

Flavonoids are Involved in Salt tolerance through ROS scavenging in the Halophyte *Atriplex canescens*

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Research Article

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Abstract

Atriplex canescens is a halophytic forage shrub with excellent adaptation to saline environment. Our previous study showed that a large number of genes related to the biosynthesis of flavonoids in *A. canescens* were significantly up-regulated by NaCl treatments. However, it remains unclear whether flavonoids are involved in *A. canescens* response to salinity. In this study, we found that the accumulation of flavonoids significantly increased in either the leaves or roots of *A. canescens* seedling under 100 mM and 300 mM NaCl treatments. Correspondingly, *AcCHS*, *AcCHI* and *AcF3H*, which encode three key enzymes (chalcone synthases, chalcone isomerase, and flavanone 3-hydroxylase, respectively) of flavonoids biosynthesis, were significantly induced in the roots or leaves of *A. canescens* by 100 mM or 300 mM NaCl. Then, we generated the transgenic *Arabidopsis thaliana* overexpressing *AcCHI* and found that transgenic plants accumulated more flavonoids through enhancing the pathway of flavonoids biosynthesis. Furthermore, overexpression of *AcCHI* conferred salt and osmotic stress tolerance in transgenic *A. thaliana*. Contrasted with wild-type *A. thaliana*, transgenic lines grew better with greater biomass, less H₂O₂ content as well as lower relative plasma permeability in either salt or osmotic stress conditions. In conclusion, our results indicate that flavonoids play an important role in *A. canescens* response to salt stress through reactive oxygen species (ROS) scavenging and the key enzyme gene *AcCHI* in flavonoids biosynthesis pathway of *A. canescens* has the potential to improve the stress tolerance of forages and crops.

Key Message

The content of flavonoids could increase in *A. canescens* under saline conditions. Overexpression of *AcCHI* in transgenic *A. thaliana* promotes flavonoid biosynthesis, thereby functioning in the tolerance of transgenic plants to salt and osmotic stress by maintaining ROS homeostasis.

Introduction

Soil salinity is one of the most influential abiotic stress indicators on growing plants (Sarker and Oba 2019) and agricultural growth (El-Agha et al. 2019), as it can disrupt ionic homeostasis that evoke the reduction of photosynthesis (Hossain et al. 2022; Sarker et al. 2018) and damage of cell structures, even plant death (Zhang and Shi 2013; Liu et al. 2020; Zhang et al. 2021). More than 3% of total soil resources throughout the world are thought to be affected by salinity (Singh et al. 2018; Feng et al. 2020). Halophytic species have thrived in saline environments for a long time, exhibiting a plethora of physiological and biochemical adaptation mechanisms to shield themselves from the harsh conditions (Flowers et al. 2015). Therefore, to cope with the challenges of salinization to global agriculture, it is necessary to comprehend and elucidate the mechanisms underlying halophytes in response to salinity (Shabala 2013; Guo et al. 2020; Kotula et al. 2020; Zhang et al. 2022).

Atriplex canescens, a C₄ perennial shrub with excellent adaptation to salt and drought conditions, is extensively distributed in saline and arid regions of the world (Hao et al. 2013; Bhatt and Santo 2016; Ma

et al. 2022). We previously found that the growth of *A. canescens* seedlings was significantly stimulated by 100 mM NaCl treatment and unaffected by 400 mM NaCl, since *A. canescens* can maintain the photosynthesis capacity, osmotic adjustment, and Na⁺/K⁺ homeostasis under saline environments (Pan et al. 2016). Additionally, there must be other important salt-resistant strategies in *A. canescens* that still need to be explored, given the salt tolerance of plants is generally a complex quantitative trait and controlled by a variety of mechanisms (Flowers 2004; Flowers and Colmer 2015). Interestingly, our previous transcriptomic data showed that the expressions of a large number of genes encoding key enzymes involved in the biosynthesis of flavonoids were significantly up-regulated in *A. canescens* under salt conditions (Feng et al. 2023), suggesting that flavonoids may be involved the process of stress tolerance in this species.

The flavonoids represent a large class of secondary metabolites found in plants, which possess the ability to effectively eliminate excessive reactive oxygen species (ROS) from plant cells (Agati and Tattini 2010; Jayaraman et al. 2021; Zhu et al. 2021). Under stress conditions, plants accumulate flavonoids through upregulating the encoding genes of key enzymes in their biosynthesis, such as chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), chalcone isomerase (CHI) and dihydroflavonol-4-reductase (DFR) (Hodaei et al. 2018; Gharibi et al. 2019). For example, the content of phenolics, flavonoids, anthocyanins and schaftosides in wheat leaves significantly increased with the upregulation of the genes related to flavonoids biosynthesis under drought stress conditions (Ma et al. 2014). The transgenic *A. thaliana* overexpressing *AeCHS* from *Abelmoschus esculentus* showed enhanced salt and osmotic stress tolerance by increasing the flavonoids accumulation (Wang et al. 2018). Similarly, overexpression of *CsF3H* from *Camellia sinensis* conferred the salt tolerance in transgenic tobacco (*Nicotiana tabacum* L.) through improving antioxidant system (Mahajan and Yadav 2014). However, little research has been conducted on the role of flavonoids in response to salt stress for *A. canescens* and its underlying regulatory mechanism.

Therefore, the objective of this study is to prove the hypothesis that flavonoids are involved in salt tolerance of *A. canescens*. The content of flavonoids in *A. canescens* was investigated under NaCl treatments, then three genes encoding key enzymes (CHS, CHI and F3H) for flavonoids biosynthesis were identified from previous transcriptomic data and their expression pattern were analyzed under NaCl treatments. Finally, the *AcCHI* gene was isolated from *A. canescens* and introduced into *A. thaliana* and the tolerance to salt and osmotic stress in transgenic plants was evaluated.

Materials and methods

Plant materials and growth conditions

The seed sources and seedlings cultivation of *A. canescens* were carried out according to the methods of our previous study (Pan et al. 2016; Guo et al. 2019). *A. canescens* seedlings with similar size were selected and cultured in plastic pots (5 cm × 5 cm × 5 cm; two seedlings/pot) containing sterilized vermiculite, and irrigated with 1/2 strength Hoagland nutrient solution (Ma et al. 2012).

A. thaliana ecotype Columbia (*Col*) was used for genetic transformation material in this study. The seeds of *A. thaliana* were sterilized with 75% (V/V) ethanol in a 1.5 mL centrifuge tube for 5 min, then treated with 1% (W/V) NaClO for 8 min, and finally rinsed with distilled water for six times. The sterilized seeds were incubated at 4°C for 4 d before germination. The temperature of the greenhouse for seedlings growth was $22 \pm 3^\circ\text{C}$, the light duration was 16 h light / 8 h dark, the light intensity was about $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the relative humidity of the air was $63 \pm 2\%$.

Fluorescence observation and content determination of flavonoids in roots and leaves of *A. canescens*

To observe the distribution of flavonoids in different tissues of *A. canescens*, four-day-old seedlings were treated with different concentrations of NaCl (0, 100, 300 mM) for 2 d, and then 2 cm of root tips were cut for diphenylboric acid-2-aminoethyl ester (DPBA) staining. Similarly, two-week-old seedlings were treated with 1/2 strength Hoagland nutrient solutions supplemented with 0 (control), 100 and 300 mM NaCl for 2 d, then the first and second true leaves were excised for DPBA staining. The DPBA staining was performed according to the method of Murphy et al. (2000) with slight modification. The epidermis of leaves and root tips were immersed in DPBA dyeing liquid (DPBA, 2.5 mg mL^{-1} ; Triton X-100, 0.005%; pH, 7) for 45 min and 15 min for staining, respectively, then were washed in deionized water and mounted on microscope slides. The fluorescence signal was visualized on a confocal microscope (Leica, DM6B, Germany) using a FITC filter set (excitation 450–490 nm, suppression long pass 520, 4 or 10X Plan-Apo objective lens).

To measure the content of flavonoids in *A. canescens*, four-week-old seedlings were treated with 1/2 strength Hoagland nutrient solutions supplemented with 0 (control), 100 and 300 mM NaCl for 2 d, the mature leaves and roots of *A. canescens* were harvested for 6 repetitions, respectively. After the samples were dried and ground, the total flavonoids content was extracted using Cominbio Plant Flavonoid Extraction kit (Suzhou Comin biotechnology Co., Ltd., Suzhou, China). Briefly, approximately 0.018 g (dry weight) of the crushed plant sample was added to 1.8 mL of 60% ethanol and combined with aluminum ions in an alkaline nitrite solution, and then incubated with oscillation at 60°C for 2 h. After that, the mixtures were centrifuged at $10,000 \times g$ for 10 min at 25°C. The absorbance (at 510 nm) of the supernatant was measured using a spectrophotometer (UV-6100PCS; Mapada Instruments Co. Ltd, Shanghai, P.R. China), and the content of flavonoids was estimated as the following formula: flavonoids content [mg/g dry weight (DW)] = $0.398 \times (\Delta A_{510} - 0.0007) / \text{DW}$ (Li et al. 2020).

Expression pattern analysis of genes encoding three key enzymes of flavonoids biosynthesis in *A. canescens*

According to our previous transcriptome data (Feng et al. 2023), three genes encoding chalcone synthase (CHS) (Gene ID CL5595.Contig2_All), chalcone isomerase (CHI) (Gene ID CL990.Contig3_All) and flavanone 3-hydroxylase (F3H) (Gene ID Unigene9407_All) were identified and found all of them were upregulated by NaCl. To determine the expression abundance of *AcCHS*, *AcCHI* and *AcF3H* in various tissues of *A. canescens* under saline conditions, four-week-old seedlings were treated with 1/2 strength

Hoagland nutrient solutions supplemented with 0 (control), 100 and 300 mM NaCl for 0, 3, 6, 12, 24 and 48 h. After that, the roots, stems, young leaves, mature leaves and old leaves were harvested, respectively (Guo et al. 2022), and the expression levels of *AcCHS*, *AcCHI* and *AcF3H* in above tissues were analyzed by real-time quantitative PCR with reference gene *AcACTIN*. The primers used in this experiment were shown in Supplementary Table S1.

Cloning of *A. canescens* *AcCHI* and sequence analysis

Based on sequence of *AcCHI* gene (Gene ID CL990.Contig3_All) in our previous transcriptome sequencing data, the open reading frame (ORF) of this gene was predicted through the NCBI ORF Finder (<https://www.ncbi.nlm.nih.gov/orffinder/>). The nested PCR method was used to amplify the full-length cDNA of *AcCHI*. All the primers for the nested amplification of cDNA ends by PCR were shown in Supplementary Table S2. Total RNA of the *A. canescens* samples was extracted following the instructions of TaKaRa Mini BEST Plant RNA Extraction Kit (TaKaRa, Beijing, China), and then was reverse-transcribed into cDNA by using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China). Then the full-length cloning was synthesized using Phusion® High-Fidelity DNA Polymerase kit (Thermo Fisher Scientific, Waltham, MA, USA). The PCRs were conducted in a total volume of 20 µL, containing 0.8 µL of cDNA, 0.8 µL of forward and reverse primers, 4 µL of 5 × Phusion GC buffer, 1.6 µL of dNTP Mixture, 0.2 µL of Phusion DNA Polymerase, and 11.8 µL of ddH₂O. PCR reactions were performed according to following procedure: initial denaturation at 98°C for 30 s min followed by 35 cycles of 10 s at 98°C, 10 s at 55°C and 40 s at 72°C, and final extension of 10 min at 72°C. Then the PCR products were collected and sequenced.

After the homologous amino acid sequences sharing high similarity with *AcCHI* being retrieved by NCBI BLAST program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), multiple-sequence alignment and phylogenetic analysis were performed according to the previously described method (Guo et al. 2022).

Generation of transgenic *A. thaliana* overexpressing *AcCHI*

The open reading frame (ORF) of *AcCHI* was amplified by using the primers of Supplementary Table S3 containing *NcoI* restriction sites and then was fused into the vector of pCambia1302-*Basta-35S* by In-Fusion® HD Cloning Kits (Takara, Beijing, China). Subsequently, the recombinant plasmid pCambia1302-*Basta-35S-AcCHI* was mobilized into *Agrobacterium tumefaciens* strain GV3101 and was transformed into *Col-0 Arabidopsis* seedlings according to floral dip method (Clough and Bent 1998). T₁ transgenic plants were selected by spraying the 0.1‰ (v/v) Basta on the seedlings three times at 3 or 4 d intervals. After screening for the next two generations, homozygous plants of T₃ generation were obtained. Then, we randomly selected two lines (named as *I1* and *I2*) for further experiments in this study.

