

RESEARCH

Open Access



Overexpression of the *Salix matsudana* *SmAP2-17* gene improves *Arabidopsis* salinity tolerance by enhancing the expression of *SOS3* and *ABI5*

Yanhong Chen[†], Yuanhao Dai[†], Yixin Li, Jie Yang, Yuna Jiang, Guoyuan Liu, Chunmei Yu, Fei Zhong, Bolin Lian and Jian Zhang^{*}

Abstract

Background: *Salix matsudana* (Koidz.) is a widely planted ornamental allotetraploid tree species. Genetic engineering can be used to enhance the tolerance of this species to soil salinization, endowing varieties with the ability to grow along coastlines, thereby mitigating afforestation and protecting the environment. The AP2/ERF family of transcription factors (TFs) plays multidimensional roles in plant biotic/abiotic stress tolerance and plant development. In this study, we cloned the *SmAP2-17* gene and performed functional analysis of its role in salt tolerance. This study aims to identify key genes for future breeding of stress-resistant varieties of *Salix matsudana*.

Results: *SmAP2-17* was predicted to be a homolog of AP2-like ethylene-responsive transcription factor ANT isoform X2 from *Arabidopsis*, with a predicted ORF of 2058 bp encoding an estimated protein of 685 amino acids containing two conserved AP2 domains (PF00847.20). *SmAP2-17* had a constitutive expression pattern and was localized to the nucleus. The overexpression of the native *SmAP2-17* CDS sequence in *Arabidopsis* did not increase salt tolerance because of the reduced expression level of ectopic *SmAP2-17*, potentially caused by salt-induced RNAi. Transgenic lines with high expression of optimized *SmAP2-17* CDS under salt stress showed enhanced tolerance to salt. Moreover, the expression of general stress marker genes and important salt stress signaling genes, including *RD29A*, *ABI5*, *SOS3*, *AtHKT1*, and *RBoHf*, were upregulated in *SmAP2-17*-overexpressed lines, with expression levels consistent with that of *SmAP2-17* or optimized *SmAP2-17*. Promoter activity analysis using dual luciferase analysis showed that *SmAP2-17* could bind the promoters of *SOS3* and *ABI5* to activate their expression, which plays a key role in regulating salt tolerance.

Conclusions: The *SmAP2-17* gene isolated from *Salix matsudana* (Koidz.) is a positive regulator that improves the resistance of transgenic plants to salt stress by upregulating *SOS3* and *ABI5* genes. This study provides a potential functional gene resource for future generation of salt-resistant *Salix* lines by genetic engineering.

Keywords: *Salix matsudana*, AP2/ERF, Salt tolerance, *Arabidopsis*, Overexpression

Background

The APETALA2/ethylene responsive factor (AP2/ERF) family transcription factors (TFs) is a plant-specific gene family with many members, which are classified into APETALA2(AP2), RELATED TO ABSCISIC ACID

*Correspondence: ntdxylzw@163.com

[†]Yanhong Chen and Yuanhao Dai contributed equally to this work.
Key Lab of Landscape Plant Genetics and Breeding, School of Life Science, Nantong University, Nantong, Jiangsu Province, China



© The Author(s) 2022, corrected publication 2022. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

INSENSITIVE3/VIVIPAROUS 1 (RAV), DEHYDRATION-RESPONSIVE ELEMENT BINDING proteins (DREBs), ETHYLENE RESPONSIVE FACTORS (ERFs), and Soloist subfamilies, according to the number or structure of AP2 and other conserved domains [1, 2]. AP2/ERF family TFs play important and multidimensional roles in plant biotic/abiotic stress tolerance and plant growth, differentiation, and development [3–8]. DREB members extensively participate in the response and regulation of abiotic stress tolerance, such as cold, drought, heat, and salt tolerance, by directly regulating the expression of stress-responsive genes [5, 9]. In addition to genes from *Arabidopsis*, many studies have illustrated the function of DREB genes from other plant species, such as maize, wheat, and soybean, by overexpressing these genes in model plants [10–16]. Members of the ERF subfamily are also involved in abiotic stress responses, and members of the ERF-VII subfamily from *Arabidopsis* and rice have been verified to play major roles in flooding and submergence tolerance (hypoxia stress) [17–19]. *Arabidopsis* AP2s play a central role in developmental processes, including axillary floral meristem development, gynoecium development, shoot regeneration, brace root development, internode elongation, and trichome formation [20–25]. Members of the AP2 subfamilies have also been reported to mediate diverse abiotic stress responses. AINTEGUMENTA (ANT) is an example of this. In addition to controlling organ cell number and size, ANT also negatively regulates salt tolerance by repressing SOS3-LIKE CALCIUM BINDING PROTEIN 8 (SCABP8/CBL10), a putative Ca^{2+} sensor that protects *Arabidopsis* shoots against salt stress and maintains ion homeostasis [26].

Salix matsudana Koidz., an allotetraploid member of *Salicaceae*, is an important and widely planted ornamental tree species with strong and wide adaptability to diverse environmental stresses, including salinity [27, 28]. The genome of *S. matsudana* was recently sequenced, and the family members and classification of the *SmAP2/ERF* gene family were identified and their expression patterns under salt stress were analyzed [28, 29]. From the sequencing results, 364 *SmAP2/ERF* members were identified and named from the *S. matsudana* genome and classified into four major subfamilies: AP2 (55 members), ERF (166 members), DREB (135 members), and RAV (6

members) [29]. The expression levels of 4 genes from the AP2 subgroup, 10 genes from the DREB subgroup, and 13 genes from the ERF subgroup were strongly induced by salt stress [29]. Considering the important roles of AP2/ERF family TFs in stress tolerance, there is an urgent need to uncover the function of the *SmAP2/ERF* genes, especially those genes with induced expression patterns under stress. These researches will promote the application of these genes in molecular assisted breeding of *Salix* plants. However, because of the difficulties in genetic transformation and the unavailability of genomic sequences, the functional characterization of *SmAP2/ERF* members remains largely unexplored in *Salix* and other woody plant species. In the present study, a putative AP2 family gene, *SmAP2-17*, whose expression was induced under salt stress, was isolated and cloned from *Salix matsudana*. To determine the function of *SmAP2-17*, this gene was transformed into *Arabidopsis* via *Agrobacterium*-mediated transformation to evaluate its role in tolerance to salt stress and uncover its downstream genes.

Results

Isolation and bioinformatics analysis of *SmAP2-17* and its homolog proteins

The coding sequence of *SmAP2-17* was 2058 bp in length and encoded a protein comprised of 685 amino acids with a molecular mass of 76.3 kDa and an isoelectric point of 6.45. In the genome annotation, *SmAP2-17* was predicted to be a homolog of AP2-like ethylene-responsive transcription factor ANT isoform X2. *SmAP2-17* shares an identity of 44.65% with *Arabidopsis* ANT protein. Phylogenetic analysis showed that *SmAP2-17* was closely related to proteins from *Salix* and *Populus*, such as SwEVM0024941.1 (96% identity), SapurV1A.0608s0160.1 (92.81% identity), and Potri.007G007400.1 (87.01% identity) from *Salix wilsonii*, *Salix purpurea*, and *Populus trichocarpa*, respectively (Fig. 1a). Other homologs shared a lower identity, in particular GRMZM2G028151_T01 and LOC_Os03g56050.1 from maize and rice, respectively. Multiple sequence alignment analysis showed that these proteins shared two conserved AP2/ERF domains (Fig. 1b). The protein sequences of *SmAP2-17* and its homologs are listed in File S1.

(See figure on next page.)

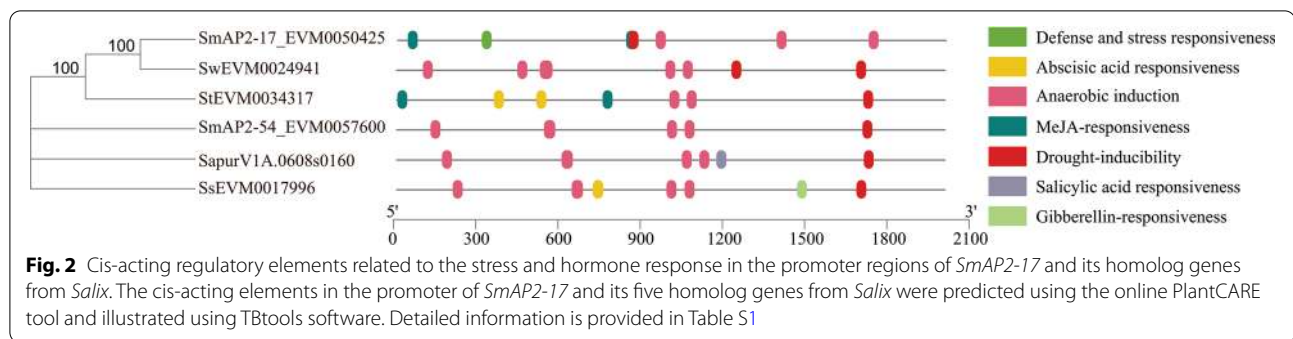
Fig. 1 Phylogenetic analysis and sequence alignment of *SmAP2-17* and its homologs. **a** Phylogenetic analysis of the *SmAP2-17* protein with its orthologous genes in other plant species. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates in MEGA 7.0 software. **b** Alignment of the *SmAP2-17* protein with its orthologous genes in *Salix*, *Populus*, maize and *Arabidopsis*. There are two AP2 domains in the proteins, which were labeled with blue and pink lines, respectively. ANT is an *Arabidopsis* protein, whose gene locus is At3g47750. SapurV1A.0608s0160.1 and Potri.007G007400.1 come from *Salix purpurea* and *Populus trichocarpa*, respectively. GRMZM2G028151_T01 and LOC_Os03g56050.1 are proteins from maize and rice. Proteins with Sw, Sm, St, and Ss denote proteins that belong to *Salix wilsonii*, *Salix matsudana*, *Salix integra* and *Salix suchowensis*, respectively

A

B

C

D



Analysis of promoter elements of *SmAP2-17* and its homolog genes

The 2000-bp promoter sequences of *SmAP2-17* and its five homologs in *Salix* were isolated (File S2) and cis-element motif analysis was performed using the PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The anaerobic induction motif had the highest number and was present in the promoter region of all genes. The drought-inducibility motif also appeared in all genes, but most genes had only one (the only exception being *SwEVM0024941*, having two). Apart from the anaerobic induction motif and drought-inducibility motif, the abscisic acid responsiveness motif, defense and stress responsiveness motif, and MeJA responsiveness are also present in the promoter region of *SmAP2-17* (the position of the abscisic acid responsiveness motif and MeJA-responsiveness motif almost overlapped, Table S1). The abscisic acid responsiveness motif was also found in *StEVM0034317* and *SsEVM0017996*. The salicylic acid responsiveness motif and gibberellin-responsiveness motif were only found in *SapurV1A.0608s0160* and *SsEVM0017996*, respectively (Fig. 2).

