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HaASR2 from *Haloxylon ammodendron* confers drought and salt tolerance in plants

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ABSTRACT

Abscisic acid (ABA), stress, and ripening-induced proteins (ASR), which belong to the ABA/WDS domain superfamily, are involved in the plant response to abiotic stresses. *Haloxylon ammodendron* is a succulent xerohalophyte species that exhibits strong resistance to abiotic stress. In this study, we isolated *HaASR2* from *H. ammodendron* and demonstrated its detailed molecular function for drought and salt stress tolerance. HaASR2 interacted with the HaNHX1 protein, and its expression was significantly up-regulated under osmotic stress. Overexpression of *HaASR2* improved drought and salt tolerance by enhancing water use efficiency and photosynthetic capacity in *Arabidopsis thaliana*. Overexpression of *HaASR2* maintained the homeostasis of reactive oxygen species (ROS) and decreased sensitivity to exogenous ABA and endogenous ABA levels by down-regulating ABA biosynthesis genes under drought stress. Furthermore, a transcriptomic comparison between wild-type and *HaASR2* transgenic Arabidopsis plants indicated that *HaASR2* significantly induced the expression of 896 genes in roots and 406 genes in shoots under osmotic stress. Gene ontology (GO) enrichment analysis showed that those DEGs were mainly involved in ROS scavenging, metal ion homeostasis, response to hormone stimulus, etc. The results demonstrated that *HaASR2* from the desert shrub, *H. ammodendron*, plays a critical role in plant adaptation to drought and salt stress and could be a promising gene for the genetic improvement of crop abiotic stress tolerance.

1. Introduction

The sessile nature of plants dictates that they must face various environmental challenges directly. Among these challenges, drought and high salinity are the major stress factors that can seriously threaten plant growth and crop productivity worldwide (Zhu, 2016). The estimated crop production damage caused by drought is about 7% (Lesk et al., 2016). Salinity affects approximately one billion hectares of soils (c. 7.5% of the land area on Earth) in over 100 countries (Li et al., 2019). Drought and soil salinity cause plant cells to suffer from hyperosmotic stress, as a result of water shortage (Maathuis, 2014). To cope with osmotic stress, plants have evolved a series of complex response mechanisms at the physiological and molecular levels, including the expression of stress-induced genes, reduction of reactive oxygen species (ROS), and maintenance of osmotic regulation capacity and metal ion homeostasis (Yang and Guo, 2018; Gupta et al., 2020).

Abscisic acid-, stress-, and ripening-induced (*ASR*) genes are plant-specific transcription factors (TFs) that play important roles in plant development, growth and abiotic stress responses. *ASR* genes are expressed in various organs and growth stages in a variety of plant species, and contribute to fruit development (Breitel et al., 2016; Jia et al., 2016), abscisic acid generation (Saumonneau et al., 2012), and tolerance to water deficit (Virlouvet et al., 2011; Hu et al., 2013; Li et al., 2017), salt (Kalifa et al., 2004), cold (Kim et al., 2009) and multiple

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abiotic stresses (Yang et al., 2005; Tiwari et al., 2015; Pérez-Díaz et al., 2019; Qiu et al., 2021). Due to their containing high levels of His, Glu and Lys, ASR proteins have a strongly hydrophilic region (Yang et al., 2005; Çakir et al., 2003; Wang et al., 2005), indicating the positive roles of plant ASRs in adaption to water deficiency at the transcriptional level. Since the first ASR protein was isolated from tomato (Iusem et al., 1993), some ASR genes have been identified in both monocot and dicot (Liang et al., 2019; Li et al., 2020; Yoon et al., 2021; Cortés et al., 2012; Zhou et al., 2017), but interestingly, not in the model plant Arabidopsis (González and Iusem, 2014). In addition, the genes responding to diverse abiotic stresses have mainly been identified from crop species, but only a few *ASR* genes involved in abiotic stresses have been isolated from xerohalophytes (Tiwari et al., 2015; Hu et al., 2014).

Haloxylon ammodendron (Chenopodiaceae) is a xerohalophytic shrub from the arid deserts of Asia (Xu et al., 2016), with a strong root system and highly keratinized or waxy rod-like leaves, allowing efficient water absorption and retention ability (Yang et al., 2014). This species can survive even when soil water content drops to 1.0% (Yang et al., 2014). Under osmotic stress of − 0.5 MPa, *H. ammodendron* stably maintained the water content of the assimilating branches (Lü et al., 2019). Seedlings of *H. ammodendron* are able to tolerate higher salinity conditions compared to xerophytes found in comparable environments (Song et al., 2005; Xue et al., 2012). The transcriptomes of *H. ammodendron* displayed a coordinated expression of genes that regulated stress tolerance and seedling development resource allocation to support survival in desert environment (Fan et al., 2018). Therefore, *H. ammodendron* contains rich genetic resources underlying the novel mechanisms for it to adapt to drought and salt stresses.

Previously, we found that *H. ammodendron HaASR2* was strongly induced by osmotic stress based on transcriptome analysis (Gao et al., 2018). In this study, to further investigate the molecular mechanisms underlying the function of *HaASR2* involved in plant drought and salt tolerance, we isolated the full-length cDNA of *HaASR2* from *H. ammodendron*, and analyzed the growth status, physiological parameters and transcriptome of *HaASR2*-overexpressing transgenic Arabidopsis plants under stress conditions. The results demonstrated that *HaASR2* from the desert shrub, *H. ammodendron*, plays a critical role in plant adaptation to drought and salt stress and could be a promising gene for the genetic improvement of crop abiotic stress tolerance.

2. Materials and methods

2.1. Growth conditions of H. ammodendron seedling and treatments

The seeds of *H. ammodendron* were obtained from Alxa region, Inner Mongolia, China. The surface-sterilized and uniform seeds germinated and grown in heat-sterilized vermiculite watered with 1/2 Hoagland nutrient solution in an artificial climate chamber under temperatures of 28 ± 2°C/23 ± 2°C (day/night), a relative humidity of 60%, and a photoperiod of 16 h. After growing for 8 weeks, the roots, stems and assimilating branches of *H. ammodendron* seedlings were collected for the *HaASR2* expression analysis. To examine the response of *HaASR2* to salt and drought stress, the roots of the remaining seedlings were immersed in 350 mM NaCl and 0.4% sorbitol (−0.75 MPa) as simulated drought stress. The roots of *H. ammodendron* seedlings were harvested at different time points after NaCl treatments (0, 0.5, 1, 3, 6 and 12 h) and sorbitol treatments (0, 0.5, 1, 3, 6, 12 and 24 h). All of the harvested samples were immediately frozen in liquid nitrogen and stored at −80 °C for the gene isolation expression analysis of *HaASR2*.

2.2. HaASR2 gene isolation and protein purification

By rapid amplification of cDNA ends (RACE), the full-length cDNA of *HaASR2* was cloned from the cDNA of mixed tissues of *H. ammodendron* seedlings using the SMART RACE cDNA amplification kit (Clontech, New Jersey, USA). The primer sequences of 5′RACE and 3′RACE are listed in Table S1. The *HaASR2* transcriptome sequences (Gao et al., 2018) and the sequences of the 5′RACE and 3′RACE products were spliced together. Based on the sequences of the 5′ and 3′ ends, a pair of primers was designed to amplify the *HaASR2* full-length sequence (Table S1). Multiple sequence alignment of ASRs was performed using the DNAMAN software. A phylogenetic tree was constructed using MEGA 11.

The coding sequence (CDS) of *HaASR2* from *H. ammodendron* was inserted into the prokaryotic expression vector pET-28a. The recombinant construct was subsequently transformed into *Escherichia coli* BL21 (DE3) strain. The His-HaASR2 fusion protein was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactoside) *E. coli* at 15 °C and purified with the Ni-NTA-Sefinose Column (Sangon, Shanghai, China). Circular dichroism (CD) analysis was used to dissect the secondary structure of HaASR2, which was carried out on a Chirascan-Plus CD spectrophotometer (Applied Photophysics, UK) at 25 °C. The data obtained from the wavelength of 190–260 nm was subjected to CNDD software (version 2.1, Applied Photophysics Ltd., Leatherhead, UK) to calculate the secondary structure compositions.

2.3. Quantitative PCR analysis

Total RNA was extracted using TRIzol reagent (TaKaRa, Japan) and converted to cDNA using the PrimeScript™ reverse transcription reagents kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Subsequently, quantitative real-time PCR (qRT-PCR) was conducted using SYBR Green dye (SYBR®Green Real-time PCR Master Mix-Plus, Code No. QPK-212) on an ABI StepOnePlus Real-Time PCR System, with *AtActin1* or *HaEF-1A* as internal controls. Relative changes in gene expression levels were quantitated based on three biological replicates via the $2^{-\Delta\Delta Ct}$ method. The primers for genes (*HaASR2, AtABA1*, *AtAPX1*, *AtCAT2*, *AtACTIN1*, *HaEF-1A*, *AtFER3, AtMIP1A*, *AT1G21140*, *AtPYL4*, *AtPYL5*, *AtPYL6*, *AtBZIP1*, *AT1G76800*, *AT5G38820*, *AtTPS11*, *AtORG2*, *AtCYP82C4*, *AT3G12900*, *AtPER59*, *AtTPS8*, *AtFLS4*, *AtCHI*, *AtPER39*, *AtNAS2*, *AtIRT1*) are shown in Table S2.