The expression of *AcCHI* in above two transgenic lines was evaluated by RT-PCR using the primers listed in Supplementary Table S4. *AtActin2* gene was used as an internal control. The PCR reaction was carried out at 94°C for 2 min for initial denaturation, followed by 35 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 90 s of extension, and 10 min of final extension at 72°C. The reaction mixture containing 1 µL of

cDNA from *A. thaliana* as template, 10 µL of Premix Taq (Takara, Beijing, China), 0.5 µL each of forward and reverse primers, and 8 µL of ddH₂O to the final volume of 20 µL. Finally, the PCR products were separated by agarose gel electrophoresis and visualized by AlphaImager (ProteinSimple Inc., Santa Clara, CA, USA).

Fluorescence observation and content determination of flavonoids in transgenic *A. thaliana* overexpressing *AcCHI*

To observe the distribution of flavonoids in *A. thaliana*, the first and second true leaves of hydroponic 6-day-old wild type (WT) and transgenic seedlings were excised and stained with diphenylboric acid-2-aminoethyl ester (DPBA). To measure the content of flavonoids in *A. thaliana*, the shoots of hydroponic 4-week-old WT and transgenic seedlings were harvested for 6 repetitions, respectively. The visualization and quantification of flavonoids were performed according to the procedures described previously.

Analysis on expression of the genes encoding key enzyme related to flavonoids biosynthesis in leaf of *A. thaliana*

To investigate whether the overexpression of *AcCHI* affected the expression of genes encoding other key enzymes related to flavonoids biosynthesis in transgenic *A. thaliana*, the leaves from hydroponic 3-week-old seedlings of WT and transgenic line *I2* were collected separately, and the relative expression levels of 8 genes that encode phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL1), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), flavonol synthase (FLS1), dihydroflavonol-4-reductase (DFR) and anthocyanidin synthase (ANS) were analyzed by qRT-PCR, respectively. *AtActin2* was used as reference gene. All the primers used in qRT-PCR for these genes were shown in Supplementary Table S5.

Real-time quantitative PCR analysis

Total RNA of all mentioned samples was obtained by the TaKaRa Mini BEST Plant RNA Extraction Kit (TaKaRa, Beijing, China) and cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China). The expression pattern of above-mentioned genes was determined using SYBR® Premix Ex Taq™ II kit (TaKaRa, Dalian, China) and finished on a StepOnePlus real-time system (ABI PRISM 7500, USA). Finally, the relative expression levels of each gene were analyzed using the $2^{-\Delta\Delta CT}$ method (Chmittgen and Livak 2008). Three replicates were conducted in each tested sample.

Assessment of salt and osmotic stress tolerance of transgenic *A. thaliana* overexpressing *AcCHI*

To evaluate the salt and osmotic stress tolerance of transgenic *A. thaliana*, a comparative analysis of growth phenotype under salt or osmotic stress conditions was conducted between WT and transgenic plants using an agar plate culture system. The seeds of WT and two transgenic lines were sown on 1/2 MS medium (1.5% sucrose and 0.8% agar, pH = 5.8), respectively. The plates were placed at 4°C for 2 d of vernalization and then germinated at 22°C for 4 d. Three uniform seedlings of each line were transferred

into 1/2 MS plates (control) and 1/2 MS plates containing 100 mM NaCl or 200 mM Sorbitol. After 10 d of vertical culture, the seedlings were photographed and their root lengths were measured using image J software (v.1.31; <https://imagej.nih.gov/ij/>). All plate experiments were repeated for six times independently.

The hydroponic experiment was conducted to verify the salt and drought tolerance of transgenic plants. For salt stress treatment, the uniform 3-week-old seedlings of WT and transgenic lines were treated with 1/2 strength Hoagland nutrient solution without (control) or with additional 50 mM NaCl for 7 d, respectively. For simulating drought stress treatment, the uniform 3-week-old seedlings of each line were treated with 1/2 strength Hoagland nutrient solution without (control) or with additional 150 mM sorbitol where the osmotic potential was -0.15 MPa for 3 d, respectively. Finally, the seedlings were photographed and harvested to measure the growth and physiological indicators.

To determine the biomass of WT and transgenic plants, the roots and shoots from hydroponic treatment were excised, and the fresh weights were weighted immediately. After that, the samples were dried in an oven (DGG-9426A, Shanghai, China) at 80°C for 48 h, and their dry weights were obtained.

Measurement of H_2O_2 content and relative plasma permeability in the leaf of transgenic *A. thaliana* overexpressing *AcCHI*

The hydroponic 3-week-old WT and transgenic seedlings were treated with 50 mM NaCl for 7 d or 150 mM sorbitol for 3 d, then the diaminobenzidine (DAB) staining was used to visualize the H_2O_2 in leaves according to the method of Daudi and O'Brien (2012). Finally, the contents of H_2O_2 in leaves were quantified by using the hydrogen peroxide content determination kit (Suzhou Comin biotechnology Co., Ltd., Suzhou, China). The experiments were repeated six times.

The fresh leaves of WT and transgenic plants from hydroponic experiment were excised, and the relative plasma permeability (RPP) of leaf cells was measured by using a conductivity meter (REX, DDSJ-319L, Shanghai, China) according to the method of Bao et al. 2014.

Data analysis

One-way analysis of variance (ANOVA) of SPSS v 26.0 (IBM, Armonk, NY, USA) was used for statistical analysis, and Duncan's multiple range tests were performed to evaluate the differences between means at a significance level of $P < 0.05$. Values were presented as means $\pm$ SE ($n = 3$ or 6).

Results

NaCl treatments promoted the accumulation of flavonoids in the roots and leaves of *A. canescens*

To investigate the potential involvement of flavonoids in the salt stress response of *A. canescens*, we employed DPBA staining to visualize and quantify flavonoid levels in both young leaves and root tips,

followed by observation using a confocal microscope. As shown in Fig. 1A, compared to control, the flavonoids fluorescence intensity in the leaves and roots of *A. canescens* was significantly increased under 100 mM and 300 mM NaCl treatments. Interestingly, the fluorescence in the leaves was found to be weaker under 300 mM NaCl compared to that observed under 100 mM NaCl. However, in contrast to the leaves, a stronger fluorescence was detected in the root tips at 300 mM NaCl as opposed to 100 mM NaCl (Fig. 1B). Correspondingly, the quantitative results demonstrated that the flavonoids levels were consistent with flavonoids fluorescence in the leaves and roots. After 4-week-old *A. canescens* seedlings being treated with 100 mM and 300 mM NaCl for 2 d, the content of total flavonoids was significantly increased by 21.7% and 16.2% in the leaves (Fig. 1C) and by 17.0% and 18.6% in roots (Fig. 1D) than for control, respectively. These results suggest that NaCl treatments can induce the accumulation of flavonoids in *A. canescens*, which may help *A. canescens* to grow well in the saline environment.

Expression of key enzyme genes involving in *A. canescens* flavonoids biosynthesis was induced by salt treatments

Our previous transcriptomic data showed that the transcriptional abundances of genes encoding key enzymes involved in flavonoid biosynthesis were significantly upregulated following treatment with 100 mM NaCl in both the leaves and roots of *A. canescens*. (Feng et al. 2023). Based on this, we screened three candidate genes (*CL5595.Contig2_All*, *CL990.Contig3_All*, and *Unigene9407_All*) from transcriptomic data and found their expression levels were increased after salt treatment for 6 h and 24 h (Supplementary Table S6). Subsequently, nucleotide sequences of these genes were blasted in NCBI with other species and the results indicated that they shared high homology with *CHS*, *CHI*, and *F3H* from other plants, thus were named as *AcCHS*, *AcCHI* and *AcF3H*, respectively.

Then, we analyzed the tissue specific expression of *AcCHS*, *AcCHI* and *AcF3H* in *A. canescens* seedlings and found that *AcCHS* mainly expressed in the roots, while *AcCHI* and *AcF3H* mainly expressed in the mature leaves (Fig. 2A-C). Moreover, the expression of *AcCHS* in the roots was rapidly induced by NaCl (especially, by 100 mM NaCl) and reached the peak at 3 h of treatment (Fig. 2D). Similarly, the expression levels of *AcCHI* and *AcF3H* in the mature leaves were significantly upregulated by 300 mM NaCl treatment to reached the peak at 6 h, which was about 4-fold and 9-fold higher than that under control condition, respectively (Fig. 2E, F). At the same time, the expression of *AcCHI* and *AcF3H* were also induced by 100 mM NaCl treatment, but their upregulation was lower than that under 300 mM NaCl treatment. Due to the expression pattern of *AcCHI* completely consistent with the transcriptome profiling results, we selected *AcCHI* in this study to further confirm its roles in the biosynthesis of flavonoids as well as in the salt and drought tolerance of plants.

Isolation and characterization of *AcCHI*

The full-length sequence of *AcCHI* was obtained using nested PCR based on our transcriptomic data (Gene ID CL990.Contig3_All, Feng et al. 2023). The open reading frame of *AcCHI* is 741 bp in length, encoding a protein composed of 246 amino acids (Supplementary Fig. S1). The amino acid sequences of *AcCHI* contains four conserved amino acid sites, Thr48, Tyr106, Asn113 and Ser192, which are unique to

type I CHI family (Supplementary Fig. S2A). The phylogenetic analysis showed that AcCHI has a close evolutionary relationship with CqCHI from *Chenopodium quinoa*, BvCHI from *Beta vulgaris*, SoCHI from *Spinacia oleracea* (Supplementary Fig. S2B).

Overexpression of *AcCHI* promoted the biosynthesis of flavonoids in *A. thaliana*

To generate the transgenic *A. thaliana* overexpressing *AcCHI*, we transferred pCAMBIA1302-*Basta* vector carrying the *AcCHI* gene under the control of CaMV35S promoter (Supplementary Fig. S3) into *A. thaliana* and obtained the T₃ transgenic lines, of which two lines were randomly selected for further assessment in this study. The RT-PCR results showed that *AcCHI* gene were significantly expressed in above two transgenic lines, but the gene expression levels varied among them, we thus named the line with lower gene expression level as *I1* and another with higher expression as *I2* (Supplementary Fig. S4).

To investigate whether the overexpression of *AcCHI* would affect the biosynthesis of flavonoids in *A. thaliana*, the leaves from 6-day-old seedlings of WT and two transgenic lines were stained with DPBA. As shown in Fig. 3A, the fluorescence in the leaves of *I1* and *I2* was more intense than for WT.

Correspondingly, we found that the contents of flavonoids in shoots of *I1* and *I2* plants were significantly higher by 26.2% and 31.0% compared to that of WT, respectively (Fig. 3B). These results indicated that overexpression of *AcCHI* increased the accumulation of flavonoids in *A. thaliana*. Interestingly, through comparing the expression levels of other key genes involving in biosynthesis pathway of flavonoids between WT and transgenic line *I2*, we found that the genes encoding 4-coumarate-CoA ligases (4CL), flavonol synthase (FLS), dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS) in transgenic plants showed significantly higher expression than that in WT; in particular, the transcript levels of *AtDFR* and *AtANS* in *I2* line were nearly 2.5-fold higher compared to those in WT (Fig. 4). This should be another important reason that resulted in more flavonoids synthase in transgenic plants.

Overexpression of *AcCHI* enhanced the salt tolerance of transgenic *A. thaliana*

To determine whether the overexpression of *AcCHI* improved the resistance of *A. thaliana* to salinity, the growth status of WT and transgenic *A. thaliana* plants were examined in 1/2 MS agar plates supplemented with 100 mM NaCl or in hydroponics with half-strength Hoagland nutrient solution containing 50 mM NaCl. In either plate or hydroponic experiments, there was no difference in the growth of WT and transgenic plants under control conditions; although the growth of all plants was significantly inhibited by salt treatments with leaf shrinkage and root shortening, the transgenic lines *I1* and *I2* performed better than WT (Fig. 5A-C). The above observations were further confirmed by biomass analysis in hydroponic experiment with 50 mM NaCl treatment: in two transgenic lines, the fresh weights of shoots and roots were higher by 53.3%-58.6% and 24.4%-32.5%, and the dry weights of shoots and roots showed higher by 26.3%-29.8% and 25.0%-31.4%, respectively, than for WT (Fig. 5D-G).

