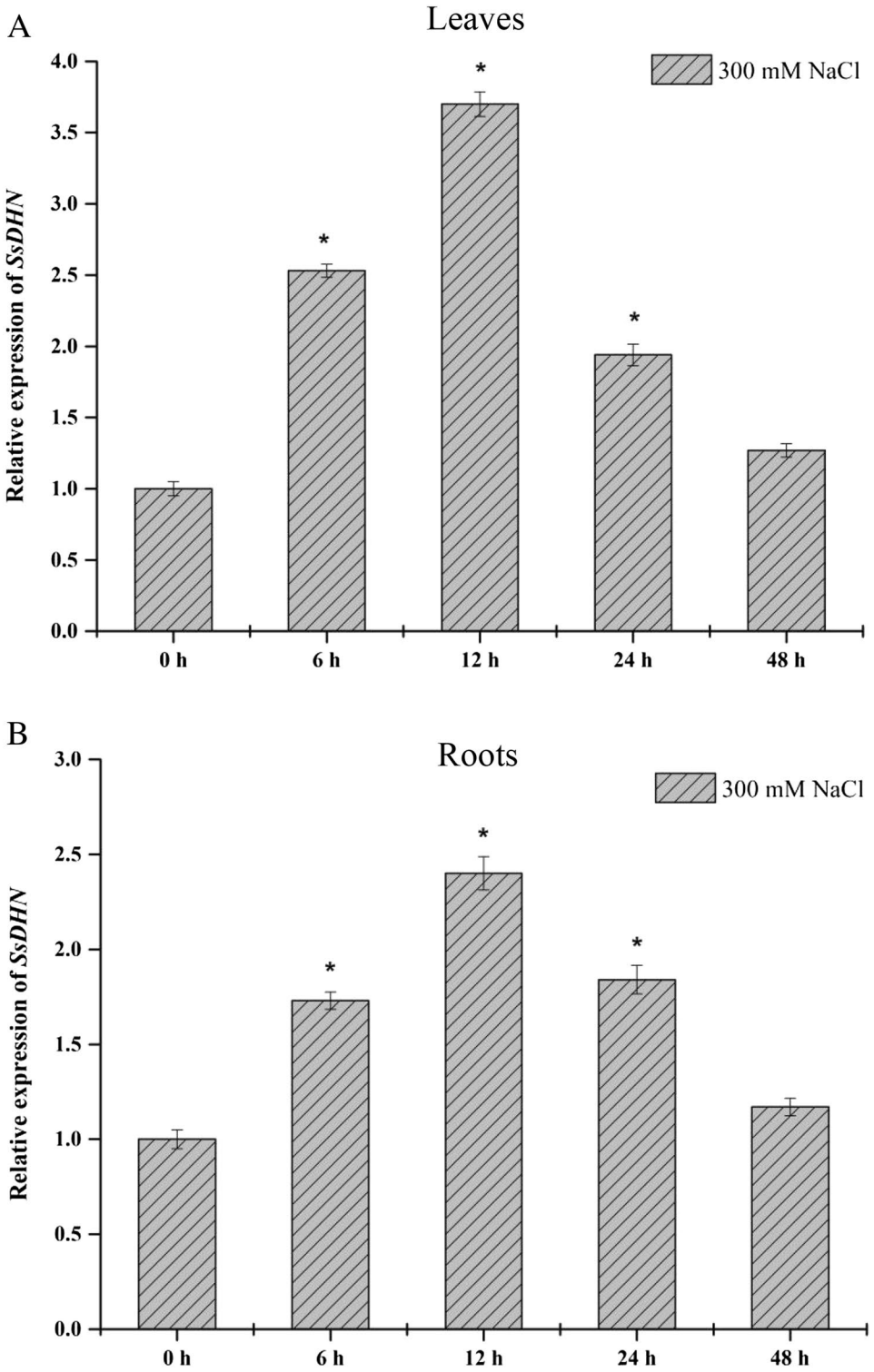

Fig. 2 Analysis of *SsDHN* expression in leaves (**A**) and roots (**B**) of *Suaeda salsa* under salt stress (300 mM NaCl) condition. *Actin* was used as an internal control. Data are means of triplicates from three independent experiments. Error bars indicate ± SD. Asterisks indicate significant differences from the control (Student's *t*-test *p*-values, $*p < 0.05$)

in roots was higher than that under control growth conditions up to 24 h of treatment and decreased with prolonged treatment time (Fig. 2B). In general, the expression of the *SsDHN* gene was upregulated under salt stress. These results suggest that the *SsDHN* gene is salt-responsive.