2.4. Subcellular localization and trans-activation activity analysis in yeast of the HaASR2 protein

The *HaASR2* coding sequence without the termination codon was fused in frame with the N-terminus of GFP in the vector PCAMBIA1300-GFP and ligated with the SACI/*Xba*I restriction site to generate a PCAMBIA1300-HaASR1-GFP recombinant plasmid. The primer sequences for constructing the HaASR2 fusion vector under the control of the CaMV35S promoter are shown in Table S1. The recombinant plasmids PCAMBIA1300-HaASR2-GFP and the control vector pCAMBIA1304-GFP were transferred separately into the epidermal cells of *Nicotiana benthamiana*. The transfected tobacco cells were incubated in darkness at 12 h and cultured for 2 days. GFP fluorescence signals were observed with a confocal laser scanning microscope (Olympus FV1000MPE2, Japan). Trans-activation activity analysis of the HaASR2 protein in yeast was performed as described previously (Li et al., 2021).

2.5. Yeast-two-hybrid and bimolecular fluorescence complementation (BiFC) assays

The two-hybrid assay was performed as described previously (Fujii et al., 2009). The *H. ammodendron* transcriptome study showed that genes related to antioxidation, betaine, proline synthesis, glucose metabolism, ion transport and aquaporin were up-regulated under osmotic stress (Gao et al., 2018). To further identify interaction between *HaASR2* and other candidate genes, we selected *HaDREB*, *HaHsfA3*, *HaABI5*, *HaAQP*, *HaBADH*, *HaPrxQ*, *HaVPP*, *HaHXT* and *HaNHX1* as candidate downstream target genes. The full-length coding sequence of *HaASR2* was cloned into pGBKT7 (binding domain [BD]) vector, and

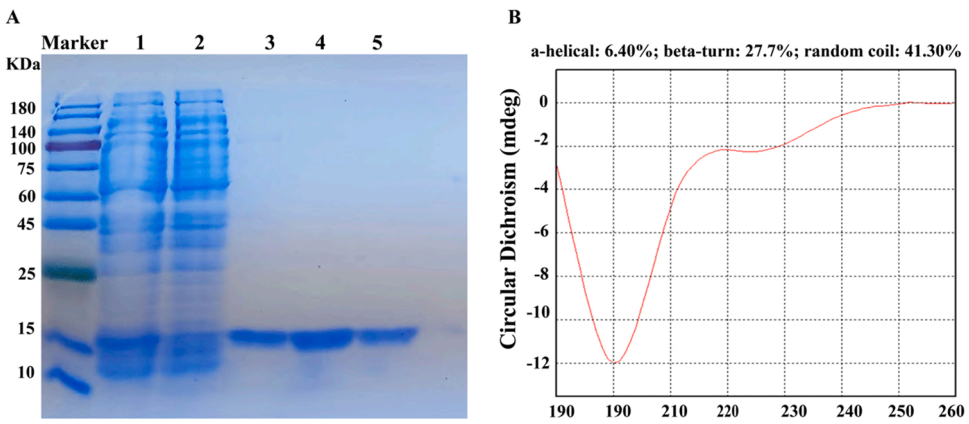

Fig. 1. Purification and circular dichroism analysis of the HaASR2 protein. **(A)** SDS-PAGE analysis of fusion protein purified by nickel agarose affinity chromatography. 1: Load; 2: Elute; 3: 20 mm imidazole eluate; 4: 50 mm imidazole eluate; 5: 500 mm imidazole eluate; **(B)** Circular dichroism analysis of the HaASR2 protein.

interaction genes into pGADT7 (activation domain [AD]) vector. The different constructs were co-transformed into the yeast strain Y2H Gold (Clontech, USA) for a two-hybrid assay. Interaction was examined by a growth assay on defined media SD (-Leu/-Trp/-Ade/-His). The primers for the constructs are shown in Table S3.

Bimolecular fluorescence complementation (BiFC) was further assayed by transient expression in tobacco leaves. The full-length coding sequence of *HaASR2* was cloned into pEarleygate201-YN (YN) and genes of interaction protein was screened by Y2H into pEarleygate202-YC (YC) vectors, respectively. The primers for the cloning are listed in Table S4. The constructs were introduced into *Agrobacterium tumefaciens* (strain GV3101) and transferred into epidermal cells of 4–5-weeks old *Nicotiana benthamiana* leaves. The YFP signal was observed by laser scanning confocal microscopy (Olympus FV1000MPE2, Japan).

2.6. Generation of HaASR2 overexpression Arabidopsis lines

The full-length coding sequence of *HaASR2* amplified from the pMD19-T-HaASR2 vector was inserted into the *Xba*I and SAC1 sites of a modified-PCAMBIA3300 vector using specific primers (Table S1). The modified-PCAMBIA3300-HaASR2 plasmid was then transformed into *Agrobacterium tumefaciens* GV3101 competent cells. The full-flowering Arabidopsis plants were used for genetic transformation by the floral dip method (Horsch et al., 1985). The T1 seeds were sprayed with 0.001% phosphinothricin, and the seedlings from the normal growth plants were collected. All homozygous lines of T4 generation seedlings were screened by phosphinothricin resistance for further experiments. The expression of *HaASR2* in the 14 independent T4 lines was analyzed by semi-quantitative RT-PCR analysis and primers are listed in Table S1. *AtActin1* was used as an internal control. Three transgenic lines (*HaASR2*-OE1, *HaASR2*-OE2 and *HaASR2*-OE3) with higher expression levels were used for further physiological and molecular characterizations under drought and salt conditions.

2.7. Exogenous ABA sensitivity assay

Seeds of the WT and *HaASR2*-OE lines were surface-sterilized in 95% ethanol and germinated on 1/2 MS medium containing 0, 3, 6 and 9 μM ABA (Sigma, USA). Germination rates were calculated at the fifth day after initiation. To test the sensitivity at post germination stage, 3-day-old seedlings were transferred to control MS plates or treated plates containing 15 μM ABA for 1 week. The root length of each seedling was measured after 7 days of ABA treatment at 28 ± 2 ℃/23 ± 2 ℃ (day/night) with a 16 h photoperiod.

2.8. Morphological, physiological and biochemical determinations of transgenic plants under drought and salt stresses

Seeds of *HaASR2*-OE and WT were sown on 1/2 MS medium supplemented with 100 mM NaCl or 150 mM mannitol. To test drought tolerance, 20-day seedlings of *HaASR2*-OE and WT grown in pots were subjected to water withdrawal for 7 days. For salt treatment, 16-day-old seedlings of *HaASR2*-OE and WT grown in pots were treated with 200 mM NaCl for 7 d. Whole seedlings were used for physiological measurements under both drought and salt stresses.

Shoots and roots of the *HaASR2*-OE and WT plants were collected to measure fresh weight, dry weight and water content. The osmotic potential of leaf sap was determined with a cryoscopic osmometer (OSMOMAT-030, GONOTEC GmbH, Germany). The readings ($mmol \cdot kg^{-1}$) were used to calculate the solute potential (s) in MPa (mega Pascal) with the formulas = -the readings × R × T, where R = 0.008314 and T = 298.8. The relative membrane permeability (RMP) of leaf cells was detected using a conductivity meter (EC215, Hanna, Woonsocket, RI, USA). For water loss rates, the fully expanded leaves of plants were detached and weighed immediately at room temperature. Subsequently, the fresh weight of detached leaves was monitored at intervals of 0.5, 1, 2, 3, 4, 5, 6 and 7 h. Water loss was calculated from the decrease in fresh weight compared to time zero. Catalase (CAT) and ascorbate peroxidase (APX) activities as well as H_2O_2, proline and betaine contents were quantified with reagent kit (Suzhou Comin Biotechnology Co., Ltd., Suzhou, China). ABA extraction and quantification were performed according to the previous method (Li et al., 2021).

The fully expanded young leaves detached from *HaASR2*-OE and WT were placed on slides abaxial side up immediately, and photographs were obtained by scanning electron microscopy (Apreo S) to examine the stomatal aperture. Transmission electron microscope (TEM) analysis of seedling leaves treated with 150 mM mannitol was performed as described previously (Gao et al., 2015). The net photosynthetic rate and stomatal conductance were measured by an automatic photosynthetic measuring apparatus (LI-6400, Li-Cor Biosciences, Lincoln, NE, USA, with a Li-Cor sample chamber). Water use efficiency was calculated with the net photosynthetic rate divided by the transpiration rate. Leaf chlorophyll content was measured by the acetone and alcohol method.

