

TsNAC1 Is a Key Transcription Factor in Abiotic Stress Resistance and Growth¹

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NAC proteins constitute one of the largest families of plant-specific transcription factors, and a number of these proteins participate in the regulation of plant development and responses to abiotic stress. *T. HALOPHILA STRESS RELATED NAC1* (*TsNAC1*), cloned from the halophyte *Thellungiella halophila*, is a NAC transcription factor gene, and its overexpression can improve abiotic stress resistance, especially in salt stress tolerance, in both *T. halophila* and *Arabidopsis* (*Arabidopsis thaliana*) and retard the growth of these plants. In this study, the transcriptional activation activity of *TsNAC1* and *RD26* from *Arabidopsis* was compared with the target genes' promoter regions of *TsNAC1* from *T. halophila*, and the results showed that the transcriptional activation activity of *TsNAC1* was higher in tobacco (*Nicotiana tabacum*) and yeast. The target sequence of the promoter from the target genes also was identified, and *TsNAC1* was shown to target the positive regulators of ion transportation, such as *T. HALOPHILA H⁺-PPASE1*, and the transcription factors *MYB HYPOCOTYL ELONGATION-RELATED* and *HOMEBOX12*. In addition, *TsNAC1* negatively regulates the expansion of cells, inhibits *LIGHT-DEPENDENT SHORT HYPOCOTYLS1* and *UDP-XYLOSYLTRANSFERASÉ2*, and directly controls the expression of *MULTICOPY SUPPRESSOR OF IRA14*. Based on these results, we propose that *TsNAC1* functions as an important upstream regulator of plant abiotic stress responses and vegetative growth.

NAC transcription factors include at least 107 members in *Arabidopsis* (*Arabidopsis thaliana*; Riechmann et al., 2000) and constitute one of the largest protein families in higher plants. NAC transcription factors have important roles in plant development (Guo et al., 2005; Petricka et al., 2012), cell apoptosis (Lee et al., 2014), abiotic stress tolerance (Tran et al., 2010), secondary cell wall formation (Yamaguchi and Demura, 2010), and endoplasmic reticulum stress resistance (Chi et al., 2017). NAC families have a conserved N terminus with a similar DNA-binding domain but highly diverse C termini, which do not contain any known protein domains (Ooka et al., 2003). *Arabidopsis RESPONSIVE TO DESICCATION26* (*RD26*; *AT4G27410*) is a NAC transcription factor belonging to the ATAF subfamily, and it is markedly up-regulated by salt, drought, and abscisic acid (ABA) treatment. In addition, *RD26* has the ability to positively

regulate ABA signaling (Fujita et al., 2004; Tran et al., 2004). In cotton (*Gossypium hirsutum*), overexpression of the rice (*Oryza sativa*) NAC gene *SNAC1* improved tolerance to drought and salt via enhanced root development and reduced transpiration rates (Liu et al., 2014). Recent studies also showed that, in maize (*Zea mays*), *STRESS RELATED NAC1* (*ZmSNAC1*) worked as a stress-responsive transcription factor in positively modulating abiotic stress tolerance (Lu et al., 2012; Shiriga et al., 2014). However, the response of the downstream networks of these NAC transcription factors to abiotic stress have not been clarified.

Arabidopsis is a salt-sensitive plant and presents certain limitations in studies of salt and drought tolerance mechanisms. *Thellungiella halophila* (and *Thellungiella salsuginea*) is a close relative of *Arabidopsis* with a similar coding sequence. However, *T. halophila* does show differences at the metabolome level, including in the metabolism of Ala and the expression patterns of certain drought- or cold-responsive genes (Amtmann et al., 2005; Benina et al., 2013). *T. halophila* is a valuable model plant for the study of plant stress response mechanisms because it has a small genome and exhibits high tolerance to drought, low temperatures, and high salinity (Zhu, 2001; Inan et al., 2004).

VACUOLAR H⁺-PYROPHOSPHATASE (*H⁺-PPASE*) plays an important role in the abiotic stress tolerance of plants. Overexpression of *V-PPASE1* (*AVP1*; *AT1G15690*) in *Arabidopsis* improved the drought and salt tolerance in this species (Gaxiola et al., 2001). The overexpression of *T. HALOPHILA H⁺-PPASE1* (*TsVP1*) also enhanced salt and drought stress resistance in different species, such as

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cotton, maize, and tobacco (*Nicotiana tabacum*; Gao et al., 2006; Li et al., 2008; Lv et al., 2009). Although the expression of *TsVP1* is apparently induced by salt stress, this is not the case for *AVP1* in Arabidopsis. Furthermore, one 130-bp (−667 to −538) region was identified as the key sequence for the salt stress response from the promoter of *TsVP1* in *T. halophila* (Sun et al., 2010).