Overexpression of *SsDHN* enhanced salt tolerance by increased germination rate and root length

To further verify whether SsDHN is related to salt stress, *SsDHN* transcription was driven by the CaMV *35S* promoter,

and six overexpressing *SsDHN* transgenic lines were generated in tobacco plants. Among these lines, we chose three representative independent ones for further analysis (abbreviated as *SsDHN*-1, *SsDHN-2*, and *SsDHN-3*), and *SsDHN* transgenic lines were examined by RT-PCR, and Southern and western blotting (Supplementary Figure S3). With increasing salt concentrations, the germination rate of the seeds decreased gradually. In the absence of stress, the germination rate of the WT and *SsDHN*-OE transgenic line seeds reached 100%. Although the germination rate decreased with increasing salt concentration, the germination rate of *SsDHN*-OE transgenic seeds was relatively higher than that of WT, indicating that *SsDHN*-OE transgenic lines exhibited greater salt resistance than WT (Fig. 3A).

We compared the root lengths of WT and overexpressing plants under salt concentrations of 0, 100, 200, and 300 mM for 20 d. The results showed that the root lengths of plants differed under different salt stress concentrations. Without salt stress, the root length of *SsDHN*-OE transgenic lines was slightly less than that of WT with the same growth time. As the salt concentration increased, the root lengths of the WT and *SsDHN*-OE transgenic lines decreased. However, the root lengths of *SsDHN*-OE transgenic lines were generally greater than that of WT plants under the same NaCl concentrations, showing higher salt tolerance than that of WT plants (Fig. 3B, C).

In MS medium with 200 mM NaCl, the leaves of WT and *SsDHN*-OE transgenic seedlings were particularly small and were severely dehydrated, curled, and wrinkled. WT leaves were also yellower than *SsDHN*-OE transgenic lines. Overall, they showed resistance to salt stress and were able to grow. After transplanting to normal MS medium for one week, *SsDHN*-OE transgenic lines returned to a normal growth state, while WT tobacco still exhibited leaf curl and wrinkling, which recovered completely after 10 days of growth (Fig. 4). These results indicated that overexpression of *SsDHN* enhances tolerance to salt stress in tobacco.

SsDHN-OX plants enhanced salt resistance with increased osmotic adjustment, enhanced ROS scavenging and maintained K^+/Na^+ balance

We investigated the relative conductivity, the MDA content, and the proline content of the WT SsDHN-OE transgenic line treated with different salt concentrations. The results indicated that the REC of *SsDHN*-OE transgenic lines was lower than that of WT plants (Fig. 5A), indicating that overexpression of *SsDHN* in tobacco can enhance salt tolerance by decreasing relative conductivity. The MDA content of *SsDHN*-OE lines was significantly lower than that of WT lines under salt treatment (Fig. 5B), indicating that increased expression level of *SsDHN* relieves cellular membrane damage. The proline content of *SsDHN*-OE transgenic lines was higher than that of WT (Fig. 5C), indicating that *SsDHN*-OE plants have increased salt tolerance.

When plants suffer from salt damage, membrane lipid peroxidation occurs and ROS are produced. Superoxide dismutase (SOD) has an antioxidant capacity because it can remove superoxide anions. Therefore, we investigated SOD activity of WT and *SsDHN*-OE transgenic lines treated with different salt concentrations. The activity of SOD in *SsDHN*-OE transgenic lines was higher than that in WT (Fig. 5D), indicating that overexpression of *SsDHN* in tobacco can enhance salt tolerance by increasing ROS scavenging capability.

The concentrations of Na^+ and K^+ in the leaves and roots of tobacco plants were determined. The results showed that the content of K^+ in the leaves and roots of *SsDHN*-OE transgenic plants was significantly higher than that of WT under different NaCl concentrations (Fig. 5E). The content of Na^+ in *SsDHN*-OE transgenic plants was lower than that in WT (Fig. 5F), indicating that the *SsDHN* gene can maintain the balance of intracellular K^+ and Na^+ and protect plants from ion toxicity.