2.9. Transcriptome analyzes

We selected *HaASR2*-OE2 plants for transcriptome sequencing. The WT and *HaASR2*-OE2 plants were grown in nutrient solution for 3 weeks and then treated with 200 mM sorbitol (abbreviated as Sb). Six hours after treatment, shoot and root samples were collected from WT or OE

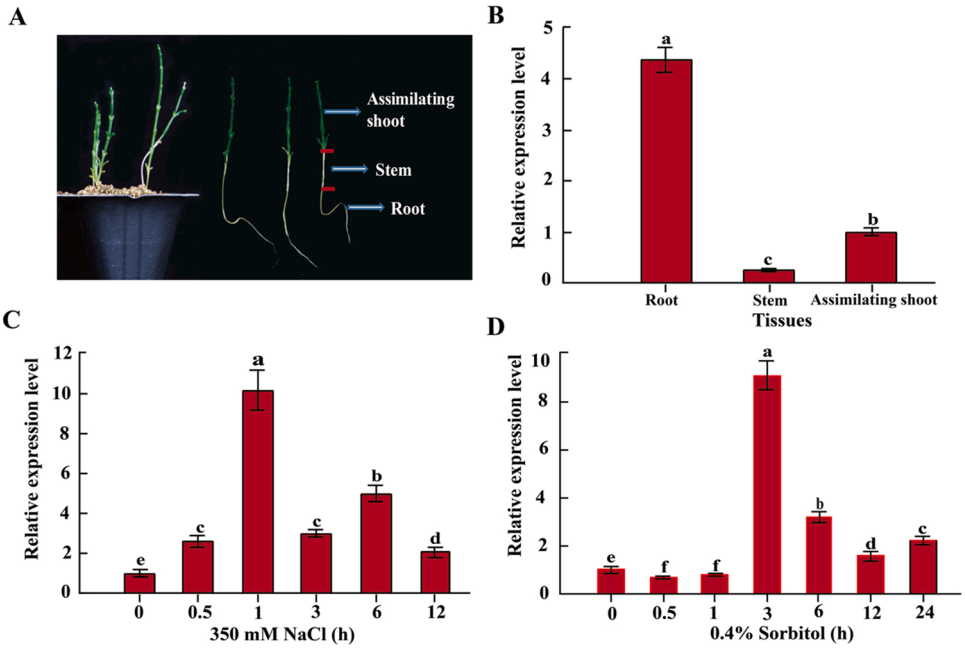

Fig. 2. Expression analysis of *HaASR2* in *H. ammodendron*. **(A)** Tissues of *H. ammodendron*. **(B)** Expression analysis of *HaASR2* in different tissues of *H. ammodendron*. **(C)** Expression analysis of *HaASR2* in roots under 350 mM NaCl treatment. **(D)** Expression analysis of *HaASR2* in roots under 0.4% sorbitol treatment. Bars indicate standard deviation (SD) ($n = 3$) and different letters indicate significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

plants for total RNA extraction. Each sample had three biological replicates. Total RNA was extracted using TRIzol reagent (Takara, Japan) according to the manufacturer's protocol. RNA-Seq was performed by Majorbio Bio-pharm Technology Co. Ltd (Shanghai, China). The sequence data was analyzed on the online platform of Majorbio Cloud Platform. Quantification of the gene expression levels was estimated by transcripts per million (TPM). Gene Ontology (GO) enrichment analysis of the DEGs were performed with $P < 0.05$, and the Arabidopsis genome was set as the background.

2.10. Statistical analysis

The statistical significance between the control and treatment groups was evaluated using one-way analysis of variance (ANOVA) and Duncan's multiple range test with SPSS version 19.0 software. Significant differences ($P < 0.05$) were indicated by different lowercase letters. All experimental results were presented as the mean value and standard deviation (SD) ($n \geq 3$).

3. Results

3.1. Cloning and molecular characterization of the HaASR2 gene

The full-length cDNA of the *HaASR2* gene (GenBank accession number: OL908904) was isolated from *H. ammodendron*. The *HaASR2* genome sequence contained an open reading frame (ORF) of 324 bp, encoding a protein of 107 amino acids. The prokaryotic expression showed that the molecular weight of the HaASR2 protein was ~17 kDa (Fig. 1A). The CD analysis showed that ~6.40%, ~27.70% and ~41.30% of the amino acid residues were present in the α-helical, beta-turn, and random coil conformations, respectively (Fig. 1B). Sequence variation analysis indicated that HaASR2 had a stretch of six His residues among eight amino acids in the N-terminal consensus sequence, which was a typical Zn-binding domain. In addition, a highly conserved

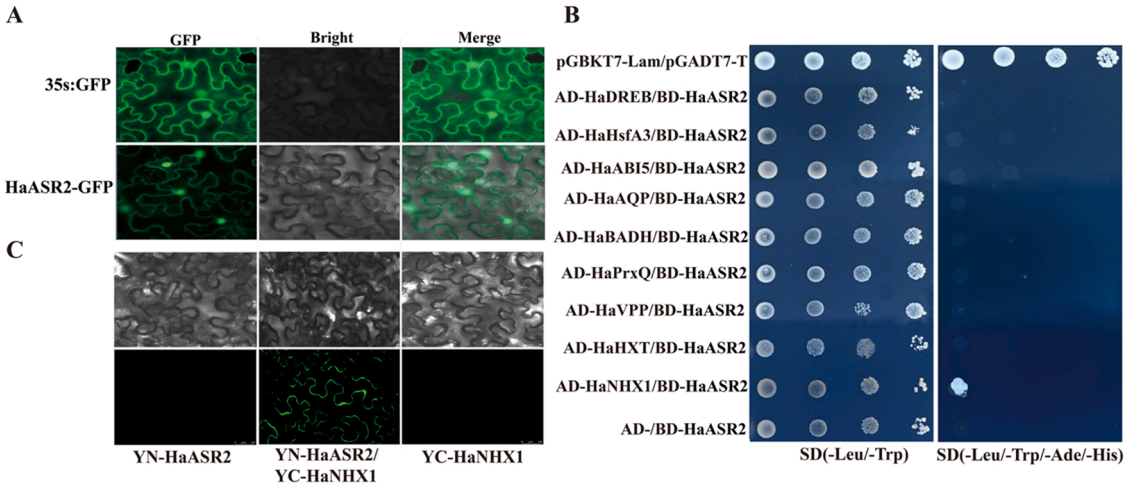

Fig. 3. Subcellular localization and interacting proteins of HaASR2. **(A)** Subcellular localization of HaASR2. **(B)** Y2H assay of HaASR2 interacting proteins. **(C)** BiFC assay in tobacco.

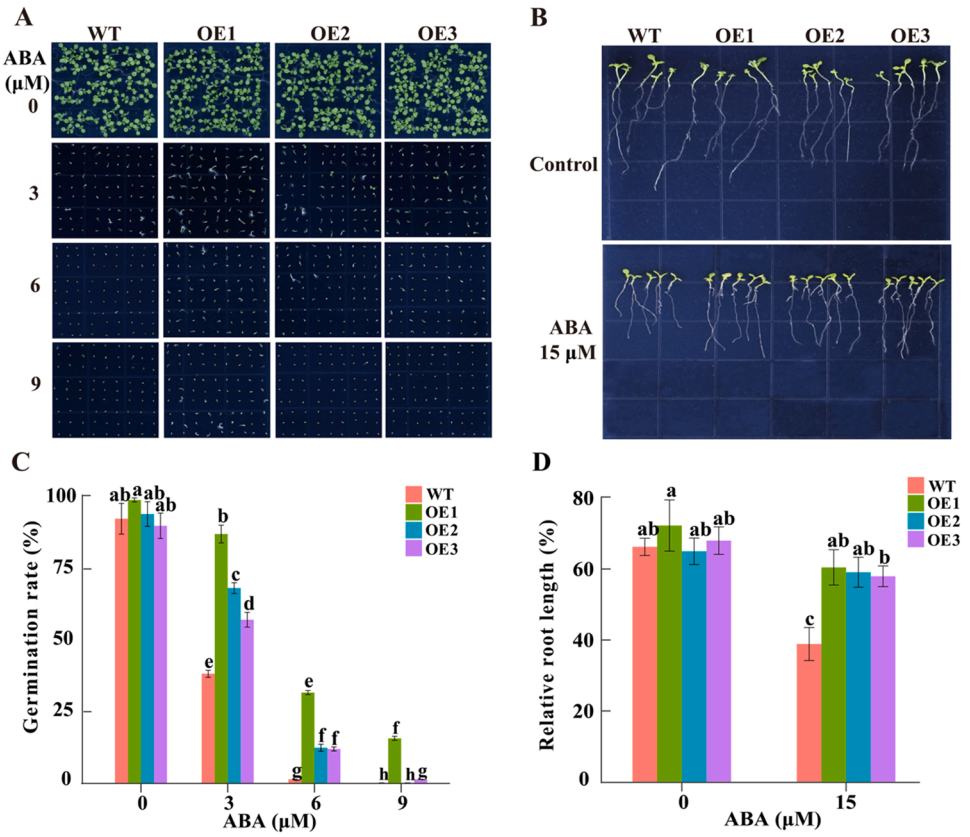

Fig. 4. The sensitivity to exogenous ABA of *HaASR2*-OE2 and WT plants. **(A, C)** Germination assay of *HaASR2* overexpression in Arabidopsis lines treated with various ABA concentrations. Photographs were taken 10 d after sowing. Seeds with emergent radicles were scored as the germination rate 5 d after sowing. The experiments were carried out in triplicate with 81 seeds in each plate. Three-day-old seedlings were transferred to plates containing 15 μM ABA and photographs **(B)** were taken and relative root length **(D)** was measured 7 d after transfer. Bars indicate standard deviation (SD) ($n = 8$) and different letters indicate significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