Transgenic *A. thaliana* overexpressing *AcCHI* showed enhanced tolerance to osmotic stress

To clarify the role of *AcCHI* in osmotic stress tolerance of plants, the phenotypes of WT and transgenic *A. thaliana* plants were observed in 1/2 MS agar plates containing 200 mM sorbitol or in 1/2 strength Hoagland nutrient solution supplemented with 150 mM sorbitol where the osmotic potential was -0.15 MPa. In either plate or hydroponic experiments, no detectable phenotype difference was observed between WT and transgenic plants under control conditions; under simulated drought conditions, however, the transgenic plants grew better than WT even though both of them exhibited growth inhibition (Fig. 6A-C). The biomass analysis of hydroponic experiment with -0.15 MPa osmotic stress treatment also confirmed above findings: in two transgenic lines, the fresh weights of shoots and roots were higher by 16.7%-39.2% and 15.0%-17.5%, and the dry weights of shoots and roots showed higher by 22.2%-30.3% and 15.2%-19.8%, compared to WT, respectively (Fig. 6D-G).

Overexpression of *AcCHI* reduced the accumulation of ROS and alleviated the damage to cell membrane system of *A. thaliana* under salt or simulated drought conditions

The experimental findings have demonstrated that exposure to stresses can elicit an excessive accumulation of ROS and impair the integrity of cell membrane systems, thereby exerting a detrimental impact on plant growth (Meng et al. 2015; Ijaz et al. 2017; Yang and Guo 2018). To investigate the ROS level in transgenic *A. thaliana*, the H_2O_2 of WT and transgenic plants was detected using DAB staining under both control and stress conditions. The results showed that the accumulation of brown deposits in transgenic plants was much less than that in WT under either salt or osmotic stress conditions (Fig. 7A, B). This was confirmed by determination of H_2O_2 content, which increased in WT as well as in transgenic plants under salt or osmotic stress conditions but was lower by 18.3%-29.9% and 13.0%-15.0% in transgenic plants compared to WT, respectively (Fig. 7C, D). Moreover, we found that the relative plasma permeability of all tested plants was significantly increased under either salt or osmotic stress conditions, but those of transgenic lines were significantly lower by 22.9%-30.3% and 13.5%-21.2% compared to WT, respectively (Fig. 8), indicating that the membrane structure of transgenic *A. thaliana* overexpressing *AcCHI* is healthier than that of WT under stress conditions.

Discussion

Flavonoids participate in the process of *A. canescens* in response to salt stress

In harsh environments, plants have the ability to regulate metabolite production in order to avoid or mitigate the damage (Xu et al. 2020; Sarker U and Ercisli 2022). Flavonoids, a class of polyphenolic secondary metabolites, are widely distributed among land plants and play essential roles in plant growth as well as response to biotic or abiotic stresses (Ni et al. 2020; Zhao et al. 2021). Li et al. (2017) observed a significant increase in flavonoids levels in the root tips of *A. centralasiatica* under salt treatment, leading to reduced H_2O_2 accumulation in the roots. This finding suggests that accumulation of flavonoids may serve as a mechanism for plants to alleviate the oxidative damage induced by salt stress (Jayaraman et al. 2021). Previous studies in different leafy *amaranth* species showed augmentation of total flavonoid content (Sarker et al. 2023; Sarker and Oba 2020) and individual flavonoids and phenolic

compounds in the leaves under both moderate and severe salinity stress (Sarker and Ercisli 2022; Sarker and Oba 2018). Similarly, Ma et al. (2020) reported that the content of total flavonoids in the leaves of *Sorghum bicolor* were increased under both moderate and severe saline stress, while the accumulation of flavonoids under moderate stress was higher than under severe stress. Consistent with previous studies, our results showed that NaCl treatments significantly induced the accumulation of the flavonoids in the roots and leaves of *A. canescens* (Fig. 1), indicating that flavonoids might play an important role in *A. canescens* response to salt stress.

Combined with our previous transcriptome data, we speculated that the increase of flavonoids content in *A. canescens* might result from a significant increase of flavonoids biosynthesis-related transcripts under NaCl treatments (Feng et al. 2023). To confirm this, we investigated the expression pattern of three genes (*AcCHS*, *AcCHI* and *AcF3H*) encoding the key enzymes of flavonoids biosynthesis in *A. canescens* and found that their expression levels were significantly induced in the roots or leaves by 100 mM or 300 mM NaCl treatments (Fig. 2). Previous studies also proposed that flavonoids biosynthesis-related genes were upregulated by salt treatments (Chen et al. 2015; Yang et al. 2019). For example, the expression of *Anoectochilus formasanus AfCHS* showed continually increased and reached about 36.2 and 19.5-fold at 2 h and 12 h compared with 0 h under 100 mM NaCl induction (Yang et al. 2019). Under NaCl treatments, similarly, the expression levels of *Solanum lycopersicum SlF3H* (Meng et al. 2015) and *Vigna unguiculata VuCHI* (Amorim et al. 2018) were significantly induced. After treating with NaCl, the total flavonoids and anthocyanin contents in *Ginkgo biloba* seedlings exhibited significantly increased with the upregulating of *GbDFR* gene (Ni et al. 2020). In *Milletia pinnata*, the transcription level of *MpCHI* was significantly enhanced in the leaves after 500 mM NaCl treatment, and the pYES2-*MpCHI* transformed yeast mutants exhibited improved salt tolerance than control (Wang et al. 2013). These results suggest that there are potential benefits of flavonoids biosynthesis-related genes in increasing flavonoids content and improving stress tolerance in plants.

AcCHI of *A. canescens* belongs to Type I CHI proteins and its overexpression promotes the biosynthesis of flavonoids in transgenic *A. thaliana* overexpressing *AcCHI*

CHI is one of the most important enzymes in the biosynthesis of flavonoids and converts chalcones into flavanones, which then gives rise to the many different classes of flavonoids compounds (Nabavi et al. 2020; Zhu et al. 2021). According to phylogenetic relationships and functions, CHIs in plants can be categorized into four distinct types (Sun et al. 2019). It has been demonstrated that only CHIs from type I and type II possess enzymatic activity for chalcone cyclization (Ngaki et al. 2012; Shimada et al. 2003). In present study, the multiple sequence alignment showed that *AcCHI* shared high similarity to CHIs from other plants (Supplementary Fig. S2), suggesting that *AcCHI* belongs to CHI family. Jez (2000) analyzed the three-dimensional structure of alfalfa (*Medicago sativa* L.) *MsCHI* and found that the active site cleft of CHI consists of Arg36, Gly37, Leu38, Phe47, Thr48, Ile50, Tyr106, Lys109, Val110, Asn113, Thr190 and Met191. In the amino acid sequence of *AcCHI*, with the exception of Ser192 in place of Thr190 and Met191, all of the residues that make up the CHI active site were shown to be conserved (Supplementary Fig. S2). It was reported that Thr190 and Met191 are the residue related to substrate preference and only

be seen in legume CHIs, which belongs to Type II CHI, whereas the Thr190 was replaced by Ser in all non-legume CHIs and identified this Type I CHI (Jez et al. 2000). These evidences confirmed that AcCHI should be a Type I CHI, and thus essential for the biosynthesis of flavonoids in plants.

Biosynthesis of flavonoids occurs through phenylpropanoid pathway, which is one of the best-known pathways in plant secondary metabolism. In this pathway, CHS catalyzes the change of 4-Coumaroyl-CoA and Malonyl-CoA to chalcone, which is then changed into flavonone by CHI. With the participation of the enzymes like DFR, FLS and ANS, chalcone and flavonone intermediates act as crucial branch sites for the biosynthesis of other flavonoids subgroups (Nabavi et al. 2020). Existing evidences indicate that overexpression of *CHI* genes could significantly lead to the increase of flavonoids accumulation. For example, Zhou et al. (2014) reported that overexpression of *Paeonia suffruticosa PsCHI* resulted in a three-fold increase of total flavonols and flavones in transgenic tobacco compared with the WT plants. Zhu et al. (2021) transferred *Dracaena cambodiana DcCHI1* into tobacco and found that the content of flavonoids in transgenic plants significantly higher compared to the control. In this study, similarly, we successfully increased the content of flavonoids in *A. thaliana* by introducing the *CHI* gene from *A. canescens* (Fig. 3), suggesting that overexpression of *AcCHI* could promote the biosynthesis of flavonoids in transgenic *A. thaliana*. Furthermore, we also found that the genes encoding several other key enzymes in flavonoids biosynthesis pathway were significantly up-regulated in transgenic *A. thaliana*, such as 4-coumarate-CoA ligases (*At4CL*), flavonol synthase (*AtFLS*), dihydroflavonol reductase (*AtDFR*) and anthocyanidin synthase (*AtANS*) (Fig. 4). Previous studies showed that overexpression of *4CL*, *FLS*, *DFR*, and *ANS* can facilitate the biosynthesis of flavonoids in plants. Transformation of *Dryopteris fragrans Df4CL* and *Grape hyacinth GhFLS* can promote the biosynthesis of flavonoids in tobacco (Liu et al. 2019; Li et al. 2020). The content of anthocyanin was significantly increased in *Brassica napus* by overexpressing *AtDFR* (Kim et al. 2017). In the same way, Zheng et al. (2019) found that the expression abundance of *AtANS* was positively correlated with anthocyanin content in *A. thaliana*. Hence, we speculate that the overexpression of *AcCHI* can up-regulate several key genes involved in flavonoids synthesis pathway of *A. thaliana*, which may also be another important reason for the increase of flavonoids content in transgenic plants (Fig. 9).

Overexpression of *AcCHI* enhances the salt and osmotic stress tolerance of transgenic *A. thaliana* by strengthening its antioxidant capacity

The recent studies have demonstrated that the accumulation of flavonoids significantly contributes to enhancing the salt and drought tolerance of plants (Kim et al. 2017; Wang et al. 2020; Jayaraman et al. 2021; Zhang et al. 2021). The overexpression of *OsCHI2* in rice leads to the accumulation of flavonoids, resulting in transgenic plants that exhibit enhanced tolerance to drought, salinity and cold stresses (Jayaraman et al. 2021). Similarly, Wang et al. (2021) found that overexpression of *Apocynum venetum AvFLS* enhanced salt tolerance in tobacco by accumulating of flavonoids and maintaining K^+/Na^+ homeostasis under salt conditions. Consistent with previous studies, we found the overexpression of *AcCHI* also conferred salt and osmotic stress tolerance in transgenic *A. thaliana*, which grew better than WT under either saline or osmotic stress conditions (Fig. 5, Fig. 6). We speculate that this may be due to

the function of flavonoids to reduce the negative effects of excessive ROS, which resulted from the abiotic stresses and could cause the oxidative stress to plants (Agati and Tattini 2010; Gao et al. 2011; Zhang et al. 2012; Jayaraman et al. 2021). Several reports have shown that the accumulation of H_2O_2 can be mediated by activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase belonging to the respiratory burst oxidase homolog protein C-like (RBOH) family, and activity of NADPH oxidases affects the release of ROS (Sewelam et al. 2016; Formentin et al. 2018). Thus, the H_2O_2 can be used as an early signaling molecule for the initiation of cellular responses to evaluate the tolerance of plants to abiotic stresses (Formentin et al. 2018). It was found that the content of H_2O_2 and O_2^- were lower in the Antarctic moss *Pohlia nutans* *PnFNS1*-overexpressing *A. thaliana* plants under drought stress and UV-B radiation compared to WT plants (Wang et al. 2020). Similarly, although the H_2O_2 and O_2^- content were significantly increased both in WT and *Nicotiana tabacum* *NtCHS*-overexpressing tobacco plants, the transgenic lines showed lower H_2O_2 or O_2^- content than WT plants under salt stress (Chen et al. 2019). In our study, *AcCHI*-overexpressing *A. thaliana* plants exhibited significantly lower H_2O_2 level compared to WT plants under saline or osmotic stress conditions (Fig. 7), suggesting that the overexpression of *AcCHI* enhanced the ROS scavenging ability of transgenic plants (Fig. 9). This conclusion was further supported by the measurement of relative plasma membrane permeability, which was significantly lower in transgenic plants than in WT plants under both salt and osmotic stress (Fig. 8, 9), since excessive accumulation of ROS can induce lipid peroxidation and thus lead to membrane damage (Meng et al. 2015). Taken together, we concluded that overexpression of *AcCHI* alleviates cell membrane damage by reducing ROS accumulation in plants, thereby evokes transgenic *A. thaliana* less vulnerable to stress conditions.