Enhanced salt resistance of *SsDHN*-OE plants with reduced damage to chloroplast membrane structures

Chloroplasts are the most significant organelles that are affected by salt stress. Therefore, we observed changes in the chloroplast ultrastructure of WT and *SsDHN*-OE transgenic lines under NaCl concentrations of 0, 100, and 200 mM for 2 months. The results showed that the chloroplasts were very clear and the distribution of chloroplast adherent to the wall without salt stress. The chloroplast structures of WT tobacco were severely damaged under salt stress of 100 mM NaCl, which was manifested by the swelling and deformation of chloroplasts, increased number of osmiophilic particles and starch grains, and swelling and deformation of matrix lamellae. However, when 100 mM salt stress was applied, chloroplast damage was also observed in the *SsDHN*-OE transgenic lines, but the damage was not significant. WT plants did not grow under salt stress at 200 mM for two months. The chloroplast membrane structures of *SsDHN*-OE transgenic lines were damaged under 200 mM salt stress. Many starch granules are produced under high salt stress, which increases the concentration of the cytoplasm and maintains the normal absorption of water by cells, thereby alleviating water shortage under salt stress (Fig. 6). These structural changes involving chloroplasts were sufficient to identify that the *SsDHN*-OE transgenic lines were more tolerant to high salt concentrations than WT tobacco because of the presence of the *SsDHN* gene in the transgenic tobacco plants.