region of at least 70 amino acids in various plant species was observed in a long C-terminal region of HaASR2 (Fig. S1), including two Ala-rich sequences and a class of plant-specific proteins with ABA/WDS domains. Furthermore, phylogenetic analysis revealed that plant ASR proteins were branched into three broad groups, and HaASR2 was very close to Chenopodiaceae species such as *Beta vulgaris*, and *Chenopodium quinoa* (Fig. S2). The hydrophilic/hydrophobic analysis showed that HaASR2 belonged to hydrophilic proteins (Fig. S3).

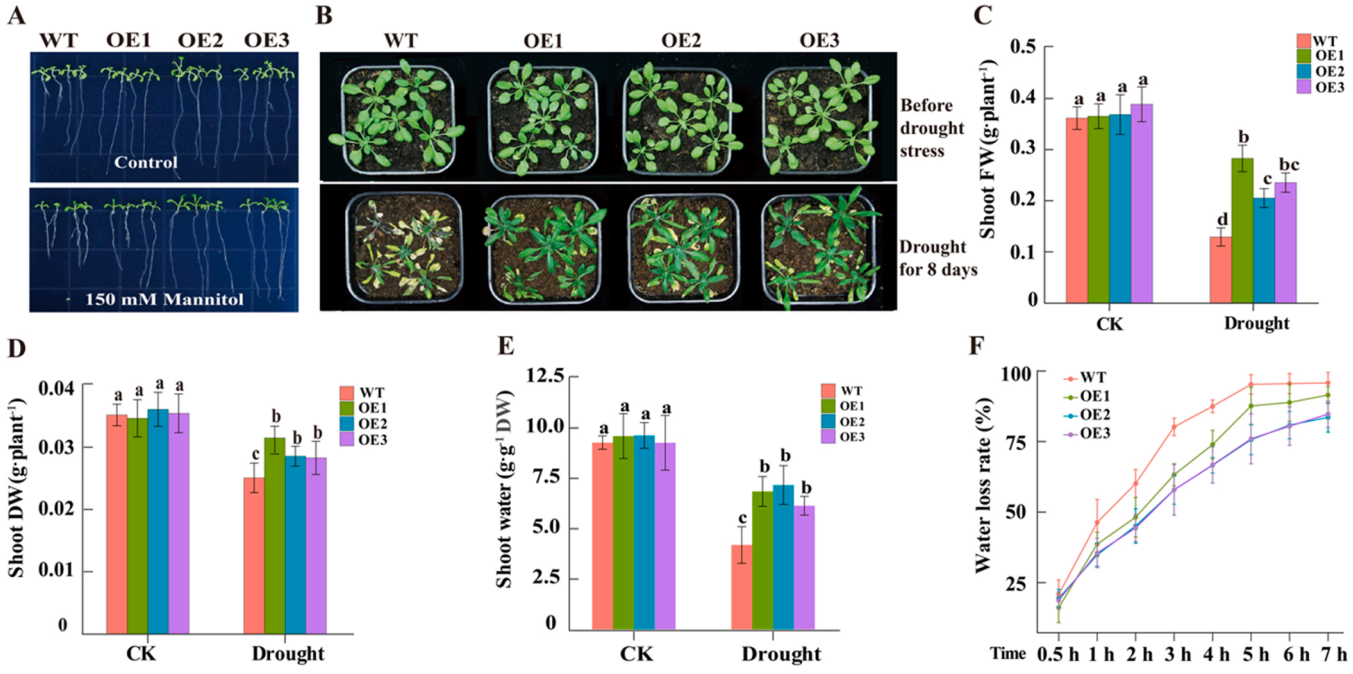

Fig. 5. Overexpression of *HaASR2* conferred drought stress tolerance in Arabidopsis. **(A)** Phenotypes of 9-day-old seedlings after normal and 150 mM mannitol treatments. **(B)** Growth performance of *HaASR2* overexpressing plants without irrigation for 7 d. Shoot fresh weight **(C)**, shoot dry weight **(D)** and shoot water content **(E)**, Water loss rates **(F)** of WT and OE plants under normal and drought conditions. Bars indicate standard deviation (SD) ($n = 8$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

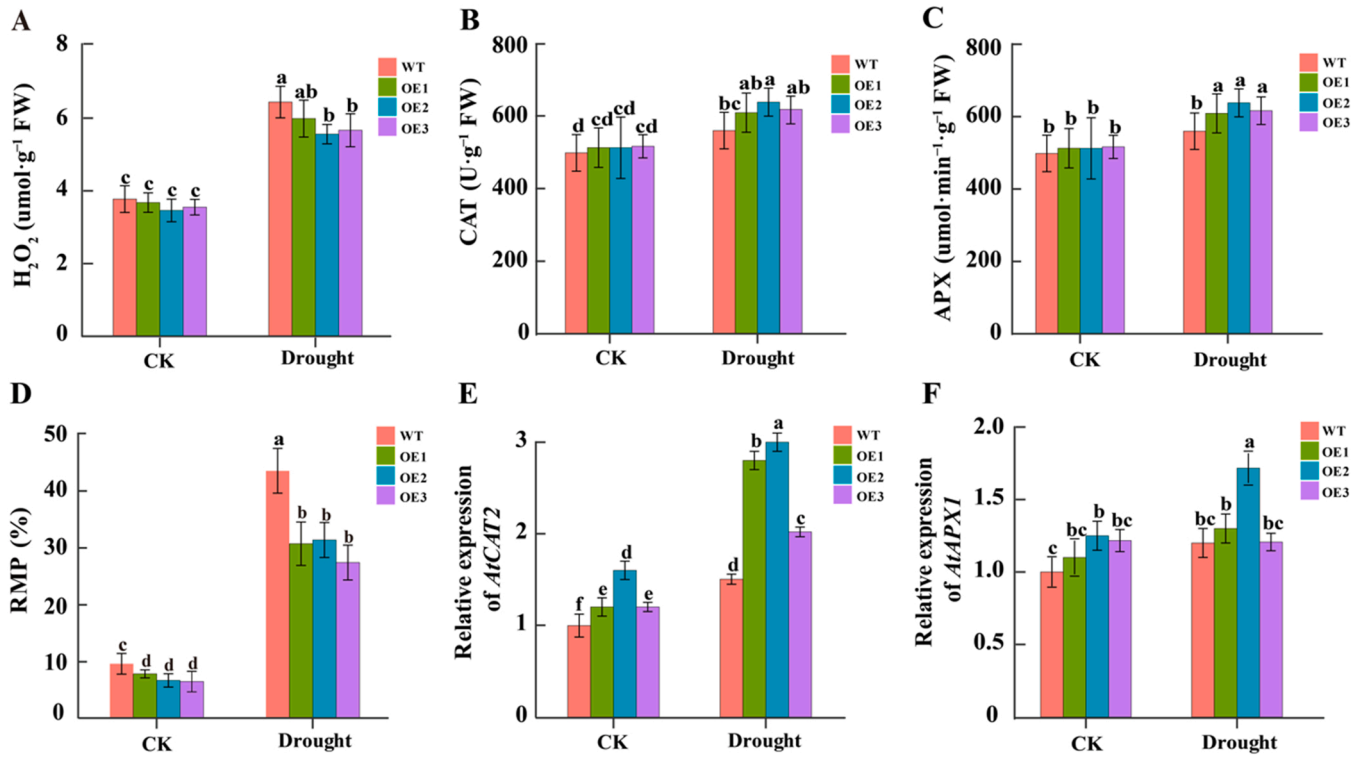

Fig. 6. H_2O_2 accumulation, CAT and APX activities, expression analysis of genes relevant in H_2O_2 scavenging and RMP in *HaASR2*-OE and WT under normal and drought stress·H_2O_2 content **(A)**, CAT activity **(B)**, APX activity **(C)**, *AtCAT2* expression level **(D)**, *AtAPX1* expression level **(E)** and RMP **(F)** in *HaASR2*-OE plants without irrigation for 7 d and normal condition. RMP, relative membrane permeability. Bars indicate standard deviation (SD) ($n = 6$) and different letters indicate a significant difference at $P < 0.05$(ANOVA and Duncan's multiple comparison test).