Expression pattern and subcellular location

Gene expression patterns can provide useful clues to gene function. To study the expression profiles of *SmAP2-17*, the transcripts of *SmAP2-17* across different tissues and under salt stress were examined using qRT-PCR. In different tissues (root, stem, leaf, shoot, and anther) of *S. matsudana*, the highest accumulation of *SmAP2-17* was observed in the stems and shoots, followed by the roots and anther, while low expression levels were detected in the leaves. Salt stress induced the expression of *SmAP2-17*, with an expression level that was eightfold higher than normal conditions (Fig. 3a).

The subcellular localization of *SmAP2-17* was examined to gain useful insights into its molecular function. WoLF PSORT (<http://www.genscript.com/wolf-psort.html>) predicted that *SmAP2-17* resides in the nucleus. To confirm this prediction, the coding sequence of

SmAP2-17 was fused to the C-terminus of green fluorescent protein (GFP) driven by the cauliflower mosaic virus 35S promoter, and the construct was transiently expressed in tobacco leaf epidermal cells. The expression of the red fluorescent protein (RFP) fusion histone protein, H₂B-RFP, was used as a nuclear localization marker. As shown in Fig. 3b, a yellow fluorescence signal from the co-localization of *SmAP2-17*-GFP construct (green) and H₂B-RFP (red) was observed in the nucleus of leaf stomata guard cells. This finding indicates that *SmAP2-17* was localized in the nucleus (Fig. 3b).

SmAP2-17 did not confer transgenic *Arabidopsis* tolerance to salt stress due to salt-induced gene silencing

To characterize the role of *SmAP2-17* in salt tolerance, the construct containing the *SmAP2-17* coding sequence (CDS) driven by the 35S enhancer was transformed into *Arabidopsis*. After screening and molecular identification, two transgenic lines were used to perform different treatments of NaCl on 1/2 MS medium to determine the germination rates of *Arabidopsis*. Upon salt treatment using 75 mM NaCl, 100 mM NaCl, and 150 mM NaCl, the germination rates of the T1 and T15 transgenic lines at 72 h were significantly lower than those of the wild-type (WT) (Fig. 4a). The delayed germination phenotype could be observed clearly in photographs (Fig. 4b), which showed germination and growth phenotypes of WT and T15 at 48 h and 72 h after planting the seeds on 1/2 MS medium or 1/2 MS medium supplemented with 100 mM NaCl (Fig. 4b).

Salt stress can induce oxidative stress through the generation and accumulation of reactive oxygen species (ROS). Antioxidant enzymes, such as peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT), play key roles in scavenging ROS and protecting plants from oxidative damage [30]. The malondialdehyde (MDA) content reflects the degree of lipid peroxidation and indirectly reflects the degree of cell damage [30]. The activities of these antioxidant enzymes and MDA content were also measured in the *SmAP2-17*-overexpression and WT

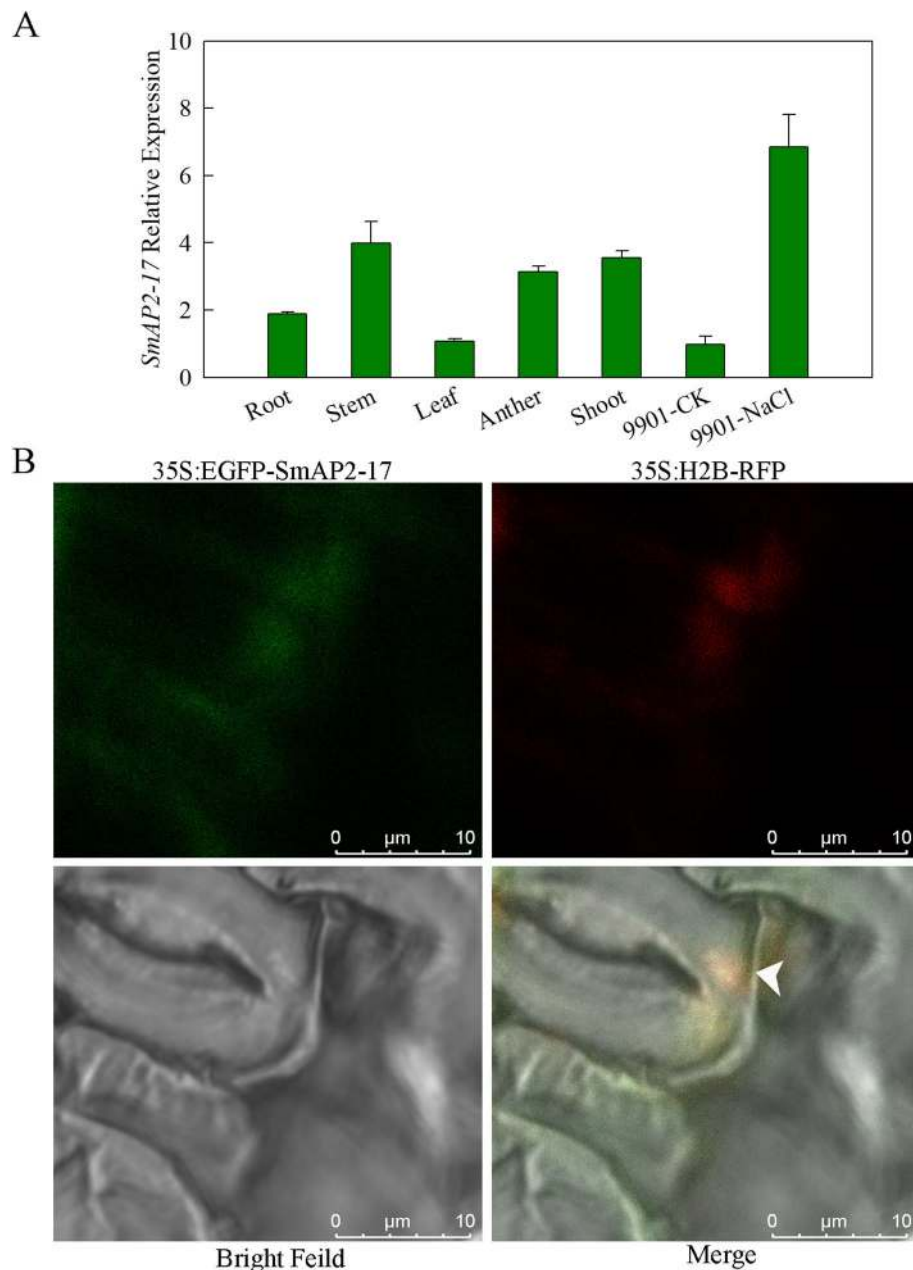


Fig. 3 Expression pattern and subcellular localization of SmAP2-17 protein. **a** Expression patterns of *SmAP2-17* in the roots, stems, leaves, anthers, and shoots of *S. matsudana* and under salt stress were measured using qRT-PCR. **b** Subcellular localization of the SmAP2-17 protein. The 35S:EGFP-SmAP2-17 fusion construct and the nucleus localization marker 35S:H₂B-RFP construct were co-transformed into tobacco epidermal leaves. The arrowhead indicates the merged signal (yellow) with EGFP (green) and RFP (red) co-located in the nucleus. Scale bar, 10 μm

plants. The results showed that there was no substantial difference in the activities of POD and SOD under normal conditions and after salt stress treatment. The MDA content was lower in T-15 under normal conditions and in T-1 under salt treatment. CAT activity was lower in T-15 than in WT and T-1, but no marked difference was observed under salt stress (Fig. 4c-f).

The data from lower CAT enzyme activity and higher MDA content were consistent with the salt sensitive phenotype of the overexpression plants, which did not exhibit increased salt tolerance. To determine the role of *SmAP2-17*, we detected the expression level of *SmAP2-17* in overexpression plants after salt stress, and found that the expression level of *SmAP2-17* decreased sharply