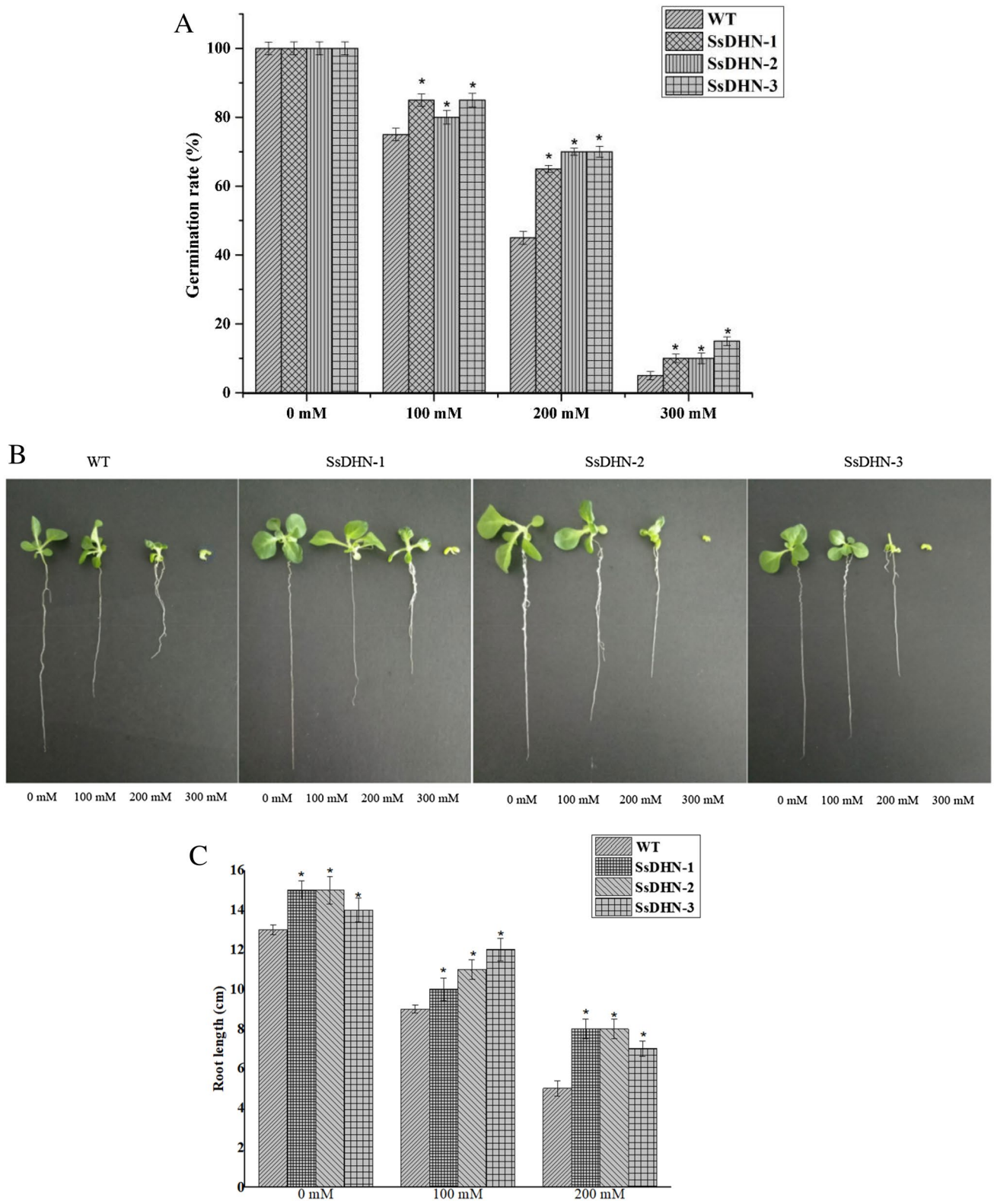

Fig. 3 Salt stress analysis of *SsDHN* transgenic plants with respect to seedling root lengths and germination rates. **A** Seed germination rates were calculated for the WT and transgenic lines grown on MS solid medium containing different concentrations of NaCl (0, 100, 200, and 300 mM) for 20 days. **B** Photographs of WT and *SsDHN*-OX seedlings on half-strength MS medium or half-strength MS medium with NaCl (100, 200, 300 mM) for 20 days. **C** Seedling root lengths of WT and transgenic lines under NaCl stress after 20 days. Error bars indicate SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's *t*-test *p*-values, $*p < 0.05$)

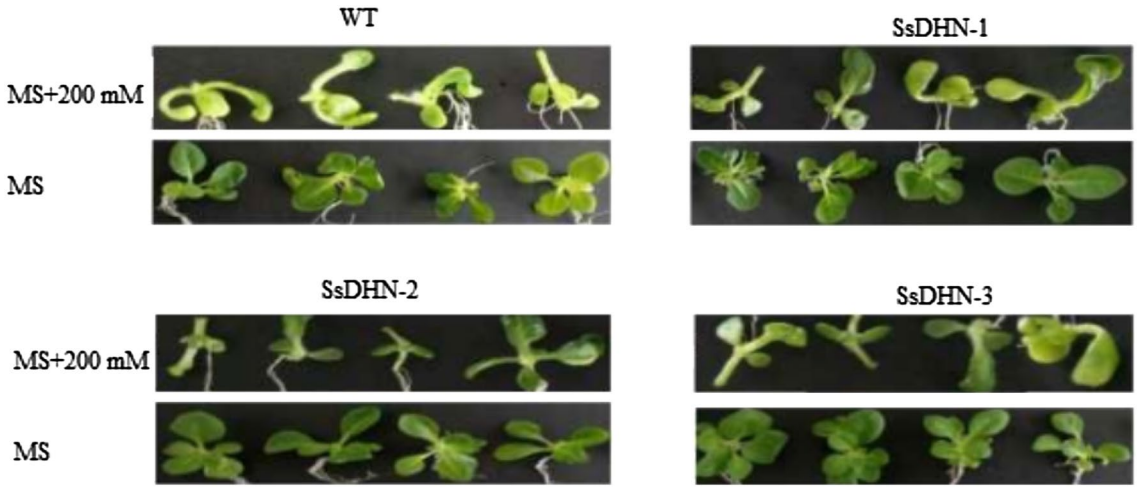

Fig. 4 Photographs of WT and transgenic lines with salt stress and recovery of tobacco seedlings. Thirty plants from the WT and from the transgenic lines were treated with 200 mM of NaCl, and then the seedlings were transferred to normal medium for recovery