Conclusion

Our results demonstrate that flavonoids are involved in salt tolerance of *A. canescens*, as their levels are increased and the key enzyme genes in their biosynthesis pathway are up-regulated under saline conditions. Moreover, the overexpression of *AcCHI* promotes the biosynthesis of flavonoids in transgenic *A. thaliana*, which facilitate to alleviate the damage of cell membrane by reducing ROS accumulation, thereby confers transgenic plant enhanced tolerance to salt and osmotic stress. This study provides a solid foundation for elucidating the mechanisms underlying salt tolerance in halophytes and improving the stress tolerance of forages and crops.

Declarations

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Contributions

A-K B conceived and designed the study. S F, Y-T Y and B-B W performed the experiments. S F wrote the manuscript. S F, Y-M L, L L and A-K B revised the manuscript. All author read and approved the final manuscript.

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Conflict of 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.

Additional information

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References

1. Agati G, Tattini M (2010) Multiple functional roles of flavonoids in photoprotection. *New Phytol* 186: 786-793. <https://doi.org/10.1111/j.1469-8137.2010.03269.x>.
2. Amorim LB, Ferreira-Neto JRC, Bezerra-Neto JP, Pandolfi V, de Araújo FT, da Silva Matos MK, Santos MG, Kido EA, Benko-Iseppon AM (2018) Cowpea and abiotic stresses: identification of reference genes for transcriptional profiling by qPCR. *Plant Methods* 14: 88. <https://doi.org/10.1186/s13007-018-0354-z>.
3. Bao AK, Wang YW, Xi JJ, Liu C, Zhang JL, Wang SM (2014) Co-expression of xerophyte *Zygophyllum xanthoxylum* *ZxNHX* and *ZxVP1-1* enhances salt and drought tolerance in transgenic *Lotus corniculatus* L. by increasing cations accumulation. *Funct Plant Biol* 41: 203-214. <https://doi.org/10.1071/FP13106>.
4. Bhatt A, Santo A (2016) Germination and recovery of heteromorphic seeds of *Atriplex canescens* (Amaranthaceae) under increasing salinity. *Plant Ecol* 217: 1069-1079. <https://doi.org/10.1007/s11258-016-0633-6>.
5. Chen L, Guo H, Lin Y, Cheng H (2015) Chalcone synthase EaCHS1 from *Eupatorium adenophorum* functions in salt stress tolerance in tobacco. *Plant Cell Rep* 34: 885-894. <https://doi.org/10.1007/s00299-015-1751-7>.
6. Chen S, Wu F, Li Y, Qian Y, Pan X, Li F, Wang Y, Wu Z, Fu C, Lin H, Yang A (2019) *NtMYB4* and *NtCHS1* are critical factors in the regulation of flavonoid biosynthesis and are involved in salinity responsiveness. *Front Plant Sci* 10: 178. <https://doi.org/10.3389/fpls.2019.00178>.
7. Chmitten T, Livak K (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3: 1101-1108. <https://doi.org/10.1038/nprot.2008.73>.
8. Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium* mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735-743. <https://doi.org/10.1046/j.1365-313x.1998.00343.x>.
9. Daudi A, O'Brien JA (2012) Detection of hydrogen peroxide by DAB staining in *Arabidopsis* leaves. *Bio protocol* 2: 263-267. <https://doi.org/10.21769/BIOPROTOCOL.263>.
10. El-Agha DE, Molle F, Rap E, Bialy ME, El-Hassan WA (2020) Drainage water salinity and quality across nested scales in the Nile Delta of Egypt. *Environ Sci Pollut Res Int* 27: 32239-32250. <https://doi.org/10.1007/s11356-019-07154-y>.
11. Feng S, Wang B, Li C, Bao AK (2023) Transcriptomic analysis provides insight into the ROS scavenging system and regulatory mechanisms in *Atriplex canescens* response to salinity. *Int J Mol Sci* 24: 242. <https://doi.org/10.3390/ijms24010242>.
12. Feng YN, Cui JQ, Zhou T, Liu Y, Yue CP, Huang JY, Hua YP (2020) Comprehensive dissection into morpho-physiologic responses, ionic homeostasis, and transcriptomic profiling reveals the systematic resistance of allotetraploid rapeseed to salinity. *BMC Plant Biol* 20: 534. <https://doi.org/10.1186/s12870-020-02734-4>.
13. Flowers TJ (2004) Improving crop salt tolerance. *J Exp Bot* 55: 307-319. <https://doi.org/10.1093/jxb/erh003>.

14. Flowers TJ, Colmer TD (2015) Plant salt tolerance: adaptations in halophytes. *Ann Bot* 115: 327-331. <https://doi.org/10.1093/aob/mcu267>.
15. Flowers TJ, Rana M, Colmer TD (2015) Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Ann Bot* 3: 419-431. <https://doi.org/10.1093/aob/mcu217>.
16. Formentin E, Sudiro C, Ronci MB, Locato V, Barizza E, Stevanato P, Ijaz B, Zottini M, De Gara L, Lo Schiavo F (2018) H₂O₂ signature and innate antioxidative profile make the difference between sensitivity and tolerance to salt in rice cells. *Front Plant Sci* 9: 1549. <https://doi.org/10.3389/fpls.2018.01549>.
17. Gao JJ, Zhang Z, Peng RH, Xiong AS, Xu Jing, Zhu B, Yao QH (2011) Forced expression of Mdmyb10, a myb transcription factor gene from apple, enhances tolerance to osmotic stress in transgenic *Arabidopsis*. *Mol Biol Rep* 38: 205-211. <https://doi.org/10.1007/s11033-010-0096-0>.
18. Gharibi S, Sayed Tabatabaei BE, Saeidi G, Talebi M, Matkowski A (2019) The effect of drought stress on polyphenolic compounds and expression of flavonoid biosynthesis related genes in *Achillea pachycephala* Rech.f. *Phytochemistry* 162: 90-98. <https://doi.org/10.1016/j.phytochem.2019.03.004>.
19. Guo H, Cui YN, Pan YQ, Wang SM, Bao AK (2020) Sodium chloride facilitates the secretohalophyte *Atriplex canescens* adaptation to drought stress. *Plant Physiol Biochem* 150: 99-108. <https://doi.org/10.1016/j.plaphy.2020.02.018>.
20. Guo H, Cui YN, Zhang L, Feng S, Ren ZJ, Wang SM, Bao AK (2022) *AcHKT1; 2* is a candidate transporter mediating the influx of Na⁺ into the salt bladder of *Atriplex canescens*. *Plant Soil* 483: 607-624. <https://doi.org/10.1007/s11104-022-05769-8>.
21. Guo H, Zhang L, Cui YN, Wang SM, Bao AK (2019) Identification of candidate genes related to salt tolerance of the secretohalophyte *Atriplex canescens* by transcriptomic analysis. *BMC Plant Biol* 19: 213. <https://doi.org/10.1186/s12870-019-1827-6>.
22. Hao GY, Lucero ME, Sanderson SC, Zacharias EH, Holbrook NM (2013) Polyploidy enhances the occupation of heterogeneous environments through hydraulic related trade-offs in *Atriplex canescens* (Chenopodiaceae). *New Phytol* 197: 970-978. <https://doi.org/10.1111/nph.12051>.
23. Hodaie M, Rahimmalek M, Arzani A, Talebi M (2018) The effect of water stress on phytochemical accumulation, bioactive compounds and expression of key genes involved in flavonoid biosynthesis in *Chrysanthemum morifolium* L. *Ind Crop Prod* 120: 295-304. <https://doi.org/10.1016/j.indcrop.2018.04.073>.
24. Hossain MN, Sarker U, Raihan MS, Al-Huqail AA, Siddiqui MH, Oba S (2022) Influence of Salinity Stress on Color Parameters, Leaf Pigmentation, Polyphenol and Flavonoid Contents, and Antioxidant Activity of *Amaranthus lividus* Leafy Vegetables. *Molecules* 27: 1821. <https://doi.org/10.3390/molecules27061821>.
25. Ijaz R, Ejaz J, Gao S, Liu T, Imtiaz M, Ye Z, Wang T (2017) Overexpression of annexin gene AnnSp2, enhances drought and salt tolerance through modulation of ABA synthesis and scavenging ROS in tomato. *Sci Rep* 7: 12087. <https://doi.org/10.1038/s41598-017-11168-2>.

26. Jayaraman K, Raman VK, Sevanthi AM, Sivakumar SR, Gayatri Viswanathan C, Mohapatra Trilochan, Mandal PK (2021) Stress-inducible expression of chalcone isomerase2 gene improves accumulation of flavonoids and imparts enhanced abiotic stress tolerance to rice. *Environ Exp Bot* 190: 104582. <https://doi.org/10.1016/j.envexpbot.2021.104582>.
27. Jez JM, Bowman ME, Dixon RA, Noel JP (2000) Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. *Nat Struct Biol* 7: 786-791. <https://doi.org/10.1038/79025>.
28. Kim J, Lee WJ, Vu TT, Jeong CY, Hong SW, Lee H (2017) High accumulation of anthocyanins via the ectopic expression of *AtDFR* confers significant salt stress tolerance in *Brassica napus* L. *Plant Cell Rep* 36: 1215-1224. <https://doi.org/10.1007/s00299-017-2147-7>.
29. Kotula L, Garcia Caparros P, Zörb C, Colmer TD, Flowers TJ (2020) Improving crop salt tolerance using transgenic approaches: An update and physiological analysis. *Plant Cell Environ* 43: 2932-2956. <https://doi.org/10.1111/pce.13865>.
30. Li J, Hossain MS, Ma H, Yang QH, Gong XW, Yang P, Feng BL (2020) Comparative metabolomics reveals differences in flavonoid metabolites among different coloured buckwheat flowers. *J Food Compos Anal* 85: 103335. <https://doi.org/10.1016/j.jfca.2019.103335>.
31. Li S, Chang Y, Li B, Shao SL, Zhen ZZ (2020) Functional analysis of 4-coumarate: CoA ligase from *Dryopteris fragrans* in transgenic tobacco enhances lignin and flavonoids. *Genet Mol Biol* 43: e20180355. <https://doi.org/10.1590/1678-4685-GMB-2018-0355>.
32. Li S, Sun L, Bai L, Wang W, Wang J (2017) Flavonoid is associated with salt stress tolerance in *Atriplex centralasiatica* seedlings. *Chinese J Ecol* 25: 1345-1350. <https://doi.org/10.13930/j.cnki.cjea.170079>.
33. Liu H, Su B, Zhang H, Gong J, Zhang B, Liu Y, Du L (2019) Identification and functional analysis of a *flavonol synthase* gene from grape hyacinth. *Molecules* 24: 1579. <https://doi.org/10.3390/molecules24081579>.
34. Liu Y, Dai XB, Zhao LK, Huo KS, Jin PF, Zhao DL, Zhou ZL, Tang J, Xiao SZ, Cao QH (2020) RNA-seq reveals the salt tolerance of *Ipomoea pes-caprae*, a wild relative of sweet potato. *J Plant Physiol* 255: 153276. <https://doi.org/10.1016/j.jplph.2020.153276>.
35. Ma D, He Z, Bai X, Wang W, Zhao P, Lin P, Zhou H (2022) *Atriplex canescens*, a valuable plant in soil rehabilitation and forage production. A review. *Sci Total Environ* 804: 150287. <https://doi.org/10.1016/j.scitotenv.2021.150287>.
36. Ma D, Sun D, Wang C, Li Y, Guo T (2014) Expression of flavonoid biosynthesis genes and accumulation of flavonoid in wheat leaves in response to drought stress. *Plant Physiol. Biochem.* 80, 60–66. <https://doi.org/10.1016/j.plaphy.2014.03.024>.
37. Ma Q, Yue LJ, Zhang JL, Wu GQ, Bao AK, Wang SM (2011) Sodium chloride improves photosynthesis and water status in the succulent xerophyte *Zygophyllum xanthoxylum*. *Tree Physiol* 32: 4-13. <https://doi.org/10.1093/treephys/tpr098>.