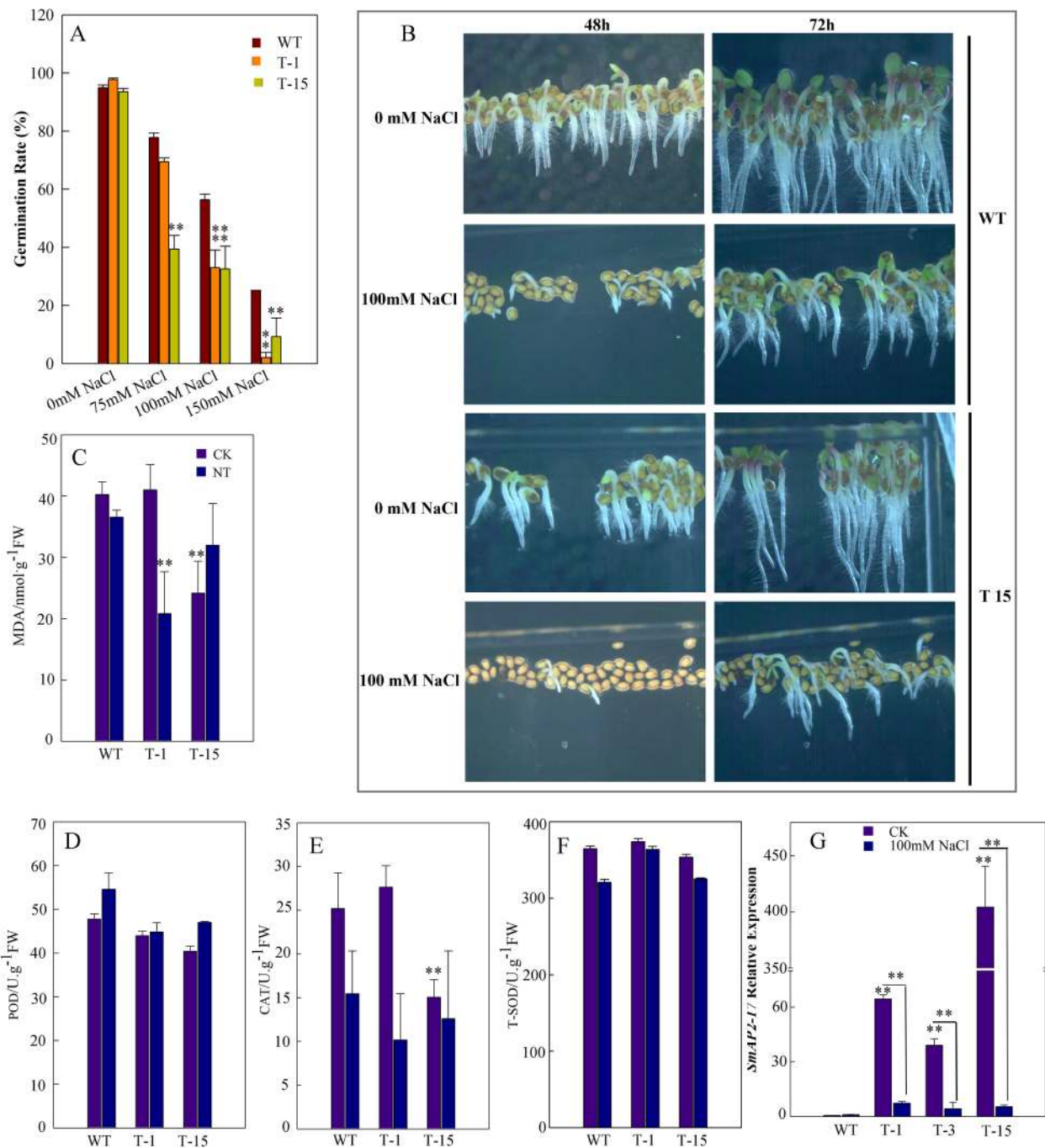


Fig. 4 *SmAP2-17*-overexpression transgenic lines did not show increased tolerance to salt stress. **a** *SmAP2-17*-overexpression transgenic line T-15 had a delayed germination phenotype under salt stress compared to the WT (wild-type) at 48 h and 72 h after sowing. **b** Statistical analysis of the germination rate of WT and transgenic lines T-15 and T-1 at 72 h after sowing under different concentrations of NaCl (75 mM NaCl, 100 mM NaCl, and 150 mM NaCl). **c** qRT-PCR analysis of the expression levels of *SmAP2-17* in WT and transgenic lines T-1 and T-15 under normal conditions and after treatment with 100 mM NaCl. **c-f** Physiological indices of transgenic and WT *Arabidopsis* seedlings under normal and salt stress conditions. The MDA content (**c**) and antioxidant enzyme activities, including peroxidase (**d**), catalase (**e**), and superoxide dismutase (**f**), were measured in WT and transgenic lines T-15 and T-1 under normal and salt stress conditions. Two-week-old seedlings were exposed to 200 mM NaCl for 4 d before the measurements. **g** qRT-PCR analysis of the expression levels of *SmAP2-17* in WT and transgenic lines T-1 T-3, and T-15 under normal and salt stress (100 mM) conditions. Data represent the mean of three replicates, and the error bars indicate the SD; * $P < 0.05$ and ** $P < 0.01$ by Student's t-test

in all lines (Fig. 4g). Considering that the higher level of *SmAP2-17* expression in the *SmAP2-17*-overexpression was silenced after salt stress, we proposed that *SmAP2-17* mRNA may be the target of a putative salt-induced miRNA molecule. Recently, a large number of abiotic stress-induced miRNAs, including the salt-induced miRNA gene *ath-MIR167d*, have been reported [31]. The secondary complementary structure of *SmAP2-17* mRNA and a salt-induced miRNA *ath-MIR167d* suggest that *SmAP2-17* mRNA may be digested through *ath-MIR167d* mediated RNAi process (Fig. S1).

Phenotypic and physiology analysis of codon-optimized *SmAP2-17* gene overexpression transgenic lines

Based on our assumption, we optimized the *SmAP2-17* mRNA codons of the *ath-MIR167d* binding site and placed the optimized CDS behind the 35S enhancer in the plant binary vector. The new *SmAP2-17*-overexpression construct was named Op- *SmAP2-17* and transformed into *Arabidopsis* via the floral dip method. The Op- *SmAP2-17*-overexpression transgenic lines were screened and verified following the same protocol as the native *SmAP2-17* gene. First, the expression level of Op- *SmAP2-17* was detected in the WT and Op-*SmAP2-17*-overexpression transgenic lines Op T-3 and Op T-6 in 1/2 MS medium and medium containing 100 mM NaCl. The qRT-PCR results showed that salt stress did not decrease the expression level of Op-*SmAP2-17* mRNA in Op T-3 and Op T-6 lines. In contrast, the expression level in Op T-3 increased by approximately tenfold when compared with that in WT control (Fig. 5a).

The Op-*SmAP2-17*-overexpression transgenic lines Op T-3 and Op T-6 were subjected to 72 h of germination. The results showed that, contrary to the germination rate of native *SmAP2-17* transgenic lines, Op T-3 and Op T-6 had higher germination rates than the WT when treated with 75 mM NaCl, 100 mM NaCl, and 150 mM NaCl (Fig. 5b). A higher difference was found after treatment with 150 mM NaCl, the germination rate of WT decreased to about 20%, while about 60% of the Op T-3 and Op T-6 seeds still could germinate (Fig. 5b). To further evaluate the salt tolerance phenotype of Op T-3 and Op T-6, the growth of these plants was assessed

on MS medium containing 100 mM NaCl. After 14 days of growth on normal MS medium, the seedlings of the WT, Op T-3, and Op T-6 lines were transplanted to MS medium containing 100 mM NaCl and grown for another eight days. The results showed that after 8 days of treatment with 100 mM NaCl, the seedlings of Op T-3 and Op T-6 grew more and longer lateral roots than the WT (Fig. 5c). The number and length of the primary and lateral roots were counted, and the total root length was calculated (primary root + [lateral root number × average length of lateral roots]) for comparison (Fig. 5d). The total root length of Op T-3 and Op T-6 was found to increase by 40–50% compared to that of the WT (Fig. 5d).

With regards to the four physiological indexes, the CAT enzyme content in the two Op lines was higher than that in the WT after treatment with 200 mM NaCl (Fig. 5e). By contrast, the MDA content was lower than in the WT under the same conditions (Fig. 5f). The SOD enzyme content in the two Op lines was higher than that in the WT under normal conditions, whereas no difference was found in these lines under salt stress (Fig. 5g). There was no difference in the POD content under both the normal and salt stress conditions (Fig. 5h).

Analysis of gene expression pattern of general stress marker genes and salt stress-related genes in *SmAP2-17*-overexpression transgenic lines

Plants can activate many transcriptional and physiological processes in order to grow and survive under conditions of salt and abiotic stress. Several genes have regulatory roles and positive effects on abiotic stress tolerance, including stress-responsive marker genes (*DREB2A*, *RD20*, *ABI5*, *RD29A*, *ATGolS2*, *ABF3*, *AtMYB2*, and *RD29B*) and salinity-responsive genes (*AtNXH1*, *AtNXH2*, *AtHKT1*, *RBoH*, and *AtSOS3*), which play important roles in helping plants to cope with and survive under conditions of salt stress [32–36].

To further explore the downstream genes regulated by *SmAP2-17* under salt stress, the expression patterns of stress/salt-responsive genes were evaluated by qRT-PCR. In the native *SmAP2-17*-overexpression transgenic lines, under normal conditions, many genes were found to be

(See figure on next page.)

Fig. 5 Effects of codon-optimized *SmAP2-17*-overexpression on salt stress tolerance in transgenic *Arabidopsis*. **a** qRT-PCR analysis of the expression levels of codon-optimized *SmAP2-17*-overexpression in WT and transgenic lines Op T-3 and Op T-6 under normal and salt stress (100 mM) conditions. **b** Statistical analysis of the germination rate of the WT and transgenic lines Op T-3 and Op T-6 at 72 h after sowing under different concentrations of NaCl (75 mM NaCl, 100 mM NaCl, and 150 mM NaCl). **c** Transgenic lines Op T-3 and Op T-6 had more and longer lateral roots than the WT. **d** Statistical comparison of the total root length (primary root + [lateral root number × average length of lateral roots]) between WT and transgenic lines Op T-3 and Op T-6. **e–h** Physiological indices of transgenic and WT *Arabidopsis* seedlings under normal and stress conditions. The MDA content (**e**) and antioxidant enzyme activities, including peroxidase (**f**), catalase (**g**), and superoxide dismutase (**h**), were measured in the WT and transgenic lines Op T-3 and Op T-6 under normal and salt stress conditions. Two-week-old seedlings were exposed to 200 mM NaCl for 4 d before the measurements. Data represent the mean of three replicates, and the error bars indicate the SD; * $P < 0.05$ and ** $P < 0.01$ by Student's t-test