3.2. Stress-responsive expression patterns of HaASR2 in H. ammodendron

To analyze the stress-response expression patterns of *HaASR2* in *H. ammodendron*, we first characterized the tissue-specific expression of *HaASR2* in roots, stems and assimilating shoots of *H. ammodendron* (Fig. 2A). The expression of *HaASR2* in roots was significantly higher than that in other tissues (Fig. 2B). Additionally, the transcript of *HaASR2* began to increase at 0.5 h after NaCl stress, peaked at 1 h and then declined (Fig. 2C). Under 0.4% sorbitol treatment, *HaASR2* transcripts induced significantly at 3, 6, 12 and 24 h, and reached a maximum level at 3 h (Fig. 2D). These results indicated that *HaASR2* was expressed primarily in roots and was strongly induced by salt and drought stress.

3.3. Subcellular localization and protein-protein interaction detection of HaASR2

Through the subcellular localization experiment, we observed that both the GFP control and HaASR2 were localized in the entire cell (Fig. 3A). As ASR proteins have previously been reported to bind to DNA motifs (Ricardi et al., 2014; Arenhart et al., 2014), it was necessary to analyze the transcription activity of HaASR2. However, the expression of the GAL4-binding domain-HaASR2 fusion protein in yeast did not lead to reporter gene expression and did not form homodimers to properly function (Fig. S4), indicating that HaASR2 had no transcriptional activity in yeast. To identify proteins interacting with HaASR2, a yeast-two-hybrid assay was performed. As shown in Fig. 3B, cells containing pGADT7(AD)-HaNHX1/BD-HaASR2 grew well in SD/-Leu/-Trp/-Ade/-His medium, suggesting HaASR2 interacted with the HaNHX1. The BiFC results further confirmed that HaASR2 positively interacted with the HaNHX1 in tobacco epidermal cells (Fig. 3C).

3.4. Overexpression of HaASR2 reduced exogenous ABA sensitivity

To identify the sensitivity of *HaASR2*-OE to ABA, we examined the germination rates and relative root length of Arabidopsis by using different concentrations of exogenous ABA treatments (Fig. 4). Under normal conditions, there were no significant differences in seed germination rates between *HaASR2*-OE lines and WT plants. However, the germination rate of *HaASR2*-OE under ABA treatment was obviously higher than that of the WT (Fig. 4A). The germination rate of the WT was almost 0 and was up by 16% in *HaASR2*-OE1 treated with 9 μM ABA (Fig. 4C). Furthermore, the relative root length of *HaASR2*-OE was significantly longer than that of WT plants under 15 μM ABA treatment (Fig. 4B, D). These results suggested that overexpression of *HaASR2* remarkably reduced ABA sensitivity of transgenic plants.

3.5. Overexpression of HaASR2 significantly enhanced drought tolerance of Arabidopsis plants

To verify the function of *HaASR2* under drought conditions, we first analyzed the main morphological phenotypes of *HaASR2* transgenic Arabidopsis plants (*HaASR2*-OE) under drought treatment. The inhibition ratio of primary root and leaf growth was significantly lower in *HaASR2*-OE lines than that in WT plants under 150 mM mannitol treatment or drought stress (Fig. 5A, B). Additionally, shoot fresh weight and dry weight and root fresh weight were significantly higher than in three OE lines those in WT plants under drought stress (Fig. 5C, D, Fig. S5). Under normal conditions, *HaASR2*-OE lines and WT plants did not differ in shoot water content, while *HaASR2*-OE lines exhibited higher shoot water content than WT plants after 7 d of drought stress (Fig. 5E). Water loss rates of *HaASR2*-OE lines from 0.5 h were significantly lower than those of WT plants (Fig. 5F).

Drought considerably induced H_2O_2 content in both *HaASR2*-OE and WT plants, but H_2O_2 accumulation in *HaASR2*-OE was significantly

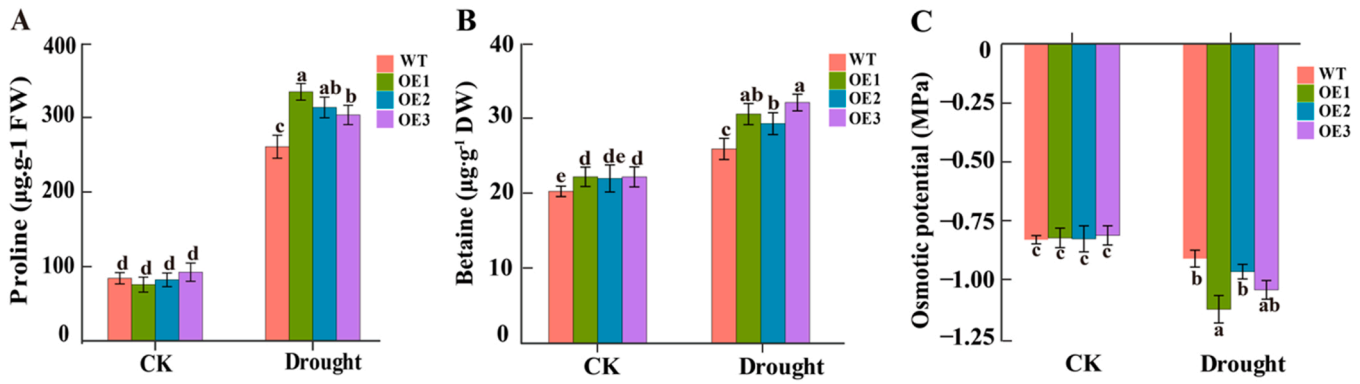

Fig. 7. Osmoregulation substances of *HaASR2* overexpressing plants of Arabidopsis under normal and drought stress. Proline content **(A)**, betaine content **(B)** and osmotic potential **(C)** in *HaASR2*-OE and WT plants without irrigation for 7 d and under normal condition. Bars indicate standard deviation (SD) ($n = 6$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

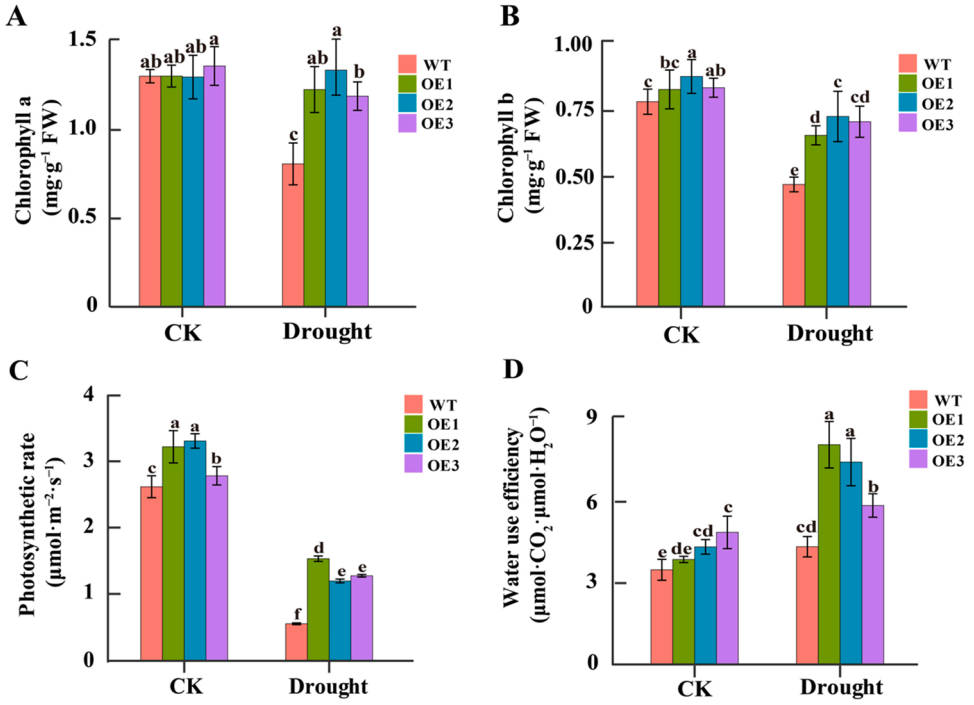

Fig. 8. Photosynthetic characteristics of *HaASR2* overexpressing plants of Arabidopsis under normal conditions and under drought stress. Chlorophyll *a* content **(A)**, chlorophyll *b* content **(B)**, photosynthesis rate **(C)**, and water use efficiency **(D)** of *HaASR2* overexpression plants without irrigation for 7 d. Bars indicate standard deviation (SD) ($n = 6$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

lower than that in the WT (Fig. 6A). No significant differences in CAT and APX activities were observed between *HaASR2*-OE and WT plants under normal conditions, but differences occurred between two types of plants after drought stress (Fig. 6B, C). Additionally, *HaASR2*-OE had significantly higher transcript levels of *AtCAT2* and *AtAPX1* than those in the WT (Fig. 6E, F). Furthermore, RMP, as an important indicator of membrane injury, was significantly higher in WT plants than that in *HaASR2*-OE lines after 7 d of drought stress (Fig. 6D). *HaASR2*-OE lines accumulated more proline and betaine and had higher osmotic potential compared to WT plants under drought stress (Fig. 7). The relative value of endogenous ABA content and the expression level of ABA biosynthesis genes *AtABA1* were significantly lower in *HaASR2*-OE than those in the WT under drought stress (Fig. S6). These results demonstrated that *HaASR2* was able to induce compound accumulation for osmotic adjustment and regulated ROS homeostasis by affecting the activity of H_2O_2-scavenging enzymes and elevating expression of genes encoding these enzymes under drought stress, thereby preventing or reducing the injury induced by drought stress.