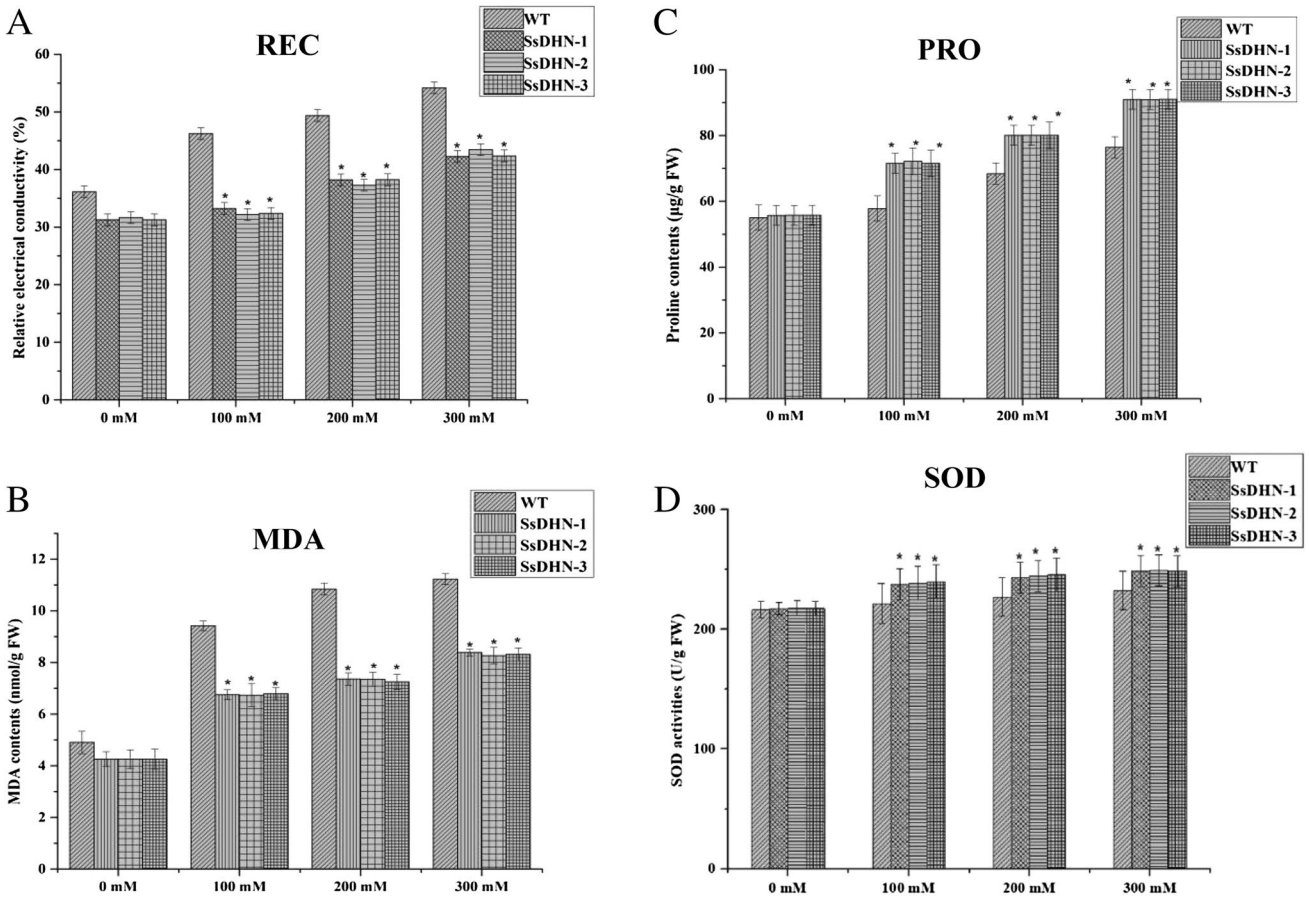

Fig. 5 Relative electrical conductivity (REC), malondialdehyde (MDA), proline, superoxide dismutase (SOD) levels, and the contents of K^+ and Na^+ in WT and *SsDHN*-overexpressing tobacco plants under normal and salt stress conditions. **A** REC content of unstressed and stressed tobacco plants. **B** MDA content of unstressed and stressed tobacco plants. **C** Proline content of unstressed and stressed tobacco plants. **D** Enzymatic activity of SOD in tobacco plants under normal and stressed conditions. **E** The content of Na^+ in tobacco plant leaves and roots under normal and stressed conditions. **F** The content of K^+ in tobacco plant leaves and roots under normal and stressed conditions. All determinations were conducted on three biological replicates. Error bars indicate SD based on three replicates. Asterisks indicate significant differences from the WT (control, Student's *t*-test *p*-values, $*p < 0.05$)