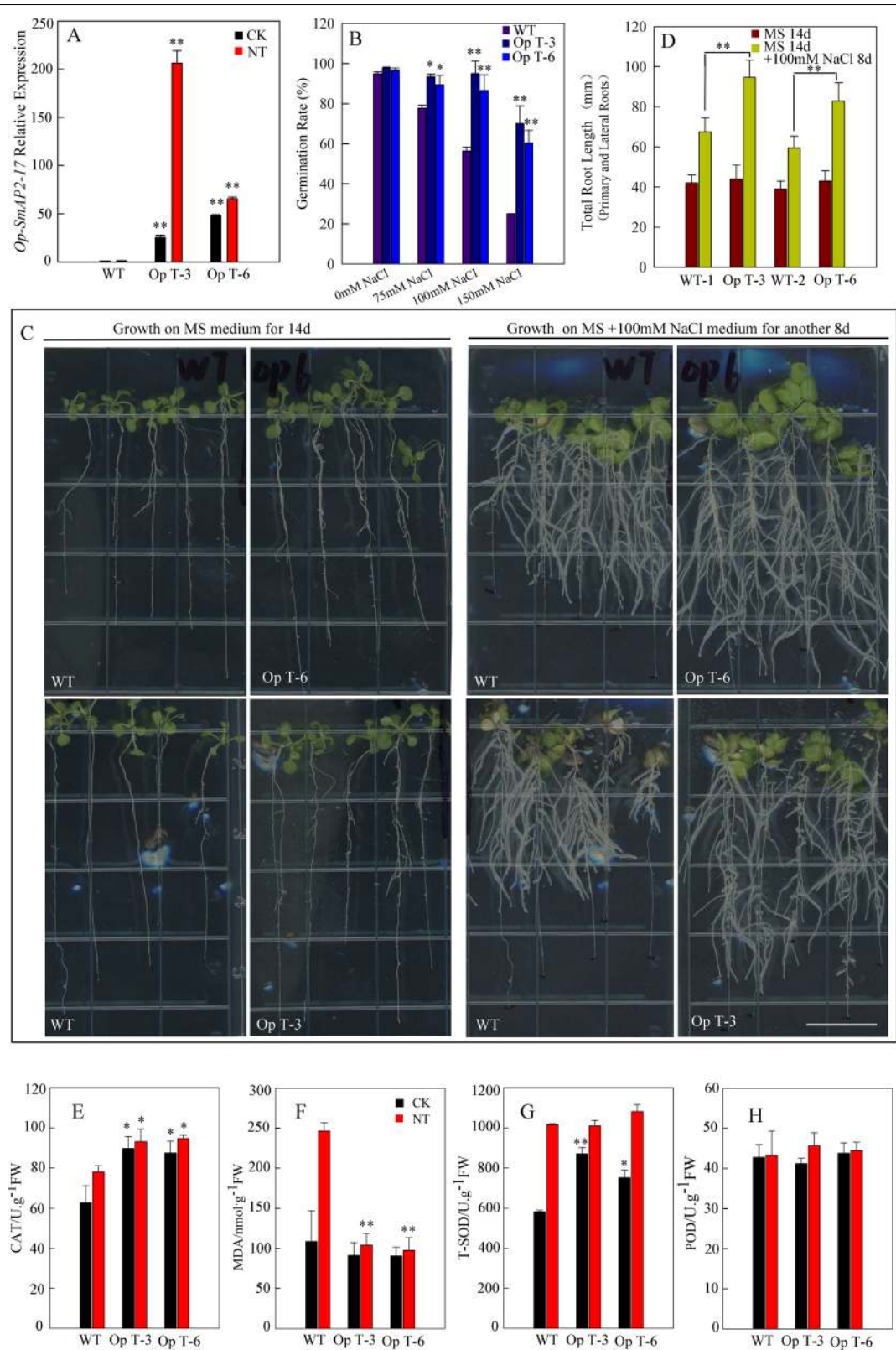


Fig. 5 (See legend on previous page.)

upregulated, especially in T-15, which expressed *SmAP2-17* at extremely high levels. The expression levels of 13 out of 15 genes we detected were upregulated by over two-fold as compared to those in the WT (Fig. 6a). The top five genes with the highest increase in expression were *RD29A*, *ABI5*, *SOS3*, *AtHKT1*, and *RbohF*. In the transgenic line T-15, the expression level of *SOS3* was over 12-fold higher than that in the WT (Fig. 6a). Interestingly, these five genes were induced with much higher expression levels than those in T-1, and the expression level of these genes was consistent with the higher expression of *SmAP2-17* in T-15 (Fig. 6a).

After treatment with 100 mM NaCl, the expression patterns of these genes in T-1 and T-15 were also detected by qRT-PCR. Following the gene silencing of *SmAP2-17* after salt stress, the expression of all genes detected was inhibited with expression levels that were lower or the same under the salt stress treatment (Fig. 6b).

In the Op T-3 and Op T-6 overexpression transgenic lines with optimized codons of *SmAP2-17* CDS, the expression patterns of 12 genes were examined after salt stress. The results showed that these genes in T-1 and T-15 were not inhibited in the Op T-3 and Op T-6 lines. All of the genes had a higher expression level than that in the WT, and the expression level of these genes was consistent with the higher expression level of Op-*SmAP2-17* in Op T-3 (Fig. 6c).

Promoter motif analysis on putative downstream gene of *SmAP2-17*

Based on the expression pattern of these genes, consistent with the expression level of *SmAP2-17* in T-15 and Op-*SmAP2-17* in OpT-3, we selected five genes as putative downstream genes of *SmAP2-17*: *ABI5*, *HKT1*, *RbohF*, *RD29A*, and *SOS3*. We downloaded the 2000-bp promoter sequence of the five genes and performed motif analysis using the PlantCARE platform. The putative binding site motifs of AP2/ERF transcription factors (including CAACA, CAA/CA/CTG, CATGCA, and ATCGAG) and coupling element 3-like (CE3-like) (CGCG) were identified in the promoters of these five genes (Fig. 7a). No AP2 binding and CE3-like motifs were found in the *RD29A* gene promoter, while only one CE3-like motif was identified in the *ABI5* gene promoter and three AP2 binding motifs were present on the *HKT1* gene promoter. *RbohF* had the highest number of these motifs, including five AP2 binding

motifs and three CE3-like motifs. The last one gene, *SOS3*, had four AP2 binding motifs and one CE3-like motif (Fig. 7a).

SmAP2-17 binds the promoter region of *SOS3* and *ABI5* and drives the expression of the *Luc* gene

Next, a dual luciferase reporter assay was used to determine whether these five genes were the direct downstream genes of *SmAP2-17*. The 2000-bp promoter sequences of *ABI5*, *HKT1*, *RbohF*, *RD29A*, and *SOS3* were cloned upstream of the *LUC* gene in the pGreenII 0800-LUC vector. *REN* was used as an internal control, and a *SmAP2-17*-overexpression vector was also constructed. The *SmAP2-17*-overexpression vector construct and each gene promoter::*LUC* construct were co-transformed into tobacco leaf cells via agrobacteria-mediated transformation. The signal intensities of *LUC* and *REN* were detected, and the *LUC*/*REN* ratios were calculated. The results showed that from the five genes analyzed, *SmAP2-17* could bind to the promoter region of *SOS3* and *ABI5* and activate the expression of the downstream *LUC* gene (Fig. 7b). In the ectopic system, these results showed that *SmAP2-17* may directly bind to the upstream regulatory region and upregulate the expression levels of *SOS3* and *ABI5*, thereby enhancing the salt tolerance of the *SmAP2-17*-overexpression transgenic lines.

Discussion

The APETALA2/ethylene response factor (AP2/ERF) transcription factor (TF) is a superfamily of plant species that plays multiple roles in plant growth and development, stress tolerance, and the integration of phytohormonal signals [3]. Since the first AP2/ERF TF AP2 was identified in *Arabidopsis* as a controller in flower and seed development, several genes of the DREB and ERF subfamily have been explored for their molecular roles and mechanisms, particularly in biotic and abiotic stresses [9, 11, 14, 37–39]. Previous studies on the function of AP2/ERF have mainly focused on model plants and crops, and some studies have explored the AP2/ERF genes of trees, mainly from *Populus* and *Broussonetia papyrifera* [40–43]. *Salix matsudana* (Koidz.) is an important and widely cultivated ornamental tree species [28]. As a result of the genome-wide analysis of AP2/ERF family members of *Salix matsudana* (Koidz.), the differential expression

(See figure on next page.)

Fig. 6 Relative expression levels of stress responsive marker genes and salinity responsive genes in WT and transgenic lines under normal conditions and after treatment with NaCl. **a** Relative expression levels of 15 genes in the WT and transgenic lines T-1 and T-15 under normal conditions. **b** Relative expression levels of 15 genes in the WT and transgenic lines T-1 and T-15 treated with 200 mM NaCl for 24 h. **c** Relative expression levels of 12 genes in the WT and transgenic lines Op T-3 and Op T-6 treated with 200 mM NaCl for 24 h. Data represent the mean $\pm$ SD of three biological replicates. * $P < 0.05$ and ** $P < 0.01$ by Student's t-test