38. Ma S, Lv L, Meng C, Zhang C, Li Y (2020) Integrative analysis of the metabolome and transcriptome of *sorghum bicolor* reveals dynamic changes in flavonoids accumulation under saline-alkali stress. J Agric Food Chem 68: 14781-14789. <https://doi.org/10.1021/acs.jafc.0c06249>.
39. Mahajan M, Yadav SK (2014) Overexpression of a tea flavanone 3-hydroxylase gene confers tolerance to salt stress and *Alternaria solani* in transgenic tobacco. Plant Mol Biol 85: 551-573. <https://doi.org/10.1007/s11103-014-0203-z>.
40. Meng C, Zhang S, Deng YS, Wang GD, Kong FY (2015) Overexpression of a tomato flavanone 3-hydroxylase-like protein gene improves chilling tolerance in tobacco. Plant Physiol Biochem 96: 388-400. <https://doi.org/10.1016/j.plaphy.2015.08.019>.
41. Murphy A, Peer WA, Taiz L (2000) Regulation of auxin transport by aminopeptidases and endogenous flavonoids. Planta 211: 315-324. <https://doi.org/10.1007/s004250000300>.
42. Nabavi SM, Samec D, Tomczyk M, Milella L, Russo D, Habtemariam S, Suntar I, Rastrelli L, Daglia M, Xiao J, Giampieri F, Battino M, Sobarzo-Sanchez E, Nabavi SF, Yousefi B, Jeandet P, Xu S, Shirooie S (2020) Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. Biotechnol Adv 38: 107316. <https://doi.org/10.1016/j.biotechadv.2018.11.005>.
43. Ngaki MN, Louie GV, Philippe RN, Manning G, Pojer F, Bowman ME, Li L, Larsen E, Wurtele ES, Noel JP (2012) Evolution of the chalcone-isomerase fold from fatty-acid binding to stereospecific catalysis. Nature 485: 530-533. <https://doi.org/10.1038/nature11009>.
44. Ni J, Ruan R, Wang L, Jiang Z, Gu X, Chen L, Xu M (2020) Functional and correlation analyses of dihydroflavonol-4-reductase genes indicate their roles in regulating anthocyanin changes in *Ginkgo biloba*. Ind Crop Prod 152: 112546. <https://doi.org/10.1016/j.indcrop.2020.112546>.
45. Ni R, Zhu TT, Zhang XS, Wang PY, Sun CJ, Qiao YN, Lou HX, Cheng AX (2020) Identification and evolutionary analysis of chalcone isomerase-fold proteins in ferns. J Exp Bot 71: 290-304. <https://doi.org/10.1093/jxb/erz425>.
46. Pan YQ, Guo H, Wang SM, Zhao B, Zhang JL, Ma Q, Yin HJ, Bao AK (2016) The photosynthesis, Na^+/K^+ homeostasis and osmotic adjustment of *Atriplex canescens* in response to salinity. Front Plant Sci 7: 848. <https://doi.org/10.3389/fpls.2016.00848>.
47. Sarker U, Ercisli S (2022) Salt eustress induction in red *Amaranth* (*Amaranthus gangeticus*) augments nutritional, phenolic acids and antiradical potential of leaves. Antioxidants (Basel) 11: 2434. <https://doi.org/10.3390/antiox11122434>.
48. Sarker U, Hossain MN, Oba S, Ercisli S, Marc RA, Golokhvast KS (2023) Salinity stress ameliorates pigments, minerals, polyphenolic profiles, and antiradical capacity in Lalshak. Antioxidants (Basel) 12: 173. <https://doi.org/10.3390/antiox12010173>.
49. Sarker U, Islam MT, Oba S (2018) Salinity stress accelerates nutrients, dietary fiber, minerals, phytochemicals and antioxidant activity in *Amaranthus tricolor* leaves. PLoS One 13: e0206388.
50. Sarker U, Oba S (2018) Augmentation of leaf color parameters, pigments, vitamins, phenolic acids, flavonoids and antioxidant activity in selected *Amaranthus tricolor* under salinity stress. Sci Rep 8: 12349. <https://doi.org/10.1038/s41598-018-30897-6>.

51. Sarker U, Oba S (2019) Salinity stress enhances color parameters, bioactive leaf pigments, vitamins, polyphenols, flavonoids and antioxidant activity in selected *Amaranthus* leafy vegetables. J Sci Food Agric 99: 2275-2284. <https://doi.org/10.1002/jsfa.9423>.
52. Sarker U, Oba S (2020) The Response of Salinity stress-induced *A. tricolor* to growth, anatomy, physiology, non-enzymatic and enzymatic antioxidants. Front Plant Sci 11: 559876. <https://doi.org/10.3389/fpls.2020.559876>.
53. Sewelam N, Kazan K, Schenk PM (2016) Global plant stress signaling: Reactive oxygen species at the cross-road. Front Plant Sci 7: 187. <https://doi.org/10.3389/fpls.2016.00187>.
54. Shabala S (2013) Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann Bot 7: 1209-1221. <https://doi.org/10.1093/aob/mct205>.
55. Shimada N, Aoki T, Sato S, Nakamura Y, Tabata S, Ayabe S (2003) A cluster of genes encodes the two types of chalcone isomerase involved in the biosynthesis of general flavonoids and legume-specific 5-deoxy(iso)flavonoids in *Lotus japonicus*. Plant Physiol 131: 941-951. <https://doi.org/10.1104/pp.004820>.
56. Singh A (2018) Alternative management options for irrigation-induced salinization and waterlogging under different climatic conditions. Ecol Indic 90: 184-192. <https://doi.org/10.1016/j.ecolind.2018.03.014>.
57. Sun W, Shen H, Xu H, Tang X, Tang M, Ju Z, Yi Y (2019) Chalcone Isomerase a key enzyme for anthocyanin biosynthesis in *Ophiorrhiza japonica*. Front Plant Sci 10: 865. <https://doi.org/10.3389/fpls.2019.00865>.
58. Wang C, Zhi S, Liu C, Xu F, Zhao A, Wang X, Tang X, Li Z, Huang P, Yu M (2017) Isolation and characterization of a novel chalcone synthase gene family from mulberry. Plant Physiol Biochem 115: 107-118. <https://doi.org/10.1016/j.plaphy.2017.03.014>.
59. Wang F, Ren G, Li F, Qi S, Xu Y, Wang B, Yang Y, Ye Y, Zhou Q, Chen X (2018) A chalcone synthase gene *AeCHS* from *Abelmoschus esculentus* regulates flavonoid accumulation and abiotic stress tolerance in transgenic *Arabidopsis*. Acta Physiol. Plant 40: 97. <https://doi.org/10.1007/s11738-018-2680-1>.
60. Wang H, Hu T, Huang J, Lu X, Huang B, Zheng Y (2013) The expression of *Millettia pinnata* chalcone isomerase in *Saccharomyces cerevisiae* salt-sensitive mutants enhances salt-tolerance. Int. J. Mol. Sci 14: 8775-8786. <https://doi.org/10.3390/ijms14058775>.
61. Wang H, Liu S, Wang T, Liu H, Xu X, Chen K, Zhang P (2020) The moss flavone synthase I positively regulates the tolerance of plants to drought stress and UV-B radiation. Plant Sci 298: 110591. <https://doi.org/10.1016/j.plantsci.2020.110591>.
62. Wang M, Ren T, Huang R, Li Y, Zhang C, Xu Z (2021) Overexpression of an *Apocynum venetum* flavonols synthetase gene confers salinity stress tolerance to transgenic tobacco plants. Plant Physiol Biochem 162: 667-676. <https://doi.org/10.1016/j.plaphy.2021.03.034>.
63. Wang N, Liu W, Yu L, Guo Z, Chen Z, Jiang S, Xu H, Fang H, Wang Y, Zhang Z, Chen X (2020) HEAT SHOCK FACTOR A8a modulates flavonoid synthesis and drought tolerance. Plant Physiol 184: 1273-

1290. <https://doi.org/10.1104/pp.20.01106>.
64. Xu N, Liu S, Lu Z, Pang S, Wang L, Wang L, Li W (2020) Gene expression profiles and flavonoid accumulation during salt stress in *Ginkgo biloba* seedlings. *Plants* 9: 1162. <https://doi.org/10.3390/plants9091162>.
65. Yang L, Zhang JC, Qu JT, He G, Yu HQ, Li WC, Fu FL (2019) Expression response of chalcone synthase gene to inducing conditions and its effect on flavonoids accumulation in two medicinal species of *Anoectochilus*. *Sci Rep* 9: 20171. <https://doi.org/10.1038/s41598-019-56821-0>.
66. Yang Y, Guo Y (2018) Unraveling salt stress signaling in plants. *J Integr Plant Biol* 60: 796-804. <https://doi.org/10.1111/jipb.12689>.
67. Zhang H, Han B, Wang T, Chen S, Li H, Zhang Y, Dai S (2012) Mechanisms of plant salt response: insights from proteomics. *J Proteome Res* 11: 49-67. <https://doi.org/10.1021/pr200861w>.
68. Zhang H, Zhu J, Gong Z, Zhu JK (2022) Abiotic stress responses in plants. *Nature Reviews Genet* 23: 104-119. <https://doi.org/10.1038/s41576-021-00413-0>.
69. Zhang JL, Shi H (2013) Physiological and molecular mechanisms of plant salt tolerance. *Photosynth Res* 115: 1-22. <https://doi.org/10.1007/s11120-013-9813-6>.
70. Zhang K, Sun Y, Li M, Long R (2021) CrUGT87A1, a UDP-sugar glycosyltransferases (UGTs) gene from *Carex rigescens*, increases salt tolerance by accumulating flavonoids for antioxidation in *Arabidopsis thaliana*. *Plant Physiol Biochem* 159: 28-36. <https://doi.org/10.1016/j.plaphy.2020.12.006>.
71. Zhao C, Liu X, Gong Q, Cao J, Shen W, Yin X, Grierson D, Zhang B, Xu C, Li X, Chen K, Sun C (2021) Three AP2/ERF family members modulate flavonoid synthesis by regulating type IV chalcone isomerase in citrus. *Plant Biotechnol J* 19: 671-688. <https://doi.org/10.1111/pbi.13494>.
72. Zheng XT, Chen YL, Zhang XH, Cai ML, Yu ZC, Peng CL (2019) ANS-deficient *Arabidopsis* is sensitive to high light due to impaired anthocyanin photoprotection. *Funct Plant Biol* 46: 756-765. <https://doi.org/10.1071/fp19042>.
73. Zhou L, Wang Y, Ren L, Shi QQ, Zheng BQ, Miao K, Guo X (2014) Overexpression of *Ps-CHI1*, a homologue of the chalcone isomerase gene from tree peony (*Paeonia suffruticosa*), reduces the intensity of flower pigmentation in transgenic tobacco. *Plant Cell Tissue Organ Cult* 116: 285-295. <https://doi.org/10.1007/s11240-013-0403-2>.
74. Zhu J, Zhao W, Li R, Guo D, Li H, Wang Y, Mei W, Peng S (2021) Identification and characterization of chalcone isomerase genes involved in flavonoid production in *Dracaena cambodiana*. *Front Plant Sci* 12: 616396. <https://doi.org/10.3389/fpls.2021.616396>.