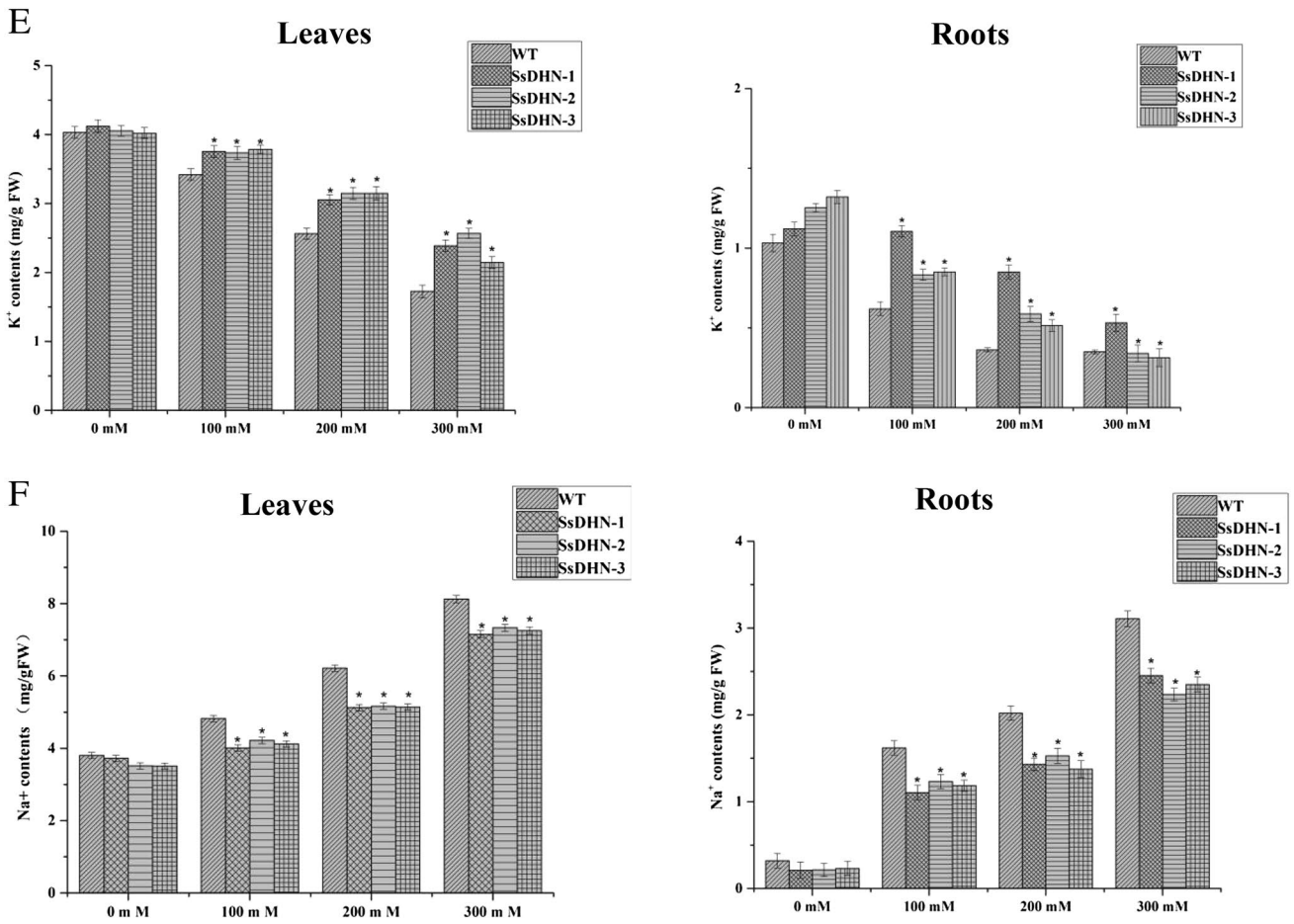

Fig. 5 (continued)

Discussion

Salt stress is a major cause of poor plant growth and crop yield reduction (Bhagi et al., 2013). Many studies have focused on functional genes that play key roles in salt tolerance, and which have attempted to elucidate the genetic and molecular basis for improving plant resistance. To date, several useful or positive genetic resources for salt stress have been identified (Ventura et al., 2015). In this study, a salt stress-related DHN from the halophyte *S. salsa*, named *SsDHN*, was identified and characterized based on sequence analyses, gene expression patterns, transgenic overexpression assays, and other physiological and biochemical tests to explore the possible mechanisms of *S. salsa* in response to salt stress. Our results showed that SsDHN positively improved salt tolerance in tobacco.

Plant DHNs are a class of highly hydrophilic proteins that have been functionally characterized and identified as being involved in responses to abiotic stresses such as drought, high salinity, and freezing (Hundertmark and Hincha, 2008; Xu et al., 2014; Zhu et al., 2014). The deduced amino acid sequence indicated that SsDHN shares typical DHN motifs, including an S-segment and two K-segments, similar to SKn-type DHNs of other plant species (Hara, 2010). In addition, *SsDHN* gene expression was significantly induced by salt stress, which is consistent with the upregulation of *DHN* gene expression in response to multiple hormones and abiotic stresses (Zhu et al., 2014; Liu et al., 2015; Aguayo et al., 2016). To elucidate the contribution of *SsDHN* to salt stress, transgenic tobacco plants overexpressing this gene were produced in our study. Our results showed that *SsDHN* transgenic lines were more tolerant to salt stress than WT plants. This enhanced tolerance was illustrated by higher seed germination and growth rates. Although the germination rate decreased as salt concentrations increased, the seed germination rate of the *SsDHN*-OE transgenic lines was higher than that of WT plants. Similarly, the root lengths of *SsDHN*-OE transgenic lines were generally longer than those of WT plants under the same NaCl concentrations, revealing greater tolerance than that of WT plants (Fig. 3). These results are consistent with previous descriptions of DHN proteins (Cao et al., 2017; Brini et al., 2007; Li et al.,