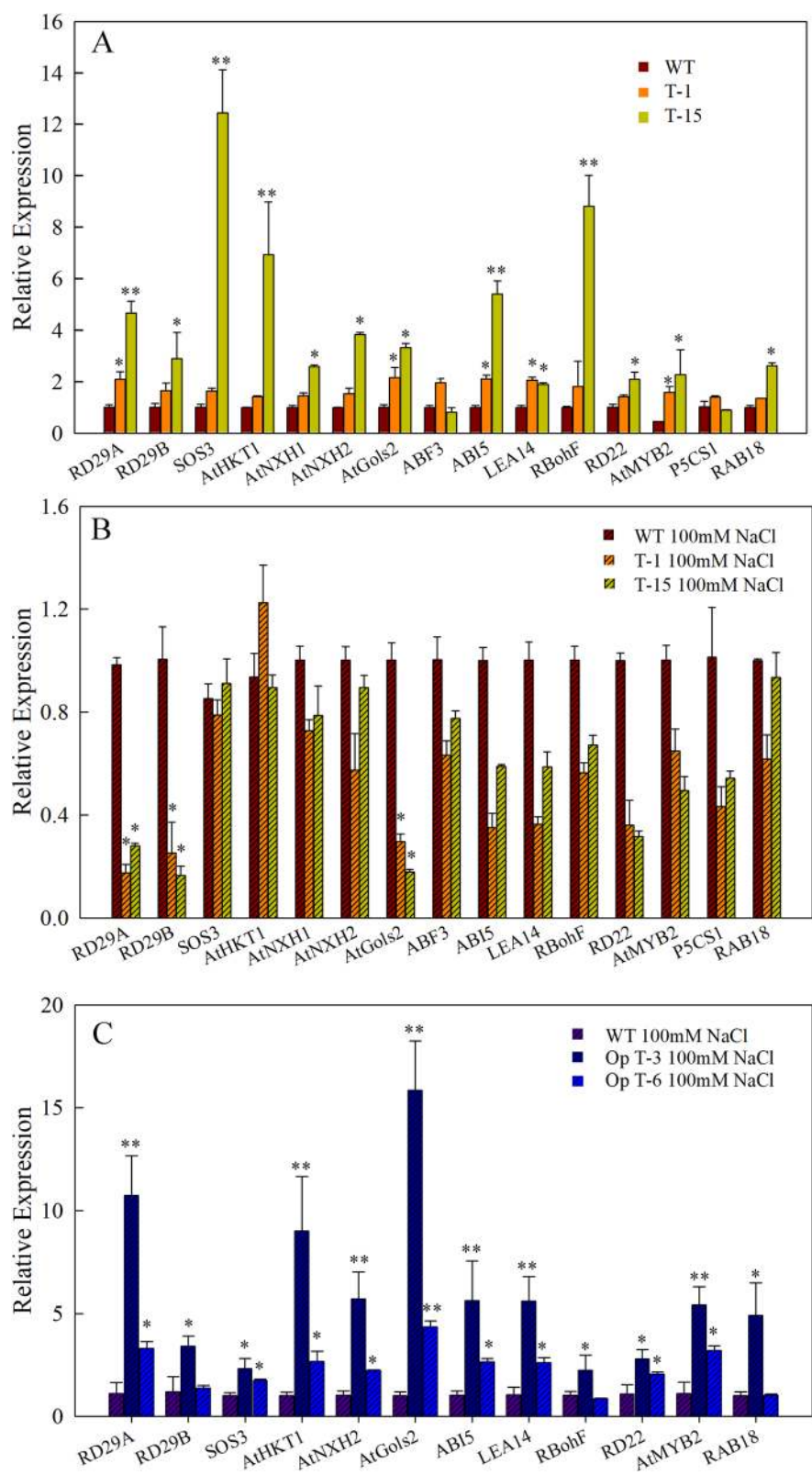


Fig. 6 (See legend on previous page.)

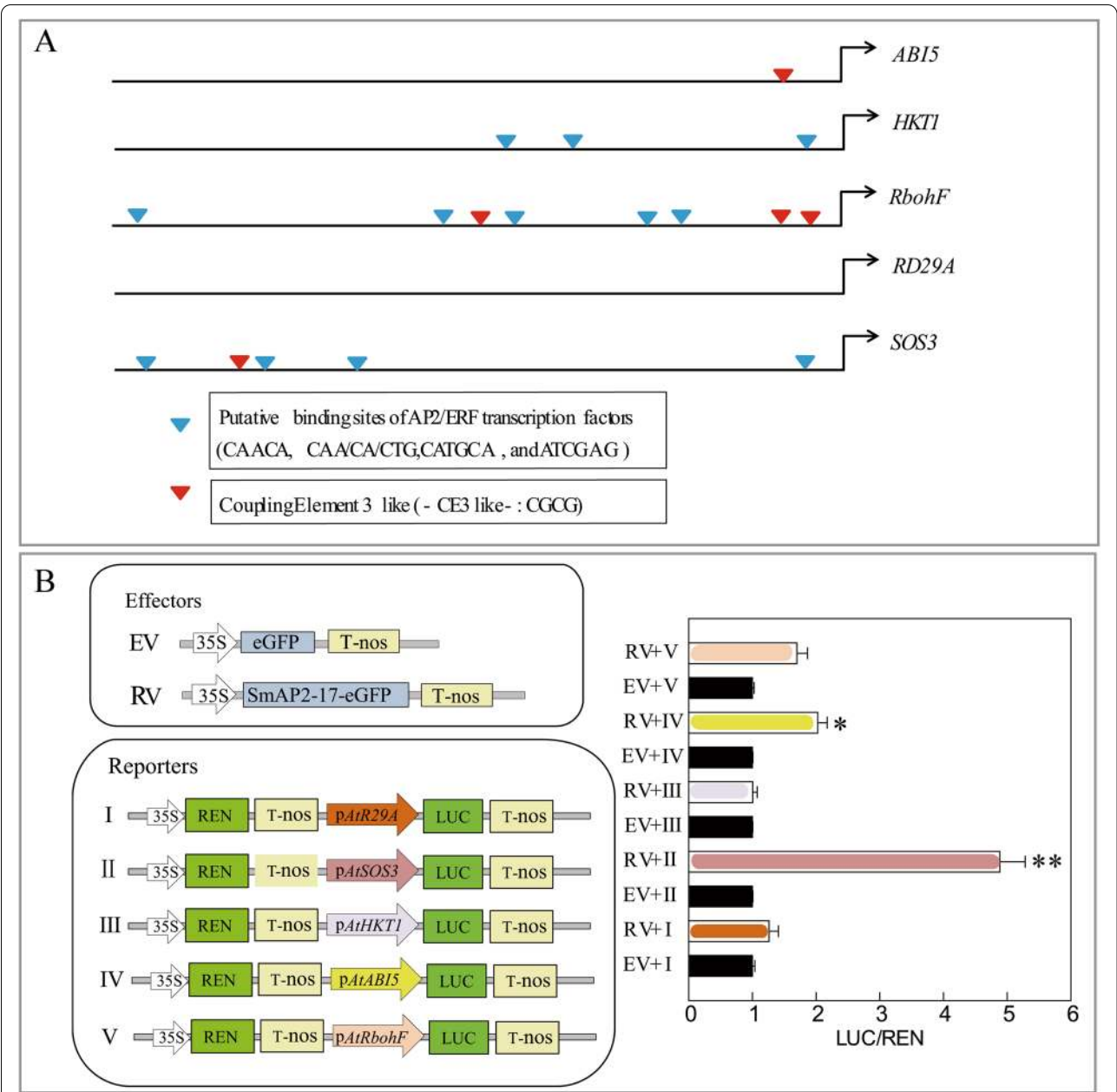


Fig. 7 *SOS3* and *ABI5* expression is activated in the presence of *SmAP2-17* as measured by the luciferase reporter assays. **a** Schematic diagrams of the putative AP2/ERF binding elements in the 2000-bp promoter region of the five candidate downstream genes. **b** Left panel: Schematic diagrams of effector and reporter plasmids used in transient transactivation assays: top, schematic diagrams of control (EV, empty vector) and effector (RV, recombination vector); bottom, schematic diagrams of reporter constructs. The promoter sequences from five candidate downstream genes were cloned upstream of the *LUC* reporter gene. The *REN* located downstream of the 35S promoter was used as an internal control. Right panel: The results showed that five candidate downstream genes, *SOS3* and *ABI5*, were regulated by *SmAP2-17*. Relative activity was calculated as *LUC/REN*, but was normalized to the reporter only. Data represent the mean $\pm$ SD of three biological replicates. * $P < 0.05$ and ** $P < 0.01$ by Student's t-test

patterns of some *SmAP2/ERF* genes under salt stress have been investigated [29]. However, little is known about the functional roles of *SmAP2/ERF* and their mechanisms in *Salix*. Transferring a single gene encoding stress-tolerance transcription factors into plants could improve plant drought, salt, and freezing tolerance. Therefore, uncovering their roles in *Salix* salt stress tolerance is important for the creation of new germplasms and the provision of environmental protection in the future [44].

SmAP2-17 may serve as a TF and play roles in salt stress tolerance

In this study, *SmAP2-17* encoding a TF protein of the SmAP2/ERF family was cloned from *S. matsudana* to determine its potential role in *Salix* resistance to salt stress. The phylogenetic tree analysis of SmAP2-17 and other AP2 subfamily proteins from maize, rice, *Arabidopsis*, and other *Salix* species showed that the SmAP2-17 protein was more similar to members of the *Salix* genus, especially SwEVM0024941 and SiEVM0034317 from *S. wilsonii* and *S. integra*, respectively (Fig. 1a). All of the members of the *Salix* genus showed 40% identity to *Arabidopsis* ANT protein, which plays roles both in plant development and abiotic stress tolerance [26, 45]. Furthermore, the multiple alignment of amino acid sequences indicated that SmAP2-17 protein had two conserved AP2 domains in the central region with characteristics similar to those of other SmAP2 proteins and *Arabidopsis* ANT (Fig. 1b). *SmAP2-17* was a ubiquitously expressed gene detected in all tissues examined, whose expression could be highly induced by salt stress (Fig. 3a). Colocalization with the nuclear marker histone H₂B showed that the SmAP2-17 protein was localized to the nucleus (Fig. 3b). Promoter motif analysis revealed TC-rich repeats and ABRE elements, which are likely to function in defense, stress responsiveness, and abscisic acid responsiveness, respectively. Among the six homolog genes from *Salix*, *SmAP2-17* was the only gene with TC-rich repeat elements (Fig. 2). These results suggest that SmAP2-17 may serve as a TF and play an important role in salt stress tolerance.

Analysis of salt resistance in *SmAP2-17*-overexpression transgenic *Arabidopsis*

To determine whether *SmAP2-17* is involved in regulating the salt stress response, overexpression transgenic lines were obtained and their salt tolerance abilities were studied. The results of phenotype analysis and physiological index showed that, under salt stress, the overexpression transgenic lines did not possess higher salt tolerance ability. These results implied that

SmAP2-17 was not a positive regulator in the salt stress response. However, the expression level of *SmAP2-17* in T-1 and T-15 after salt stress did not support this conclusion (Fig. 4). The qRT-PCR results showed that, after salt treatment, the high levels of *SmAP2-17* expression in T-1 and T-15 fell sharply; therefore, SmAP2-17 could not work properly after salt stress (Fig. 4g). The reduced expression level of *SmAP2-17* may not be the consequence of post-transcriptional silencing because of its higher expression level, since under normal conditions, silence phenomena did not occur. Thus, we proposed that *SmAP2-17* may be the target of a salt-induced microRNA, with studies previously reporting several salt-induced microRNA genes in *Arabidopsis* [31]. Through the analysis of RNA secondary structures, we found that ath-MIR167d can bind to *SmAP2-17* mRNA to initiate mRNA degradation (Fig. S1).