Figures

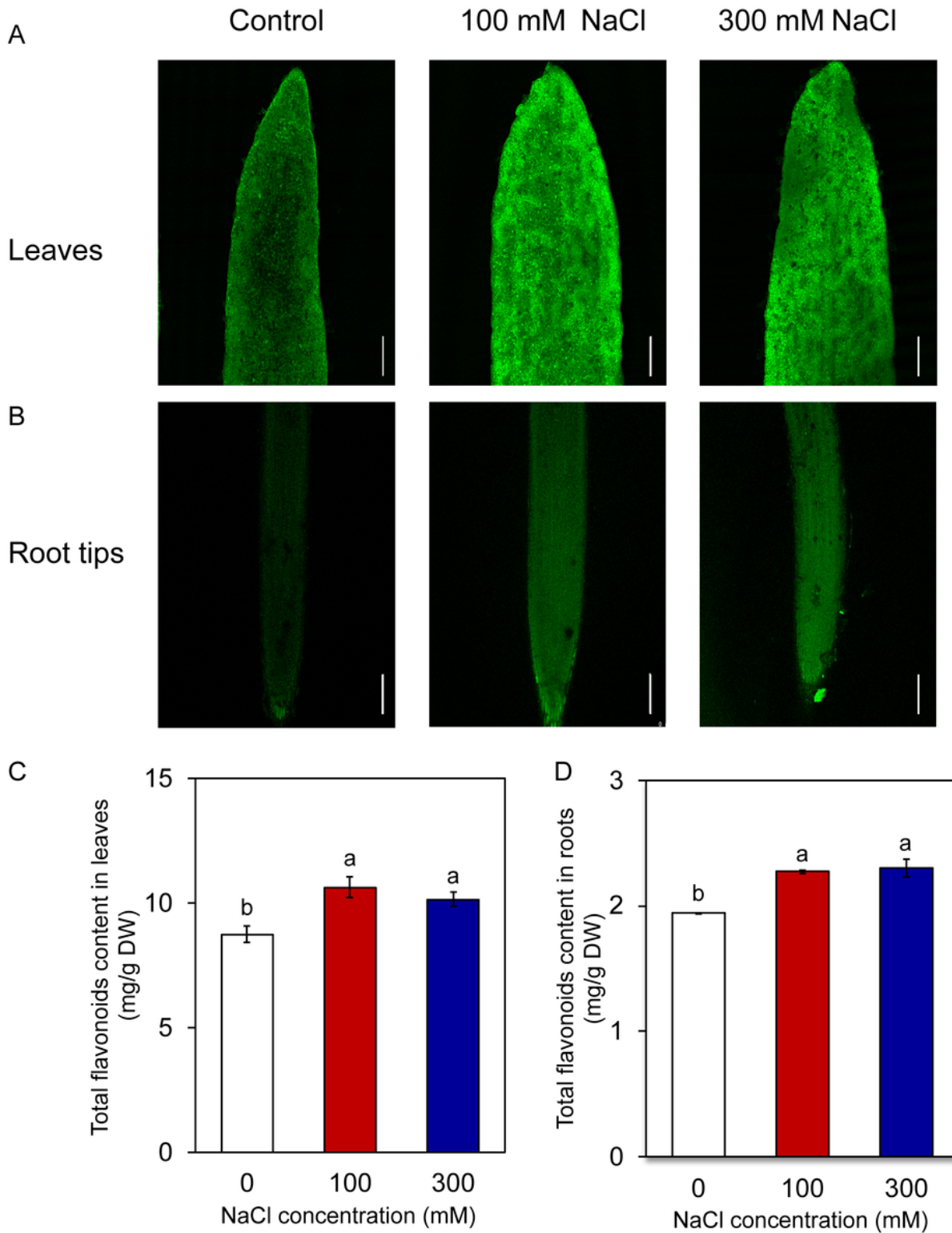


Figure 1

Accumulation of flavonoids in *A. canescens* under NaCl treatments. (A) Two-week-old seedlings were treated with 0 (control), 100 and 300 mM NaCl for 2 d, then the first and second true leaves were excised for DPBA staining and visualized by the fluorescence signal. (B) Four-day-old seedlings were treated with 0 (control), 100 and 300 mM NaCl for 2 d, then the root tips were cut for DPBA staining and the accumulation of flavonoids was visualized by the fluorescence signal. (C) and (D) Total flavonoids

content in the leaves and roots of four-week-old seedlings treated with 0, 100 and 300 mM NaCl for 2 d. The scales in (A) and (B) are equal to 0.05 mm. Different letters in (C) and (D) indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE (n=6).

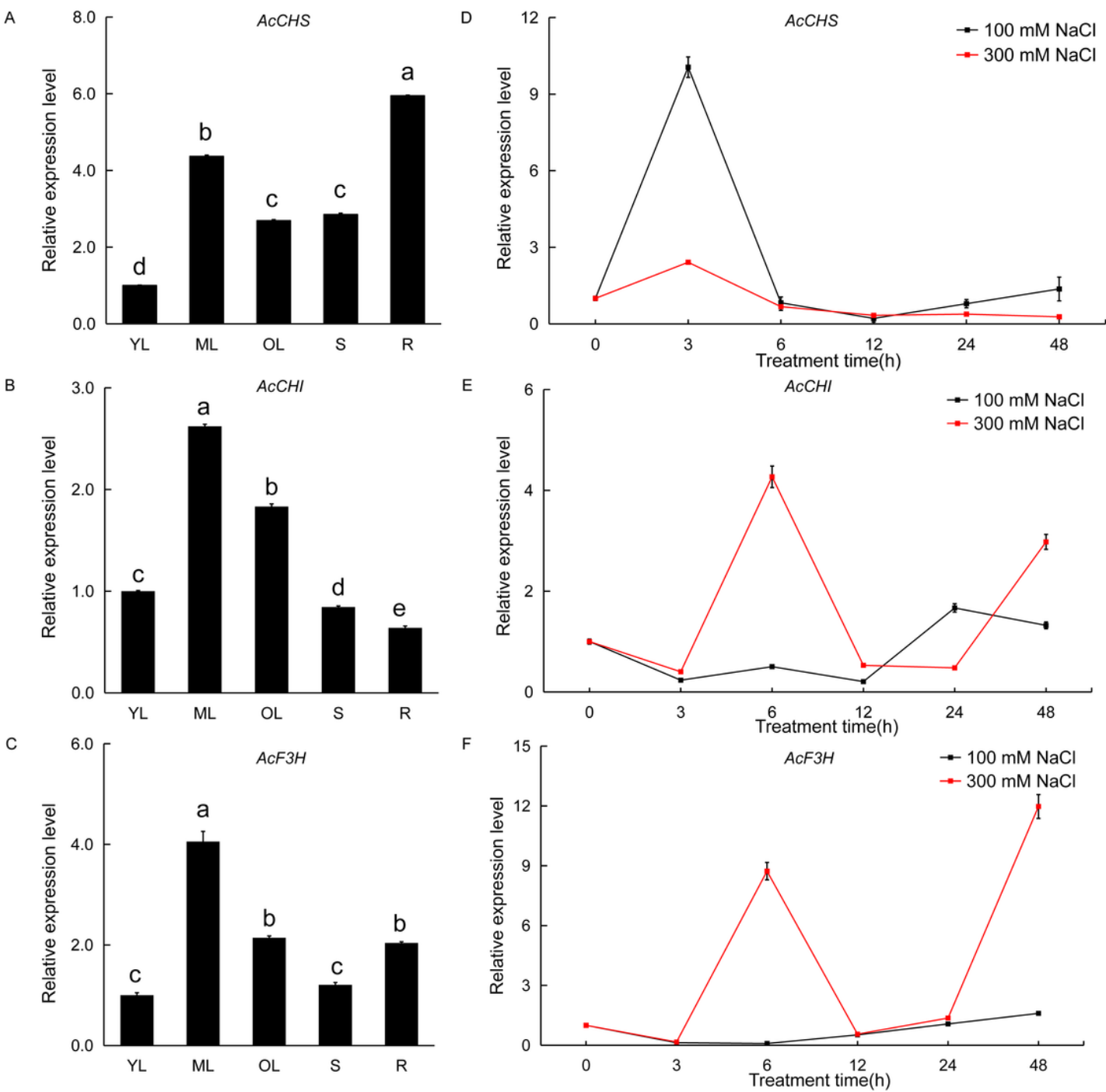


Figure 2

The expression analysis of *AcCHS*, *AcCHI* and *AcF3H* in *A. canescens*. (A)-(C) Tissue specific expression analysis of *AcCHS* (A), *AcCHI* (B) and *AcF3H* (C) in four-week-old *A. canescens* seedlings. (D)-(F) The

relative expression level of *AcCHS* (D) in the roots, *AcCHI* (E) and *AcF3H* (F) in the mature leaves of four-week-old *A. canescens* seedlings treated with 100 and 300 mM NaCl for 0-48 h. YL: young leaves (the first and second layers of leaves), ML: mature leaves (the third and fourth layers of leaves), OL: old leaves (the fifth layer of leaves and below), S: stem, R: root. Different letters in (A) to (C) indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE (n=3).

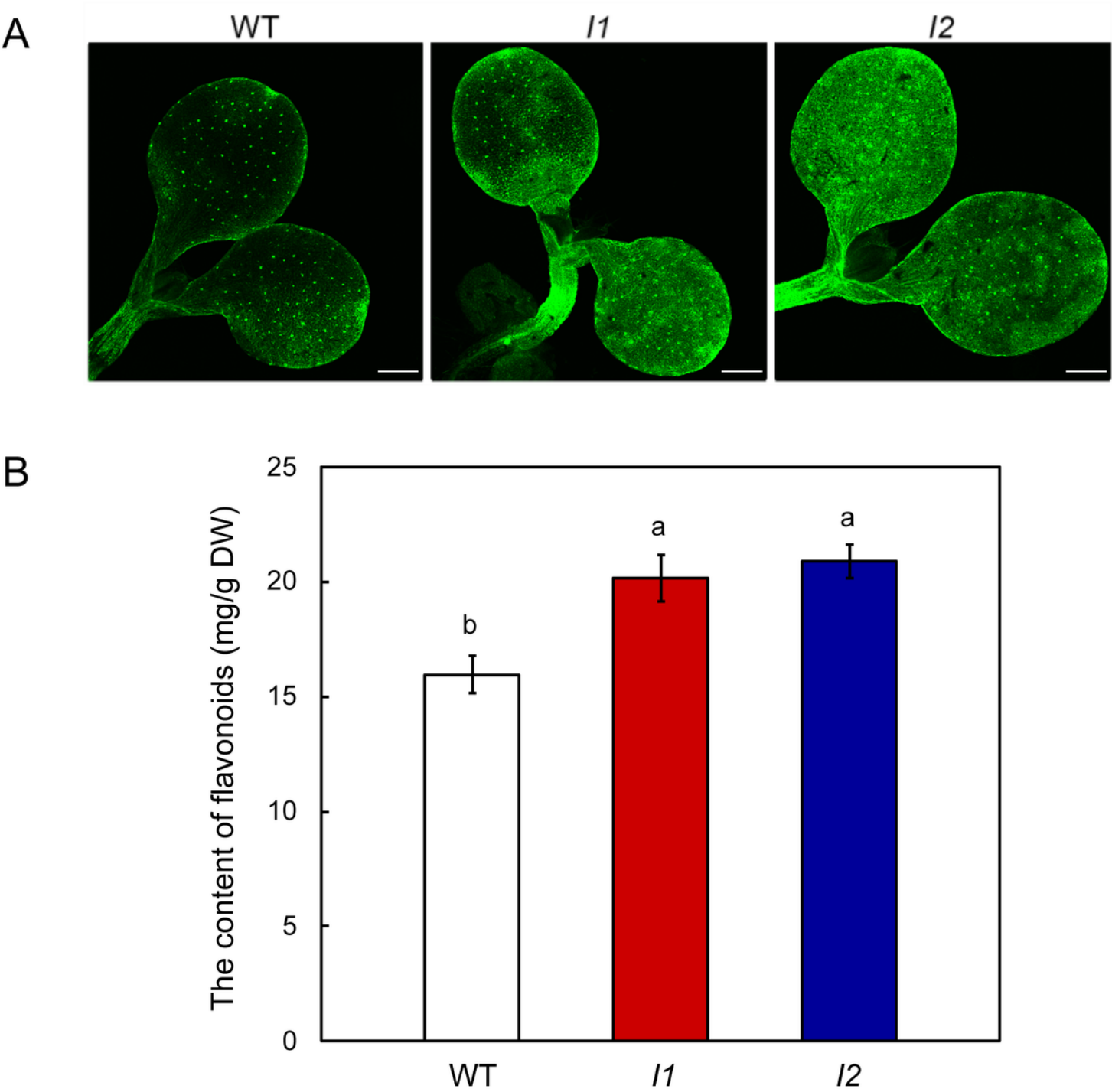


Figure 3

Accumulation of flavonoids in wild-type (WT) and transgenic *A. thaliana* overexpressing *AcCHI*. (A) The first and second true leaves of hydroponic 6-day-old seedlings were excised for DPBA staining and visualized by the fluorescence signal. (B) Total flavonoids content in the shoots of hydroponic 4-week-old seedlings. Bars in (A) is equal to 400 μ m. Different letters in (B) indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE (n=6).