To explore the effects of HaASR2 on photosynthesis, we analyzed the changes in leaf mesophyll cell ultrastructure and photosynthetic parameters of *HaASR2*-OE lines. Under normal condition, the ultrastructural changes were similar between *HaASR2*-OE2 and WT plants. Under drought stress, the cellular structure of the WT was severely destroyed, but the membrane structure of *HaASR2*-OE2 was basically intact, and its stromal lamellae of chloroplasts were only slightly dissolved (Fig. S7). Furthermore, chlorophyll *a* and *b* content, photosynthetic rate, and water use efficiency were significantly higher in *HaASR2*-OE than those in WT plants after drought stress (Fig. 8). The stomata closed completely in the WT and partially opened in *HaASR2*-OE under drought stress (Fig. S8A). Subsequently, higher stomatal conductance was observed in *HaASR2-OE* than in WT plants (Fig. S8B). These results indicated that overexpression of *HaASR2* was able to maintain photosynthesis in plants under drought stress.

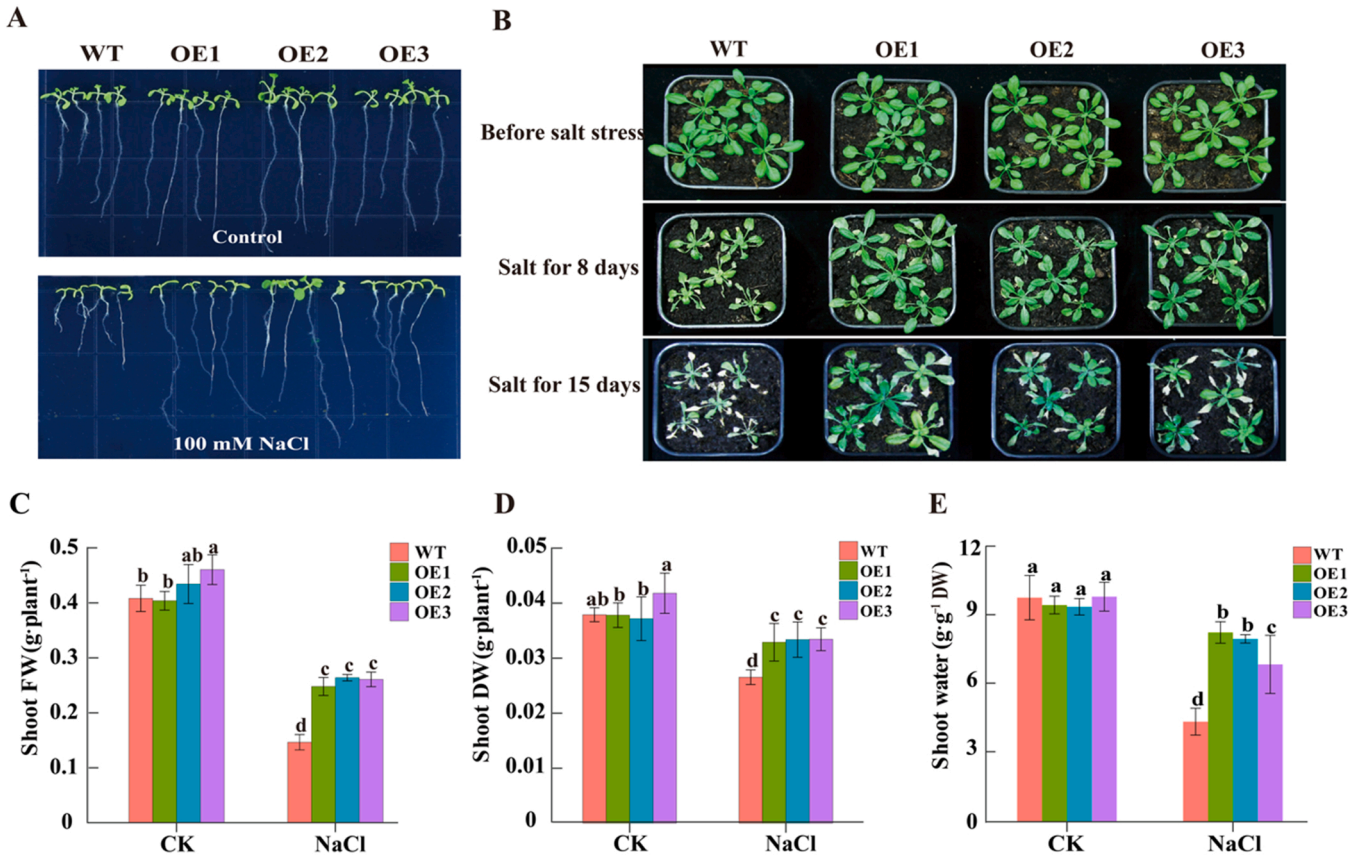

Fig. 9. Overexpression of *HaASR2* conferred salt stress tolerances in Arabidopsis. **(A)** Phenotypes of 9-day-old seedlings under normal and 150 mM mannitol treatments. **(B)** Growth performance of *HaASR2* overexpressing plants with salt treatment for 7 d and 15 d. Shoot fresh weight **(C)**, shoot dry weight **(D)** and shoot water content **(E)** of WT and OE plants under normal condition and NaCl stress. Bars indicate standard deviation (SD) ($n = 8$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

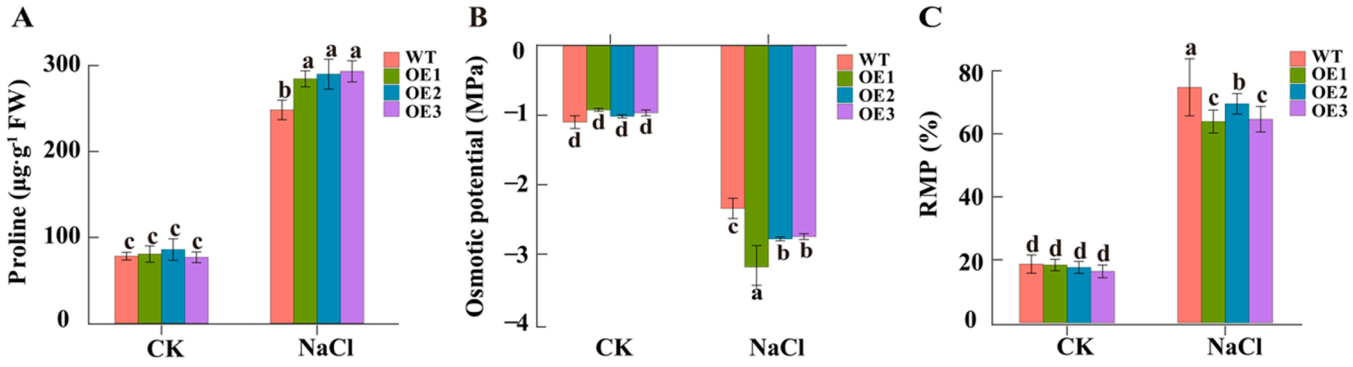

Fig. 10. Osmoregulation and RMP assay of *HaASR2* overexpressing plants of Arabidopsis under normal condition and under salt stress. Proline content **(A)**, osmotic potential **(B)** and RMP **(C)** in *HaASR2*-OE and WT plants under normal and NaCl conditions. RMP, relative membrane permeability. Bars indicate standard deviation (SD) ($n = 8$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

3.6. Overexpression of HaASR2 significantly enhanced salt tolerance of Arabidopsis plants

To evaluate the effects of *HaASR2*-OE on salt tolerance, we tested the performance of the WT and *HaASR2*-OE lines under NaCl treatment. The primary root and leaf growth of WT and *HaASR2*-OE plants were inhibited under salt stress (Fig. 9A, B). However, all three OE lines displayed higher root and leaf growth, biomass and shoot water content than WT after 7 d of salt stress (Fig. 9C, D, S9, 9E). Moreover, *HaASR2*-OE plants showed a significant increase in proline content and osmotic potential compared with WT plants (Fig. 10A, B). Additionally, higher RMP was found in WT than that in *HaASR2*-OE under salt stress (Fig. 10C).