To provide support for our proposition and to further clarify the function of *SmAP2-17*, another version of *SmAP2-17* with an optimized codon at the binding site was introduced into *Arabidopsis*, revealing two new overexpression transgenic lines, Op T-3 and Op T-6. Phenotype analysis and physiological index measurement provided evidence that SmAP2-17 played a positive role in salt stress tolerance and that the overexpression of *SmAP2-17* in *Arabidopsis* could lead to a higher tolerance to conditions of salt stress (Fig. 5).

To obtain the overexpression transgenic lines, the cauliflower mosaic virus (CaMV) 35S promoter was used. The 35S promoter is the most frequently used promoter, with a strong and constitutive expression pattern [46]. Although it functions as a constitutive expression pattern, the 35S promoter has variable expression patterns in different organs of *Arabidopsis thaliana* and in response to abiotic stress [46, 47]. However, the phenomena reported in our study on transgenic lines, namely that salt stress induced a sharp miRNA-mediated reduction in the expression of *SmAP2-17*, has not been previously reported.

With regards to the increased sensitivity of the T-1 and T-15 transgenic lines to salt stress, although we were unable to provide a reasonable explanation, the data of four physiological indexes and the downregulation of stress marker genes were all in line with the salt-sensitive phenotype (Fig. 4, Fig. 6b).

SmAP2-17 may directly regulate the expression of *SOS3* and *ABI5*

Some studies have demonstrated that several AP2 TF genes enhance tolerance to salt stress through the regulation of stress/ABA-responsive genes and genes from the salt stress signaling regulatory network [11, 26, 41,

48]. To gain insights into the molecular functions of SmAP2-17 in salt stress tolerance, we detected several well-characterized stress-responsive marker genes and salt stress in transgenic lines (Fig. 6). The qRT-PCR results of gene expression showed that 13 out of the 15 genes detected were upregulated in transgenic lines. Five genes, including *RD29A*, *ABI5*, *SOS3*, *AtHKT1*, and *RBoH1*, were significantly upregulated, and the expression levels of these genes were consistent with the expression levels of *SmAP2-17* in different transgenic lines and lines treated with salt stress (Fig. 6a, Fig. 4g). In T-15, which had the highest *SmAP2-17* expression level, the expression levels of five genes were also the highest. After salt stress in T15, the expression of *SmAP2-17* decreased sharply, followed by that of the marker genes mentioned above (Fig. 6b). In the Op T-3 and Op T-6 plants, salt treatment did not influence the expression level of *SmAP2-17*, and the expression of *RD29A*, *ABI5*, *SOS3*, *AtHKT1*, and *RBoH1* remained at a higher level (Fig. 6c).

The expression levels of these genes were consistent with those of *SmAP2-17*, which suggested that SmAP2-17 may regulate the expression of these genes directly or indirectly. In the promoter/regulatory sequence of representative target genes of the AP2/ERF proteins, many conserved binding motifs, including GCC-box (AGC CGCC), DRE/CRT elements, and coupling elements 3-like (CE3-like) (CGCG) exist [48–50]. We detected the cis-elements in the promoter region of five genes and found that, except for the *RD29A* gene promoter, at least one or several motifs were found in the regulatory region of four other genes (Fig. 7a). The results of a dual luciferase reporter assay showed that SmAP2-17 binds to the promoter region of *SOS3* and *ABI5* and activates the expression of the downstream *LUC* gene (Fig. 7b). These results indicate that *SOS3* and *ABI5* are the direct downstream genes of SmAP2-17 in transgenic *Arabidopsis*. *SOS3* is a component of the salt-overly sensitive (SOS) pathway; it could physically interact with the Thr/Ser protein kinase SOS2. Then SOS2 phosphorylated SOS1 after being recruited to the plasma membrane (PM). SOS1 is a PM Na⁺/H⁺ antiporter responsible for excluding Na⁺ from the cytosol to the apoplast and the soil solution to mitigate salt stress [51, 52]. *ABI5* encodes a member of the basic leucine zipper transcription factor family. It involves in ABA signaling during seed maturation and germination and also plays a positive role in drought and high-salinity conditions [53, 54]. By upregulating the expression levels of *SOS3* and *ABI5*, the overexpression of *SmAP2-17* in *Arabidopsis* improves the tolerance of transgenic plants to salt stress by inducing ABA signaling and regulating salt stress-related genes.

Conclusions

In this study, *SmAP2-17* was cloned from *Salix matsudana*, and its function in salt tolerance was determined. By upregulating *SOS3* and *ABI5*, SmAP2-17 was found to play a positive role in salt stress tolerance, and the overexpression of *SmAP2-17* in *Arabidopsis* could increase the tolerance of *Arabidopsis*. Our results provide a basis for the further functional characterization of *SmAP2-17* in *Salix* and the potential use of this salt-resistant gene in the creation of new *Salix* germplasms.

Methods

Plant materials, growth conditions, and treatments

Arabidopsis thaliana ecotype *Col-0* was obtained from Arabidopsis Biological Resource Center with permission and used for the genetic transformation of the *SmAP2-17* gene. Sterilized *Arabidopsis* seeds of the *Col-0* (WT) and transgenic lines were sown on the agar medium of 1/2 Murashige and Skoog (MS) agar medium, and vernalized in the dark for 2 days at 4 °C. The seedlings were then cultured under long-day conditions (16 h light/8 h dark cycle) at 22 °C. For transgenic line screening, 25 mg/L hygromycin was added to the medium; for salt stress treatment with different concentrations of NaCl, different amounts of NaCl were dissolved into the medium. After growing for 2 weeks on medium, the seedlings were transplanted into soil and cultured in a greenhouse at 22 °C under long-day conditions. The 5-week-old plants were watered with 200 mM NaCl to mimic the salt stress treatment. After treatment for 72 h, leaf samples of *Arabidopsis* were collected and used to measure the levels of antioxidant enzyme activity and the MDA content.

The *salix matsudana* cultivar ‘9901’ was used in this study. ‘9901’ willow, a fine and widely planted variety of willow, was selected by many willow experts from Shandong Academy of Forestry. ‘9901’ willow was introduced and cultivated in the botany Garden of Nantong University and authorized for only scientific research purpose. One-year-old, 10-cm long cuttings of *Salix matsudana* ‘9901’ clone were planted in Hoagland solution for 20 days to induce shoots and roots. The cuttings with shoots and roots were transferred to a solution of 150 mM NaCl and cultured for 24 h. Willow samples from different organs and root samples after salt stress were collected and used for RNA extraction.

Cloning and sequence analysis of *SmAP2-17*

The coding sequence of *SmAP2-17* was amplified from the cDNA of *S. matsudana* using gene-specific primers (Table S2) and ligated into the pGEM-T vector through T-A cloning. Lasergene software was used to deduce the amino acid sequence of SmAP2-17. *Arabidopsis* ANT protein and other homologous proteins from *Salix*

purpurea, *Populus trichocarpa*, maize, rice and other *Salix* genus were obtained by blasting against proteins datasets downloaded from the Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) and NCBI (<https://www.ncbi.nlm.nih.gov/>) websites. The sequence alignment of these proteins was performed using the ESPript website (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). MEGA 6.0 was used to construct a phylogenetic tree of SmAP2-17 and other plant ANT-Like proteins using the neighbor-joining (NJ) method [55]. The cis-acting elements in the promoter of *SmAP2-17* and its five homolog genes from the *Salix* genus were predicted using the online PlantCARE tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and illustrated using TBtools software [56, 57]. The coding sequences and promoter sequences of *SmAP2-17* and its homologs are provided in File S1 and S2.

Plasmid construction and transformation of *SmAP2-17* in *Arabidopsis*

The full-length coding sequence of *SmAP2-17* (File S1) amplified from pGEM-T-SmAP2-17 using specific primers listed in Table S2 were inserted into the pWM101 vector using Hieff Clone™ Plus One Step Cloning recombination Kit (Yeasen, Shanghai). The *SmAP2-17* cDNA was cloned under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter, the construct was named pWM101-35S:SmAP2-17. The optimized codon of the *SmAP2-17* CDS sequence (File S1) was synthesized by General Biology Company (Hefei, China), and the same protocol was used to obtain the pWM101-35S:Op-SmAP2-17 construct.

The pWM101-35S:SmAP2-17 and pWM101-35S:Op-SmAP2-17 plasmids were then transformed into *Agrobacterium tumefaciens* GV3101 competent cells by electroporation, and *Arabidopsis* genetic transformation was performed using the floral dip method [58]. All *SmAP2-17*/Op-*SmAP2-17*-overexpression transgenic seedlings were screened using 25 mg/L hygromycin to obtain positive plants. These plants were then further verified by genotyping and expression level detection.

Subcellular localization of *SmAP2-17*

Using the one-step cloning method, the complete coding sequence of *SmAP2-17* amplified by PCR using specific primers was inserted into the pCAMBIA2300-35S:NEGFP vector under the control of the CaMV 35S promoter to generate an in-frame fusion construct, 35S:EGFP-SmAP2-17. As a positive nuclear marker, the cDNA of a previously characterized nuclear histone protein, H₂B, was fused to the mCherry gene to generate

35S:H2B-mCherry. The constructs of 35S:H2B-mCherry and 35S:EGFP-SmAP2-17 were co-transformed into *Agrobacterium tumefaciens* strain GV3101 (pSoup-19) using the heat shock method. Agroinfiltration was performed as previously reported [59]. Recombination agrobacteria were suspended in 10 mM MgCl₂ and 150 mg/l acetosyringone. The cells were allowed to stand in this medium for 3 h, and then infiltrated into the abaxial air spaces of young leaves of 2- to 4-week-old *N. benthamiana* plants using a syringe with the needle removed. The agroinfiltrated tobacco leaves after 48 h agro-infiltration were photographed using a confocal laser scanning microscope (Leica, Germany).