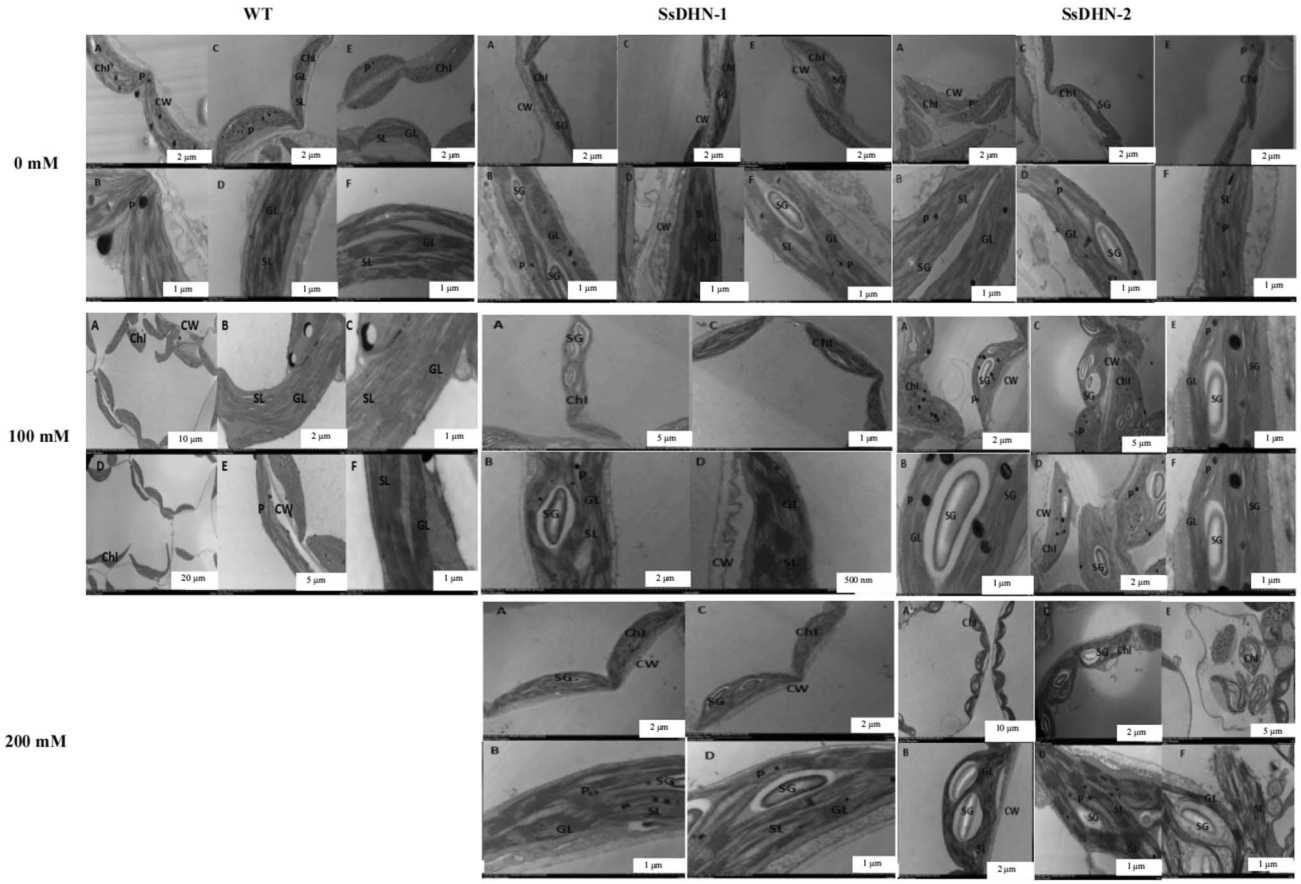

Fig. 6 Changes to chloroplast ultrastructure in WT and *SsDHN*-overexpressing tobacco plants under normal and NaCl stress conditions. WT plants did not grow under salt stress at 200 mM for two months. Chl, chloroplast; GL, grana lamella; SL, stroma lamella; SG, starch granules; P, osmiophilic particles; CW, cell wall

2017). In MS medium with 200 mM NaCl, the leaves of WT and transgenic tobacco plants exhibited water loss and shrinkage, and the leaves of WT tobacco showed bleaching. When transferred to standard growth conditions, the transgenic lines showed more rapid recovery than WT plants. This difference in recovery linked to DHN function has been reported previously (Saavedra et al. 2006). This indicates the importance of studying plant responses during stress treatment as well as during recovery conditions.