Photosynthesis-related parameters were also assessed in *HaASR2*-OE and WT plants. The results showed that chlorophyll content, water use efficiency and photosynthetic rate in *HaASR2*-OE were all significantly higher than those in WT plants after salt stress (Fig. 11). These results indicated that overexpression of *HaASR2* was able to confer salt stress tolerance by improving the photosynthetic capacity of Arabidopsis plants.

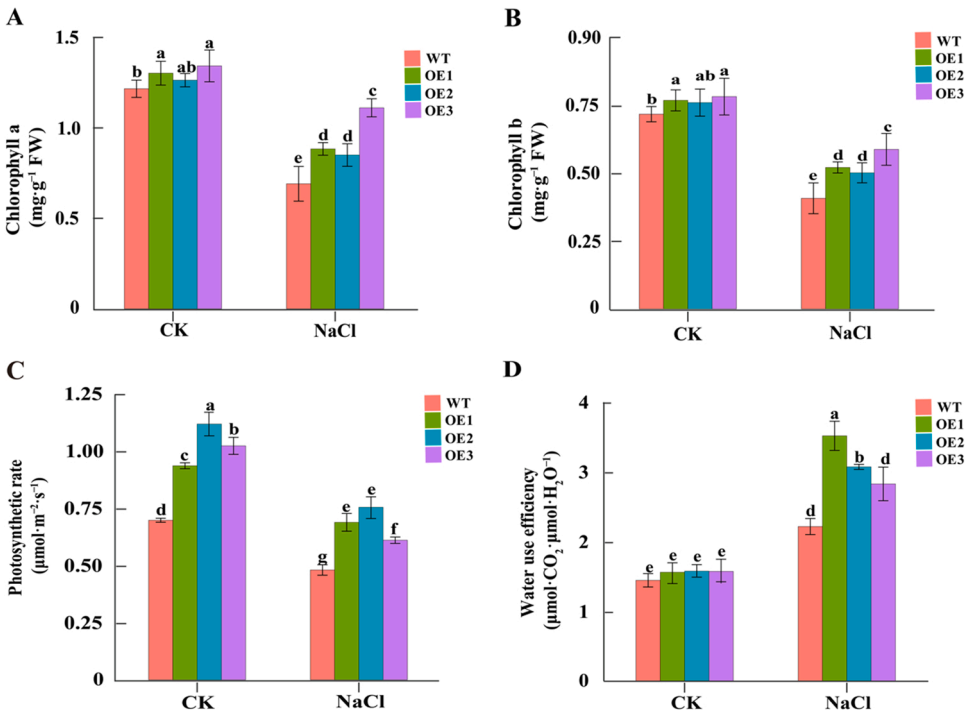

Fig. 11. Photosynthetic characteristics of *HaASR2* overexpressing plants of Arabidopsis under normal condition and under salt stress. Chlorophyll *a* content **(A)**, chlorophyll *b* content **(B)**, photosynthesis rate **(C)** and water use efficiency **(D)** of *HaASR2* overexpression plants under normal and salt stress. Bars indicate standard deviation (SD) ($n = 6$) and different letters indicate a significant difference at $P < 0.05$ (ANOVA and Duncan's multiple comparison test).

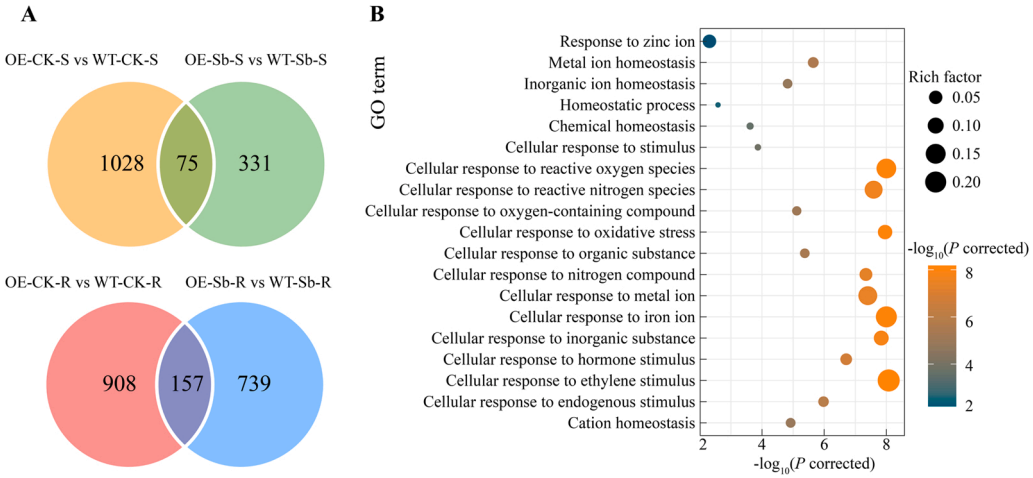

Fig. 12. Transcriptome profiling showing comparisons between *HaASR2*-OE and WT plants under normal condition and under 200 mM sorbitol. **(A)** Venn diagrams show statistically significant DEGs in OE-CK-S vs WT-CK-S and OE-Sb-S vs. WT-Sb-S, OE-CK-R vs. WT-CK-R and OE-Sb-R vs. WT-Sb-R. **(B)** GO enrichment categories of up-regulated DEGs in OE-Sb-S vs. WT-Sb-S and OE-Sb-R vs. WT-Sb-R with *P* corrected < 0.01.

3.7. Transcriptomic comparisons between HaASR2-OE and WT plants under osmotic stress

To elucidate possible molecular mechanisms of *HaASR2* improving drought and salt resistance, the transcriptomes of roots and shoots in *HaASR2*-OE2 and WT plants under normal conditions and under 200 mM sorbitol treatment were analyzed by RNA sequencing. Shoots or roots of OE and WT plants under normal conditions (abbreviated as OE-CK-S, OE-CK-R, WT-CK-S and WT-CK-R), and under 200 mM sorbitol treatment (abbreviated as OE-Sb-S, OE-Sb-R, WT-Sb-S and WT-Sb-R) were compared pair-wise, respectively. A total of 1103, 1065, 406 and 896 differentially expressed genes (DEGs) were found in the pair-wise comparison of transcriptomes of OE-CK-S vs. WT-CK-S, OE-CK-R vs. WT-CK-R, OE-Sb-S vs. WT-Sb-S and OE-Sb-R vs. WT-Sb-R, respectively ($P < 0.05$) (Fig. 12A, Table S5). However, only 75 and 157 DEGs overlapped between control and stress conditions in OE-S vs. WT-S and OE-R vs. WT-R, respectively. To validate the RNA-Seq results, the relative expression levels of 20 selected DEGs were further analyzed using qRT-PCR. The results from RNA-Seq and qRT-PCR were consistent for these 20 DEGs. The similar expression patterns observed between these two methods confirmed the reliability of the RNA-seq results (Fig. S10).

To identify the biological function of the above DEGs involved in osmotic tolerance in *HaASR2*-OE plants, we performed a GO analysis on two DEG sets in comparative analyses (OE-Sb-S vs. WT-Sb-S and OE-Sb-R vs. WT-Sb-R) (Fig. 12B, Table S6). Under osmotic stress, most DEGs mainly participated in "response to reactive oxygen species", "response

to reactive nitrogen species", "metal ion homeostasis" and "response to hormone stimulus" in the root (Fig. 12B), while a large number of genes were enriched in related to "carbohydrate metabolic process", "anthocyanin metabolic process" and "red, far-red light phototransduction" pathways in the shoot (Table S6). These results indicated that *HaASR2* might mainly regulate different expression of ROS scavenging, metal ion homeostasis, response to hormone stimulus and carbohydrate metabolic process-related genes under osmotic stress.

4. Discussion

As a stress-inducible gene, most *ASRs* were cloned and reported in several glycophytes exposed to abiotic stresses (Hu et al., 2013; Qiu et al., 2021). However, there was little information on *ASR* genes in xerohalophytes that can survive in the harshest environments. In the present study, we isolated the *HaASR2* gene from the xerohalophytic *H. ammodendron*, which encoded a 17 kDa protein in both the cytosol and the nucleus. After comparing the ASR2 from *H. ammodendron* and glycophytic plants, we found that their N-terminal sequence was quite different (Fig. S1).