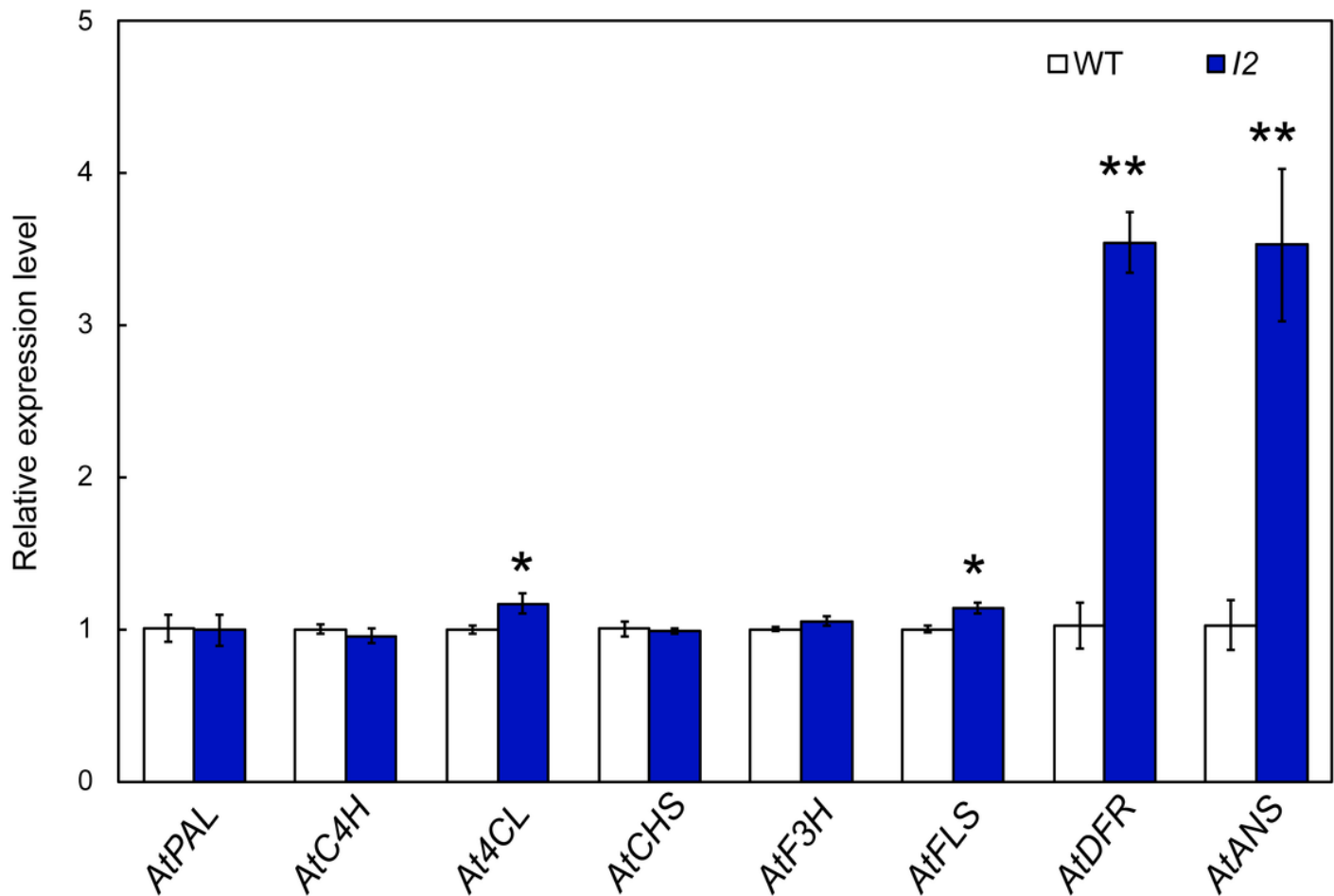


Figure 4

The expression analysis of key genes involving in flavonoids biosynthesis pathway in the leaves of hydroponic 3-week-old seedlings of wild-type (WT) and transgenic line *I2*. Asterisks indicate a significant difference (* $p < 0.05$; ** $p < 0.01$). Values are means $\pm$ SE (n=3).

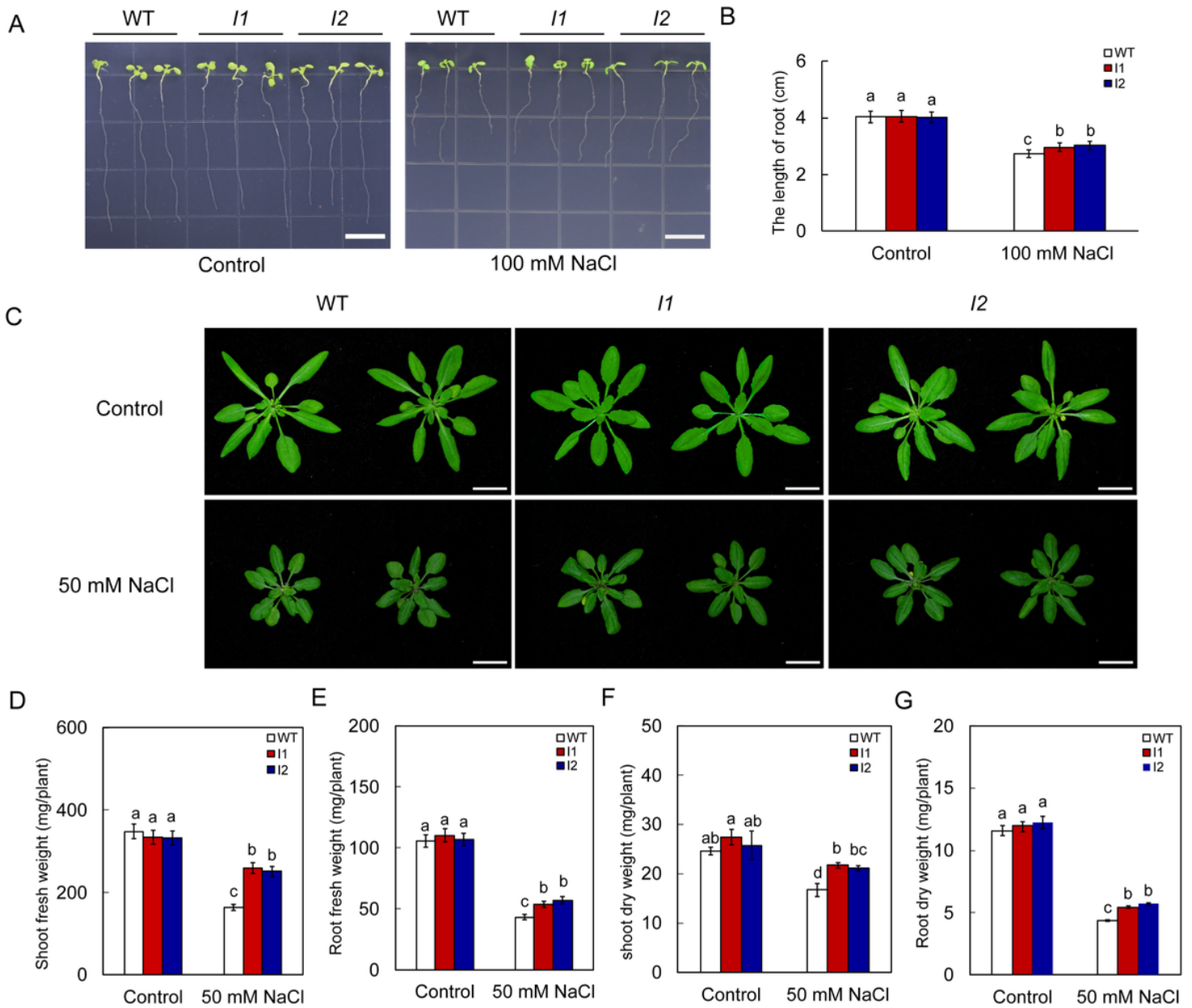


Figure 5

Growth of wild-type (WT) and transgenic *A. thaliana* overexpressing *AcCHI* under NaCl treatments. Four-day-old seedlings were treated with 0 (control) and 100 mM NaCl on 1/2 MS medium for 10 d, then the phenotypes were photographed (A) and the root lengths were measured (B). Three-week-old WT and transgenic lines were treated with 1/2 strength Hoagland nutrient solution without (control) or with additional 50 mM NaCl for 7 d in hydroponics and the phenotypes were photographed (C), then the shoot fresh weight (D), root fresh weight (E), shoot dry weight (F), and root dry weight (G) were determined, respectively. The scales in (A) and (C) are equal to 1 cm and 2 cm, respectively. Different letters in (B) and (D) to (G) indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE ($n=6$).

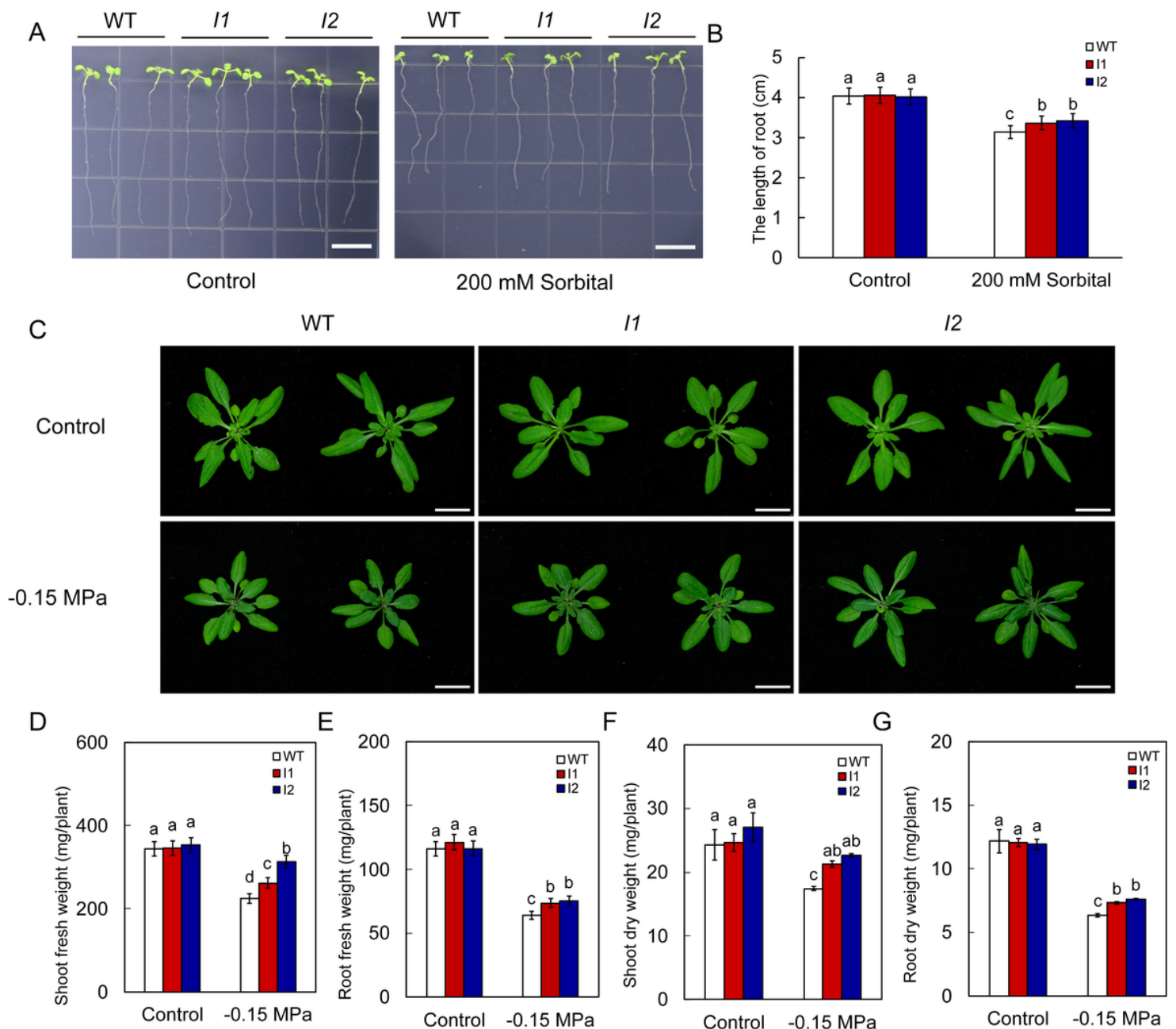


Figure 6

Growth of wild-type (WT) and transgenic *A. thaliana* overexpressing *AcCHI* under osmotic stress conditions. Four-day-old WT and transgenic seedlings were treated with 0 (control) and 200 mM Sorbital on 1/2 MS medium for 10 d, then the phenotypes were photographed (A) and the root lengths were measured (B). Three-week-old WT and transgenic lines were treated with 1/2 strength Hoagland nutrient solution without (control) or with additional 150 mM Sorbital (-0.15 MPa) for 3 d in hydroponics and the phenotypes were photographed (C), then the shoot fresh weight (D), root fresh weight (E), shoot dry weight (F), and root dry weight (G) were determined, respectively. The scales in (A) and (C) are equal to 1 cm and 2 cm, respectively. Different letters in (B) and (D) to (G) indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE ($n=6$).