Physiological assessment showed that *SsDHN* overexpressing tobacco plants exhibited superior salt tolerance than WT. REC is an important tool for measuring osmotic stress tolerance in plants. The REC of *SsDHN*-OE transgenic lines was lower than that of WT plants. MDA is the final product of lipid peroxidation caused by ROS and is, therefore, a key indicator of osmotic stress injury in plants (Moore and Roberts, 1998). Our research indicated that the MDA content in *SsDHN*-OE lines was significantly lower than that in WT plants under salt stress. Free proline commonly accumulates in cells under stress (Ben Rejeb et al., 2014). Proline is both an osmoprotectant and an effective non-enzymatic antioxidant that maintains cell viability and prevents oxidative damage otherwise caused by ROS. Elevated accumulation of proline was measured here in *SsDHN* overexpressing lines, indicating that *SsDHN* overexpression provided better protection by regulating proline metabolism to maintain the growth of plants under osmotic stress. The activities of antioxidant-related enzymes were also measured in this study. The SOD activity of transgenic plants was significantly higher than that of WT plants. Under salt stress, plants are easily poisoned by metal ions; therefore, maintaining the balance of Na^+ and K^+ in cells is key for plants to survive in salty environments (Bojórquez-Quintal et al., 2016; Zepeda-Jazo et al., 2008). It has been reported that the increase in K^+ content in plants under salt stress is often regarded as a manifestation of tolerance traits (Adem et al. 2014; Rascio et al. 2001; Hariadi et al. 2011). The levels of K^+ in the leaves and roots of *SsDHN*-OE transgenic lines were significantly higher than that of WT plants under different NaCl concentrations. In contrast, the content of Na^+ in *SsDHN*-OE transgenic lines was lower than that in WT plants, indicating that *SsDHN* can promote ion isolation in

the vacuoles of transgenic plants, prevent Na^+ poisoning, and promote K^+ uptake. The resulting elevated vacuolar solute content enables greater water retention, allowing plants to survive under low soil water potential conditions.

The internal structure of plant cells is often closely associated with salt tolerance. The tissue structure of leaves is often different owing to different growth conditions. In recent years, the submicroscopic structures of plants have been studied under salt stress (Guntzer et al., 2012; Anwaar et al., 2015). However, there are few reports regarding changes to the submicroscopic structures of the leaves of *DHN* transgenic tobacco plants under salt stress. Due to differences in the environmental conditions modulating plant growth, the morphological characteristics, structure, size, number, and distribution of chloroplasts will also change differently, and such changes involving structural characteristics and physiological functions will be more prominent under stress (Vani et al., 2001). Therefore, these changes are often used as evidence for plant tolerance to adverse stress conditions. In this study, the chloroplast structural damage in *SsDHN*-OE transgenic lines was significantly lower than that of WT tobacco plants (Fig. 6). Simultaneously, many starch grains were produced in *SsDHN*-OE transgenic lines under high salt stress conditions, which increased cell fluid concentrations and kept cells absorbing water normally, thus alleviating the phenomenon of cell water shortage under salt stress. Taken together, these results indicated that *SsDHN*-OE transgenic lines had greater salt tolerance than WT tobacco.

In conclusion, we report the isolation and characterization of a *DHN* gene, *SsDHN*, from the halophyte *S. salsa*. Our research showed that the full-length *SsDHN* cDNA is 847 bp long and that SsDHN belongs to an SKn-type DHN. Expression of *SsDHN* in *S. salsa* was induced by salt stress. Ectopic expression of *SsDHN* in tobacco plants enhanced resistance to salt stress by increasing seedling root length, germination rate, protection of the cell membrane, maintenance of the K^+/Na^+ balance, and reducing damage to chloroplast membranes. In summary, *SsDHN* may be a valuable resource for future crop improvement programs. Further studies should focus on the regulatory mechanisms surrounding *SsDHN* to better understand the molecular mechanisms of salt stress tolerance.

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Data availability All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest The authors have not disclosed any competing interest.

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