RNA isolation and real-time qRT-PCR analysis

Total RNA was extracted from different organs of *S. matsudana* or *Arabidopsis* seedlings using the TaKaRa MiniBEST Plant RNA Extraction Kit (Takara, Beijing, China). Reverse transcription for the first strand cDNA synthesis was performed with 3 µg of total RNA using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Beijing, China). The qRT-PCR experiments were performed on an ABI 7500 Real-Time PCR system (Applied Biosystems, USA) with TB Green™ Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Beijing, China). The actin genes *AtActin2* (AT3G18780) and *SpActin1* (*SapurV1A.0655s0050.1*) from *Arabidopsis* and *S. matsudana* were used as internal reference genes to normalize the data. The expression level differences between different tissues, samples, and treatments were evaluated using the $2^{-\Delta\Delta Ct}$ method, as previously described [60]. Three independent biological repeats were performed to ensure an accurate statistical analysis. The specific primers used are listed in Table S2.

Dual luciferase reporter assays

To verify the interaction between SmAP2-17 and the putative AP2/ERF binding sites in the promoter sequences of five genes, including *RD29A*, *ABI5*, *SOS3*, *AtHKT1*, and *RBoH*, luciferase reporter assays were conducted in the model plant species tobacco via transient expression as previously reported [61, 62]. The PCR-amplified 2000-bp promoter sequences from five genes were cloned into pGreenII 0800-LUC vectors to obtain reporter plasmids. The cloning procedure was the same as the *SmAP2-17* CDS cloning procedure, that is, through T-A cloning and one-step recombination cloning. The construct pCAMBIA2300-35S:SmAP2-17-NEGFP was used as an effector construct, while the pCAMBIA2300-35S:NEGFP construct was used as a negative effector control. The effector/negative effector control and reporter constructs were subsequently transferred into *Agrobacterium tumefaciens*

strain GV3101 (P19). The recombinant *A. tumefaciens* strains were cultured to an OD₆₀₀ of 1.0, and strains with effector/negative effector control and reporter constructs were mixed in a 1:1 proportion. After sedimentation, the confluent bacterium was re-suspended in 10 ml of infiltration media (10 mM MgCl₂, 0.5 M acetosyringone) to an OD₆₀₀ of 0.2, and incubated at room temperature without shaking for 2 h. The tobacco leaves of 2- to 4-week-old plants were then infiltrated using the virus genes activated GV3101 (P19) using a needle-free syringe. After infiltration, the tobacco plants were incubated in an illuminated chamber for 48–72 h at 25 °C, a Dual-Luciferase Reporter Assay System (Promega, USA) was used to determine *LUC* and *REN* luciferase activities, according to the manufacturer's instructions.

Analysis of salt tolerance

Sterilized seeds from the WT, *SmAP2-17*-overexpression, and codon-optimized *SmAP2-17*-overexpression plants were sown on 1/2 MS solid medium (control) or 1/2 MS medium containing different concentrations of NaCl (75 mM, 100 mM, 150 mM) to measure the germination rate after 72 h of culture. For the root length phenotype, the seeds of the WT and codon-optimized *SmAP2-17*-overexpression plants were sown on 1/2 MS solid medium and cultured for 14 days. Then, seedlings with the same length were transferred to 1/2 MS solid medium containing 100 mM NaCl. After 8 days, the lengths of the roots of the WT and transgenic seedlings were compared. Two-week-old seedlings of the WT, *SmAP2-17*-overexpression, and codon-optimized *SmAP2-17*-overexpression lines were transplanted into soil from 1/2 MS medium and watered for two weeks before the stress treatment experiments. For salt stress, the seedlings were irrigated with 30 mL of 200 mM NaCl solution once every two days, or with pure water as a control. After 96 h, the physiological parameters, including MDA content and levels of antioxidant enzyme activity (e.g. peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT)) were measured using the corresponding kits, according to the manufacturer's instructions (Solarbio, Beijing, CN). All experiments included three biological replicates.

Abbreviations

AP2/ERF: APETALA2/ethylene responsive factor; ORF: Open reading frame; CDS: Coding sequence; TF: Transcription factor; GFP: Green fluorescent protein; RFP: Red fluorescent protein; SOD: Superoxide dismutase; POD: Peroxidase; MDA: Malondialdehyde; CAT: Catalase; WT: Wild-type; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; LUC: Luciferase; REN: Renilla; CaMV: Cauliflower mosaic virus; MS: Murashige and Skoog.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-022-03487-y>.

Additional file 1: Table S1. Cis-element motifs in the promoter region of *SmAP2-17* and its homologous genes.

Additional file 2: File S1. Protein sequences of *SmAP2-17* and its homolog genes.

Additional file 3: File S2. Promoter sequences of *SmAP2-17* and its homolog genes.

Additional file 4: Table S2. List of primers used.

Additional file 5: Fig. S1. The secondary complementary structure of *SmAP2-17* mRNA and salt-induced miRNA ath-MIR167d.

Acknowledgements

We thank editing service from Editage Company (<https://www.editage.cn>) for professional scientific editing the English text of a draft of this manuscript.

Authors' contributions

Y.C. and J.Z. designed the study. Y.C., Y.D., Y.L., J.Y., Y.J., G.L., C.Y., F.Z., and B.L. performed the experiments. Y.C. generated all the constructs used in the *Arabidopsis* studies. Y.D. and Y.J. participated in generating and screening all the transgenic materials. Y.D. and Y.J. participated in phenotype and physiological analyses; Y.D. participated in the expression pattern analysis and subcellular localization. G.L. and C.Y. performed sequence analysis, alignment, and motif prediction; F.Z. performed the qRT-PCR experiments; Y.C. and B.L. generated all constructs used in promoter activity analysis; Y.J. participated in the luciferase reporter assays. Y.L. carried out tissue culture and willow transformation; J.Y. performed genotype identification and expression pattern analysis on transgenic calli. Y.C. and J.Z. wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported financially by the National Natural Science Foundation of China (31971681). The supporters had no role in study design, data collection, data analysis, data interpretation, the writing of the manuscript or decision to publish.

Availability of data and materials

Proteins and nucleotides sequences in this study were downloaded from the Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) and NCBI (<https://www.ncbi.nlm.nih.gov/>) websites and analyzed using ESPript (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) and PlantCARE tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) websites. Sequence data of proteins and promoters described in this article are available using the accession numbers listed in Supplementary File S1 and S2 respectively. The datasets supporting the conclusions of this article are included within the article and its additional files, and available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

There are no ethical issues to report.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 29 July 2021 Accepted: 21 February 2022