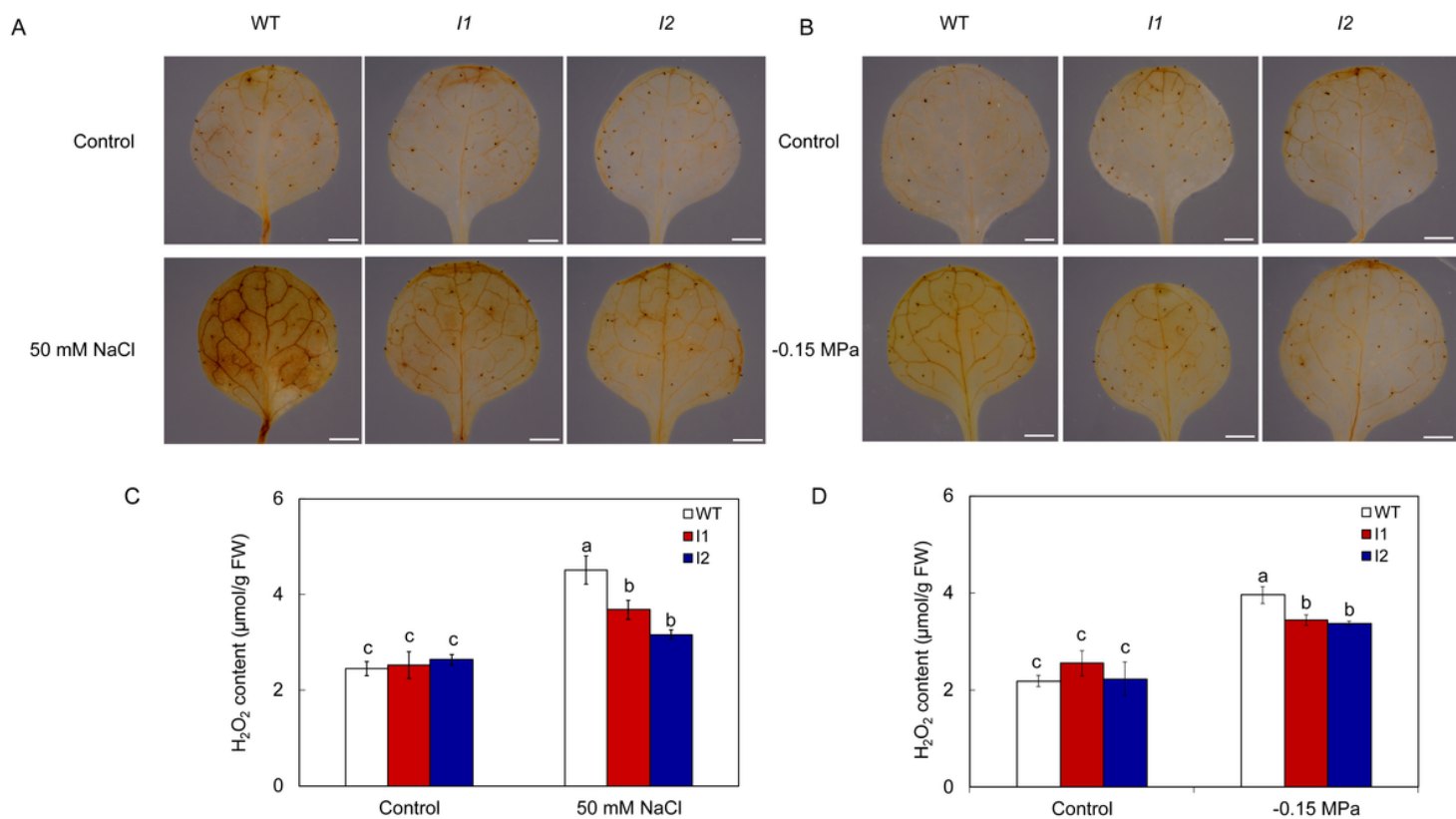


Figure 7

Accumulation of H₂O₂ in wild-type (WT) and transgenic *A. thaliana* overexpressing *AcCHI* under salt and osmotic stress conditions. (A) and (B) Hydroponic 3-week-old seedlings of WT and transgenic lines were treated with 0 (control) and 50 mM NaCl for 7 d or 150 mM Sorbital (-0.15 MPa) for 3 d, then the H₂O₂ of leaves were detected by 3,3-diaminobenzidine (DAB). (C) and (D) The content of H₂O₂ in the leaves of hydroponic 3-week-old WT and transgenic lines treated with 50 mM NaCl 7 d or 150 mM Sorbital (-0.15 MPa) for 3 d. The scales in (A) and (B) are equal to 400 μm. Different letters indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE (n=6).

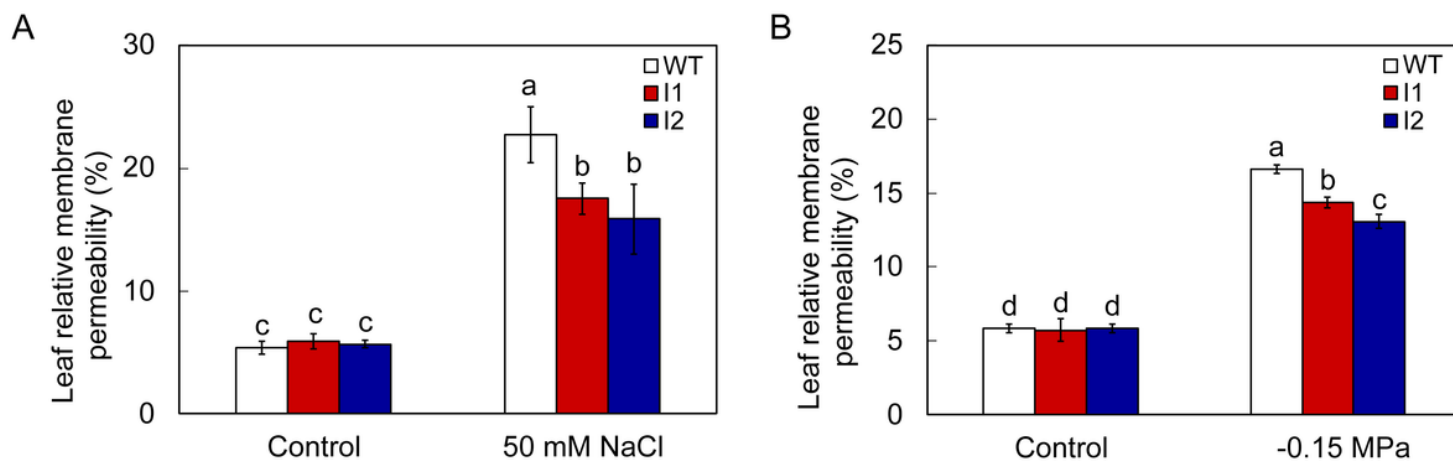


Figure 8

Page 27/29

The leaf relative membrane permeability in wild-type (WT) and transgenic *A. thaliana* overexpressing *AcCHI* under salt and osmotic stress conditions. Hydroponic 3-week-old seedlings were treated with 0 (control) and 50 mM NaCl 7 d (A) or 150 mM Sorbital (-0.15 MPa) for 3 d (B), then the leaf relative membrane permeability was measured. Different letters indicate significant differences at $P < 0.05$ (Duncan's test). Values are means $\pm$ SE (n=6).

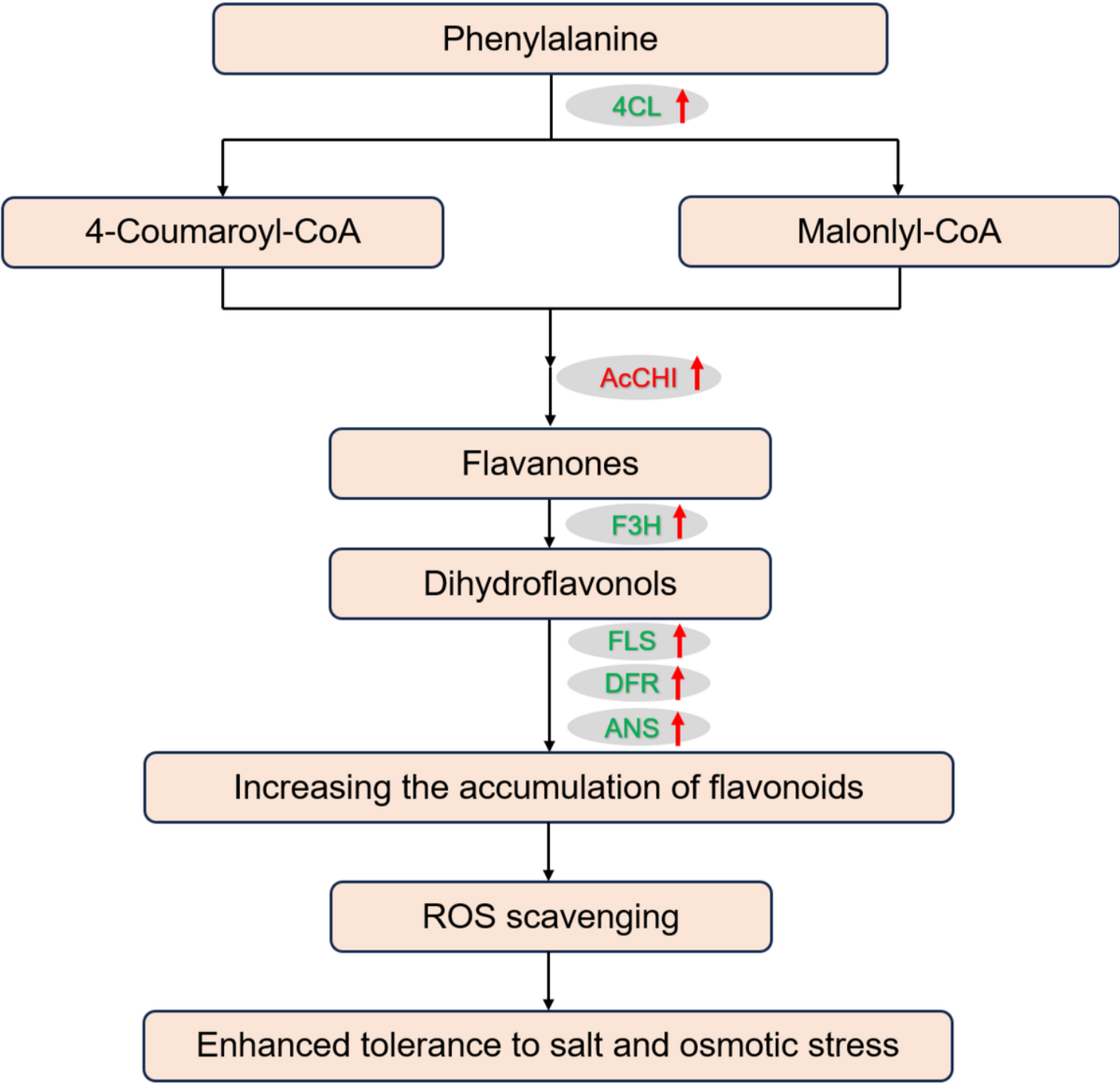


Figure 9

The supposed model pathway related to the increase of flavonoids content in transgenic *A. thaliana* overexpressing *AcCHI*. Overexpression of *AcCHI* up-regulates the genes involved in the flavonoid

biosynthesis and improves ROS scavenging, leading to the enhanced salt and osmotic tolerance of transgenic *A. thaliana*. 4CL, 4-coumaric acid: CoA ligase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; FLS, flavonol synthase; ANS, anthocyanidin synthase. The red arrow indicates up-regulated expression.

Supplementary Files

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