ASR proteins bind to DNA motifs (Ricardi et al., 2014; Arenhart et al., 2014) and interact with a variety of proteins that play positive roles in abiotic stress tolerance. For example, tomato SlASR1 interacted with ARF2A, which regulated ethylene-dependent ripening process (Breitel et al., 2016). OsASR5 in rice interacted with HSP40 and ferredoxin to prevent their degradation in response to water stress (Li et al., 2017). In this study, we found that *H. ammodendron* HaASR2 interacted with NHX1, a new ASR-interacting protein (Fig. 3). NHX1 is a Na^+/H^+ antiporter that can use the transmembrane proton gradient produced by the vacuolar membrane H^+-ATPase and H^+-PPiase as the driving force to compartmentalize Na^+ from the cytoplasm into vacuoles, avoiding ion toxicity and maintaining intracellular ion balance (Ma et al., 2016). The role of NHX1 in salinity or drought tolerance have been extensively studied in plant species (Wu et al., 2011; Huang et al., 2018; Zhang et al., 2019; Solis et al., 2022; Tang et al., 2013; Du et al., 2010). Overexpression of NHX1 improved drought and salt tolerance by increasing plant growth, SOD activity, proline content, and cell membrane stability, and reduced ROS accumulation and MDA content, as well as regulating Na^+ compartmentalization (Zhang et al., 2019; Faïc Xal Brini et al., 2007; Asif et al., 2011). Given that NHX1 is strongly associated with the stress tolerance, our results suggested that the interaction between HaASR2 and HaNHX1 contributed to drought and salt tolerance of Arabidopsis. Moreover, ASR proteins have shown a molecular chaperone function and are able to prevent protein degradation and inactivation in several plant species (González and Iusem, 2014; Hand et al., 2011; Konrad and Bar-Zvi, 2008; Dai et al., 2011; Hsu et al., 2011). Therefore, we proposed that HaASR2 could act as a molecular chaperone to protect HaNHX1 from degradation to increase plant adaptation to drought and salt stress.

Many ASRs are functionally involved in abiotic stress responses, acting as molecular chaperons (Dai et al., 2011), osmotic adjustment proteins (Zhang et al., 2019), metal-binding protein (Li et al., 2017), and antioxidation or detoxification proteins (Hu et al., 2013; Ye et al., 2019). In this study, the expression of *HaASR2* was significantly induced by NaCl or sorbitol. The results were consistent with those *ASR* genes found in *Canavalia rosea* (Lin et al., 2021), chickpea (Liang et al., 2019) and wheat (Li et al., 2020) in response to drought or salt stress, suggesting that increased *ASR* expression might enable genotypes to perform better under stress conditions. Furthermore, the overexpression of *HaASR2* improved water-retention capacity through osmotic adjustment by a greater accumulation of proline and betaine in the *HaASR2*-OE lines of Arabidopsis (Figs. 7, 10). In this case, the stomata were kept partially opened in *HaASR2*-OE plants under drought stress, resulting in an improved photosynthesis rate under drought and salt treatments (Figs. 8, 11). Overexpression of *HaASR2* also reduced electrolyte leakage and H_2O_2 content and enhanced the capacity of the ROS-scavenging

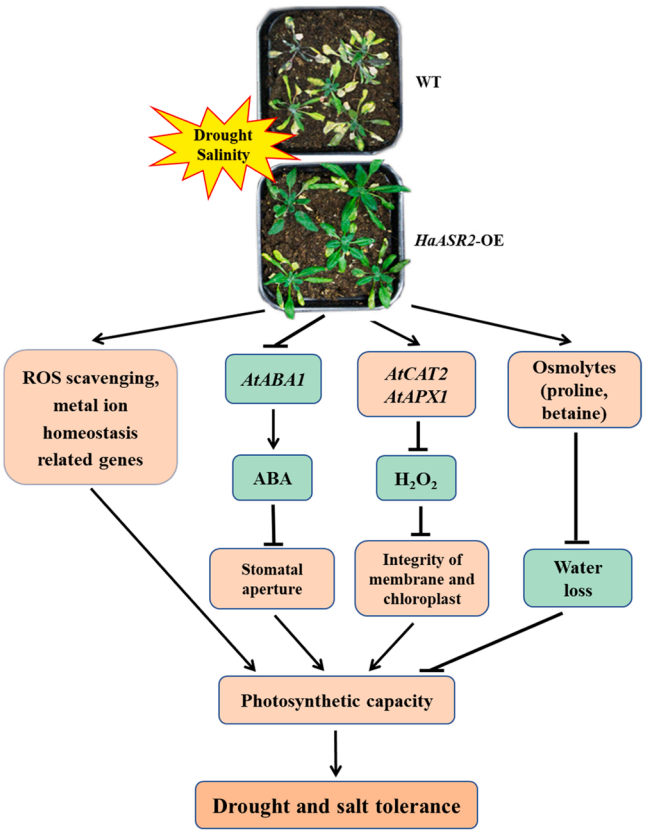

Fig. 13. A proposed model depicting the roles of *HaASR2* overexpression in plants. The overexpression of *HaASR2* in Arabidopsis caused the plants to be partially defective in ABA signaling under drought condition, resulting in lower expression levels of *AtABA1* expression and endogenous ABA level compared to WT plants, therefore, the stomatal aperture was maintained. The overexpression of *HaASR2* also improved the ROS scavenging ability by up-regulating the expressions of *AtCAT2* and *AtAPX1*, resulting in reduced H_2O_2 accumulation, therefore, the integrity of plasmalemma and chloroplasts was maintained. The open stoma and integrity of plasmalemma and chloroplasts guaranteed the operation of photosynthesis to some degree. Also, the overexpression of *HaASR2* elevated the concentrations of osmoregulation substances (proline and betaine), thus increasing plant water-retaining capacity and resulting in reduced water loss. Besides, the overexpression of *HaASR2* induced the expression of genes correlated with ROS scavenging, metal ion homeostasis, response to hormone stimulus, etc. Therefore, the photosynthetic capacity was maintained. Taken together, the overexpression of *HaASR2* significantly improved drought and salt tolerance of Arabidopsis. Note: Light red and light green fillings of the frames indicate the increase and decrease of the parameters compared to the WT, respectively.

system such as peroxidase activity under stress conditions. Other transgenic studies also confirmed that overexpression of *ASR* genes could be involved in increased stomatal closure, higher water content and grain yields, lesser accumulation of malondialdehyde (MDA), ion leakage and reactive oxygen species (ROS), and enhanced antioxidant activities of superoxide dismutase and catalase in Arabidopsis, tobacco, and rice under drought or salt condition (Hu et al., 2013; Kalifa et al., 2004; Liang et al., 2019; Park et al., 2020). Above results demonstrated that *ASR* genes improved plant drought and salt tolerance by promoting growth, osmotic adjustment, photosynthesis, and antioxidant capacity.

Transcriptome analysis provide insights into molecular mechanisms of plant adaptation to abiotic stress (Cheng et al., 2019; Chang et al., 2017; Yu et al., 2020; Arshad et al., 2018; Zhu et al., 2013). More than twice amount of DGEs expressed in the roots compared to the shoots in transgenic plants indicated that *HaASR2* gene regulated molecular responses of roots to drought or salt stress. This might result in a better

stress tolerance of transgenic plants than the WT plant. Furthermore, through GO analysis, a number of genes related to ROS scavenging, metal ion homeostasis, response to hormone stimulus were identified in the transgenic plants. Response to oxidative process and peroxidase activity are closely related to drought tolerant phenotypes (Farooq et al., 2009). Metal ions are involved in the biosynthesis of chlorophyll and the regulation of peroxidase activity (Khobra et al., 2014; Schmidt et al., 2020; Kumar et al., 2010; Ravet and Pilon, 2013) (Fig. 12). These results indicated the role of *HaASR2* in conferring drought and salt tolerance by mediating metabolic and cellular pathways, including but not limited photosynthetic and antioxidant metabolism.

5. Conclusion

HaASR2 was cloned from a desert shrub, *Haloxylon ammodendron*, and its function was analyzed in both yeast and Arabidopsis. HaASR2 interacted with the HaNHX1 protein, and its expression was significantly up-regulated under osmotic stress. The overexpression of *HaASR2* improved plant drought and salt tolerance of Arabidopsis mainly through making plants partially defective in ABA signaling under drought condition, enhancing ROS-scavenging ability, elevating the concentrations of osmoregulation substances and inducing the expression of genes related to ROS scavenging, metal ion homeostasis and responses to hormone stimulus, therefore, maintaining the photosynthetic capacity (Fig. 13). The results demonstrated that *HaASR2* from the desert shrub, *H. ammodendron*, plays a critical role in plant adaptation to drought and salt stress and could be a promising gene for the genetic improvement of crop abiotic stress tolerance.

Author contributions

JZ, CR and HZ designed the experiments. YC, W*R*, HG, and XL conducted the research and analyzed the results. YC wrote the original draft of the manuscript. JZ, CR, QZ and HZ revised the manuscript. All authors approved the submitted version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2022.111572.

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