Published online: 07 March 2022

References

- Xie Z, Nolan TM, Jiang H, Yin Y. AP2/ERF Transcription Factor Regulatory Networks in Hormone and Abiotic Stress Responses in Arabidopsis. *Front Plant Sci.* 2019;10:228.
- Nakano T, Suzuki K, Fujimura T, Shinshi H. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol.* 2006;140(2):411–32.
- Gu C, Guo ZH, Hao PP, Wang GM, Jin ZM, Zhang SL. Multiple regulatory roles of AP2/ERF transcription factor in angiosperm. *Bot Stud.* 2017;58(1):6.
- Gutterson N, Reuber TL. Regulation of disease resistance pathways by AP2/ERF transcription factors. *Curr Opin Plant Biol.* 2004;7(4):465–71.
- Agarwal PK, Agarwal P, Reddy MK, Sopory SK. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep.* 2006;25(12):1263–74.
- Harrop TWR, Mantegazza O, Luong AM, Béthune K, Lorieux M, Jouannic S, Adam H. A set of AP2-like genes is associated with inflorescence branching and architecture in domesticated rice. *J Exp Bot.* 2019;70(20):5617–29.
- Akhtar M, Jaiswal A, Taj G, Jaiswal JP, Qureshi MI, Singh NK. DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. *J Genet.* 2012;91(3):385–95.
- Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim Biophys Acta.* 2012;1819(2):86–96.
- Agarwal PK, Gupta K, Lopato S. Agarwal P Dehydration responsive element binding transcription factors and their applications for the engineering of stress tolerance. *J Exp Bot.* 2017;68(9):2135–48.
- Zhou Y, Chen M, Guo J, Wang Y, Min D, Jiang Q, Ji H, Huang C, Wei W, Xu H, et al. Overexpression of soybean DREB1 enhances drought stress tolerance of transgenic wheat in the field. *J Exp Bot.* 2020;71(6):1842–57.
- Fu J, Zhu C, Wang C, Liu L, Shen Q, Xu D, Wang Q. Maize transcription factor ZmEREB20 enhanced salt tolerance in transgenic Arabidopsis. *Plant Physiol Biochem.* 2021;159:257–67.
- Zhang XX, Tang YJ, Ma QB, Yang CY, Mu YH, Suo HC, Luo LH, Nian H. OsDREB2A, a rice transcription factor, significantly affects salt tolerance in transgenic soybean. *PLoS One.* 2013;8(12):e83011.
- Donde R, Gupta MK, Gouda G, Kumar J, Vadde R, Sahoo KK, Dash SK, Behera L. Computational characterization of structural and functional roles of DREB1A, DREB1B and DREB1C in enhancing cold tolerance in rice plant. *Amino Acids.* 2019;51(5):839–53.
- Mao L, Deng M, Jiang S, Zhu H, Yang Z, Yue Y, Zhao K. Characterization of the DREBA4-Type Transcription Factor (SIDREBA4), Which Contributes to Heat Tolerance in Tomatoes. *Front Plant Sci.* 2020;11:554520.
- Tang Y, Liu K, Zhang J, Li X, Xu K, Zhang Y, Qi J, Yu D, Wang J, Li C. JcDREB2, a Physic Nut AP2/ERF Gene, Alters Plant Growth and Salinity Stress Responses in Transgenic Rice. *Front Plant Sci.* 2017;8:306.
- Liang Y, Li X, Zhang D, Gao B, Yang H, Wang Y, Guan K, Wood AJ. ScDREB8, a novel A-5 type of DREB gene in the desert moss *Syntrichia caninervis*, confers salt tolerance to Arabidopsis. *Plant Physiol Biochem.* 2017;120:242–51.
- Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, Matsumoto T, Yoshimura A, Kitano H, et al. The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. *Nature.* 2009;460(7258):1026–30.
- Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ. Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. *Nature.* 2006;442(7103):705–8.
- Hinz M, Wilson IW, Yang J, Buerstenbinder K, Llewellyn D, Dennis ES, Sauter M, Dolferus R. Arabidopsis RAP2.2: an ethylene response transcription factor that is important for hypoxia survival. *Plant Physiol.* 2010;153(2):757–72.
- Iwase A, Mitsuda N, Koyama T, Hiratsu K, Kojima M, Arai T, Inoue Y, Seki M, Sakakibara H, Sugimoto K, et al. The AP2/ERF transcription factor WIND1 controls cell dedifferentiation in Arabidopsis. *Curr Biol.* 2011;21(6):508–14.
- Patil V, McDermott HI, McAllister T, Cummins M, Silva JC, Mollison E, Meikle R, Morris J, Hedley PE, Waugh R, et al. APETALA2 control of barley internode elongation. *Development.* 2019;146(11):dev170373.
- Duran-Medina Y, Serwatowska J, Reyes-Olalde JL, de Folter S, Marsch-Martinez N. The AP2/ERF Transcription Factor DRNL Modulates Gynoecium Development and Affects Its Response to Cytokinin. *Front Plant Sci.* 2017;8:1841.
- Li J, Chen F, Li Y, Li P, Wang Y, Mi G, Yuan L. ZmRAP2.7, an AP2 transcription factor, is involved in maize brace roots development. *Front Plant Sci.* 2019;10:820.
- Sun W, Gao D, Xiong Y, Tang X, Xiao X, Wang C, Yu S. Hairy Leaf 6, an AP2/ERF Transcription Factor, Interacts with OsWOX3B and Regulates Trichome Formation in Rice. *Mol Plant.* 2017;10(11):1417–33.
- Debernardi JM, Greenwood JR, Jean Finnegan E, Jernstedt J, Dubcovsky J. APETALA 2-like genes AP2L2 and Q specify lemma identity and axillary floral meristem development in wheat. *Plant J.* 2020;101(1):171–87.
- Meng LS, Wang YB, Yao SQ, Liu A. Arabidopsis AINTEGUMENTA mediates salt tolerance by trans-repressing SCABP8. *J Cell Sci.* 2015;128(15):2919–27.
- Zhang J, Yuan H, Yang Q, Li M, Wang Y, Li Y, Ma X, Tan F, Wu R. The genetic architecture of growth traits in *Salix matsudana* under salt stress. *Hortic Res.* 2017;4:17024.
- Zhang J, Yuan H, Li Y, Chen Y, Liu G, Ye M, Yu C, Lian B, Zhong F, Jiang Y, et al. Genome sequencing and phylogenetic analysis of allotetraploid *Salix matsudana* Koidz. *Hortic Res.* 2020;7(1):201.
- Zhang J, Shi SZ, Jiang Y, Zhong F, Liu G, Yu C, Lian B, Chen Y. Genome-wide investigation of the AP2/ERF superfamily and their expression under salt stress in Chinese willow (*Salix matsudana*). *Peer J.* 2021;9:e11076.
- Choudhury S, Panda P, Sahoo L, Panda SK. Reactive oxygen species signaling in plants under abiotic stress. *Plant Signal Behav.* 2013;8(4):e23681.
- Sharma R, Upadhyay S, Bhat B, Singh G, Bhattacharya S, Singh A. Abiotic stress induced miRNA-TF-gene regulatory network: A structural perspective. *Genomics.* 2020;112(1):412–22.
- Chen YH, Cao YY, Wang LJ, Li LM, Yang J, Zou MX. Identification of MYB transcription factor genes and their expression during abiotic stresses in maize. *Biol Plantarum.* 2018;62(2):222–30.
- Park HJ, Kim WY, Yun DJ. A New Insight of Salt Stress Signaling in Plant. *Mol Cells.* 2016;39(6):447–59.
- Ullah A, Sun H, Hakim, Yang X, Zhang X. A novel cotton WRKY gene, GhWRKY6-like, improves salt tolerance by activating the ABA signaling pathway and scavenging of reactive oxygen species. *Physiol Plant.* 2018;162(4):439–54.
- He L, Wu YH, Zhao Q, Wang B, Liu QL, Zhang L. Chrysanthemum DgWRKY2 Gene Enhances Tolerance to Salt Stress in Transgenic Chrysanthemum. *Int J Mol Sci.* 2018;19(7):2062.
- Yoshida T, Mogami J, Yamaguchi-Shinozaki K. ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Curr Opin Plant Biol.* 2014;21:133–9.
- Jofuku KD, den Boer BG, Van Montagu M, Okamoto JK. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. *Plant Cell.* 1994;6(9):1211–25.
- Debbarma J, Sarki YN, Saikia B, Boruah HPD, Singha DL, Chikkaputtaiah C. Ethylene Response Factor (ERF) Family Proteins in Abiotic Stresses and CRISPR-Cas9 Genome Editing of ERFs for Multiple Abiotic Stress Tolerance in Crop Plants: A Review. *Mol Biotechnol.* 2019;61(2):153–72.
- Lv K, Li J, Zhao K, Chen S, Nie J, Zhang W, Liu G, Wei H. Overexpression of an AP2/ERF family gene, BpERF13, in birch enhances cold tolerance through upregulating CBF genes and mitigating reactive oxygen species. *Plant Sci.* 2020;292:110375.
- Sun J, Peng X, Fan W, Tang M, Liu J, Shen S. Functional analysis of BpDREB2 gene involved in salt and drought response from a woody plant *Broussonetia papyrifera*. *Gene.* 2014;535(2):140–9.
- Chen N, Tong S, Tang H, Zhang Z, Liu B, Lou S, Liu J, Liu H, Ma T, Jiang Y. The PaERF109 transcription factor positively regulates salt tolerance via PalHKT1;2 in *Populus alba* var. *pyramidalis*. *Tree Physiol.* 2020;40(6):717–30.
- Chen J, Xia X, Yin W. A poplar DRE-binding protein gene, PeDREB2L, is involved in regulation of defense response against abiotic stress. *Gene.* 2011;483(1–2):36–42.
- Chen J, Xia X, Yin W. Expression profiling and functional characterization of a DREB2-type gene from *Populus euphratica*. *Biochem Biophys Res Commun.* 2009;378(3):483–7.

44. Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nat Biotechnol*. 1999;17(3):287–91.
45. Krizek BA, Blakley IC, Ho YY, Freese N, Loraine AE. The Arabidopsis transcription factor AINTEGUMENTA orchestrates patterning genes and auxin signaling in the establishment of floral growth and form. *Plant J*. 2020;103(2):752–68.
46. Boyko A, Molinier J, Chatter W, Laroche A, Kovalchuk I. Acute but not chronic exposure to abiotic stress results in transient reduction of expression levels of the transgene driven by the 35S promoter. *New Biotech*. 2010;27(1):70–7.
47. Kiselev KV, Aleynova OA, Ogneva ZV, Suprun AR, Dubrovina AS. 35S promoter-driven transgenes are variably expressed in different organs of Arabidopsis thaliana and in response to abiotic stress. *Mol Biol Rep*. 2021;48(3):2235–41.
48. Qu Y, Nong Q, Jian S, Lu H, Zhang M, Xia K. An AP2/ERF Gene, HuERF1, from Pitaya (*Hylocereus undatus*) Positively Regulates Salt Tolerance. *Int J Mol Sci*. 2020;21(13):4586.
49. Mishra S, Phukan UJ, Tripathi V, Singh DK, Luqman S, Shukla RK. PsAP2 an AP2/ERF family transcription factor from *Papaver somniferum* enhances abiotic and biotic stress tolerance in transgenic tobacco. *Plant Mol Biol*. 2015;89(1–2):173–86.
50. Cheng MC, Liao PM, Kuo WW, Lin TP. The Arabidopsis ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiol*. 2013;162(3):1566–82.
51. Lou L, Yu F, Tian M, Liu G, Wu Y, Wu Y, Xia R, Pardo JM, Guo Y, Xie Q. ESCRT-I Component VPS23A Sustains Salt Tolerance by Strengthening the SOS Module in Arabidopsis. *Mol Plant*. 2020;13(8):1134–48.
52. Rolly NK, Imran QM, Lee IJ, Yun BW. Salinity Stress-Mediated Suppression of Expression of Salt Overly Sensitive Signaling Pathway Genes Suggests Negative Regulation by AtbZIP62 Transcription Factor in Arabidopsis thaliana. *Int J Mol Sci*. 2020;21(5):1726.
53. Chang HC, Tsai MC, Wu SS, Chang IF. Regulation of ABI5 expression by ABF3 during salt stress responses in Arabidopsis thaliana. *Bot Stud*. 2019;60(1):16.
54. Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K. Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci U S A*. 2000;97(21):11632–7.
55. Kang HG, Kim J, Kim B, Jeong H, Choi SH, Kim EK, Lee HY, Lim PO. Overexpression of FTL1/DDF1, an AP2 transcription factor, enhances tolerance to cold, drought, and heat stresses in Arabidopsis thaliana. *Plant Sci*. 2011;180(4):634–41.
56. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol Plant*. 2020;13(8):1194–202.
57. Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouzé P, Rombauts S. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res*. 2002;30(1):325–7.
58. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J*. 1998;16(6):735–43.
59. Voinnet O, Rivas S, Mestre P, Baulcombe D. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J*. 2003;33(5):949–56.
60. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nature Protoc*. 2008;3(6):1101–8.
61. Sun S, Hu C, Qi X, Chen J, Zhong Y, Muhammad A, Lin M, Fang J. The AaCBF4-AaBAM3.1 module enhances freezing tolerance of kiwifruit (*Actinidia arguta*). *Hortic Res*. 2021;8(1):97.
62. Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, Karunairetnam S, Gleave AP, Laing WA. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*. 2005;1:13.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