Using the above-mentioned 130-bp region as a starting point, we identified *T. HALOPHILA STRESS RELATED NAC1* (*TsNAC1*), which is an ortholog of *RD26* in Arabidopsis. The overexpression of *TsNAC1* substantially promoted the abiotic stress tolerance of plants and retarded the growth of *T. halophila* plants. We compared the differences between *TsNAC1* and *RD26* to determine the function of *TsNAC1* in growth regulation and abiotic responses. A chromatin immunoprecipitation sequencing (ChIP-Seq) assay was used to reveal the downstream genes and regulation network. This study provides evidence that the growth and abiotic stress resistance responses of the halophyte *T. halophila* are regulated by the key transcription factor *TsNAC1* in a dose-dependent manner.

RESULTS

TsNAC1 Is an Upstream Regulator of *TsVP1*

A plasmid containing a 3× tandemly repeated 530-bp DNA fragment of the *TsVP1* promoter (nucleotides −667 to −138) was constructed and introduced into the yeast strain Y187. This strain was then transformed with the pGADT7-cDNA library comprising cDNAs from *T. halophila* plants exposed to a 12-h high-salt stress treatment. One target protein was identified from the strains grown on synthetic dextrose (SD)/-His/-Trp medium with 5 mM 3-aminotriazole. The candidate gene was cloned from *T. halophila*, and the BLAST results indicated that the target protein was *TsNAC1*, a member of the NAC superfamily. *TsNAC1* has an open reading frame with a length of 918 bp, shares approximately 86% similarity with *RD26* at the nucleotide level and 92% similarity at the amino acid level (Supplemental Data S1), and includes the A to E subdomains of the NAC DNA-binding domain (Fig. 1A). Evolutionary tree analysis indicated that *TsNAC1* is the closest gene to *RD26* (*ANAC072*) in *T. halophila* (Supplemental Fig. S1).

The coding region of *TsNAC1* was fused to the GAL4 activation domain (AD), and the promoter of *TsVP1* was cloned into the reporter plasmid pLacZi. Both of the plasmids were transformed into the yeast strain YM4271. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (X-gal) staining for β-galactosidase activity showed a blue color in strains with the promoter region of *TsVP1* (Fig. 1B), indicating that *TsNAC1* was bound to the promoter region to activate the transcription of *TsVP1* in yeast.

The *TsNAC1*-GST fusion protein was expressed in *Escherichia coli*, and the 130-bp fragments of the *TsVP1* promoter were labeled with digoxigenin-ddUTP for an

EMSA. *TsNAC1* was bound to the labeled probe, and the 200× molar excess unlabeled probe could competitively inhibit the combination (Fig. 1C). These results demonstrated that *TsNAC1* could bind to the 130-bp sequence of the *TsVP1* promoter in vitro.

Expression Profile of *TsNAC1*

To evaluate the expression pattern of *TsNAC1* in *T. halophila*, real-time reverse transcription (RT)-PCR was performed with diverse organ samples collected from embryogenesis to senescence. The results suggested that the senescent organs, including senescent cauline leaves, rosette leaves, and roots, always had higher expression levels than the other stages. Parts of the floral organs, such as sepals and petals, also exhibited a clear expression pattern (Supplemental Fig. S2A). These data were consistent with the public microarray data for *RD26* from the e-FP browser (Schmid et al., 2005).

Wild-type plants of *T. halophila* were sprayed and watered with 100 μM ABA or methyl jasmonate (MeJA). The roots were sensitive to ABA and showed a 600-fold expression peak after 24 h of treatment, whereas the shoots were sensitive to MeJA and showed a 720-fold expression peak after 24 h of treatment. The expression of *TsNAC1* also was induced by cold, drought, and active oxygen stress in the shoots and by drought and salt stress in the roots (Supplemental Fig. S2B).

Overexpression of *TsNAC1* Retards the Growth of *T. halophila*

Transgenic plants overexpressing *TsNAC1* (OX; 35S:*TsNAC1*) and *TsNAC1* RNA interference (RNAi) plants (NR; harboring hairpin-shaped *TsNAC1* mRNA fragments [411–882 bp] driven by the cauliflower mosaic virus 35S promoter) were generated from *T. halophila*, and Arabidopsis overexpressing *TsNAC1* plants (OXA; 35S:*TsNAC1*) also were generated (Fig. 2A). Western-blot analyses indicated that the protein levels of these independent lines were consistent with the transcription levels of *TsNAC1* (Fig. 2C). The overexpressing lines OX7 and OX10 and the RNAi lines NR10 and NR11 of *T. halophila* were selected to determine the effects of *TsNAC1* transcript levels on growth and abiotic stress responses. Seeds were germinated on one-half-strength Murashige and Skoog-agar medium, and seeds of the overexpressing lines OX7 and OX10 germinated later than the wild-type and RNAi seeds (Fig. 2B). After 6 weeks of growth, the OX7 and OX10 plants were much smaller than the wild-type plants (Fig. 2B), and the dry weights of the shoots and roots were substantially lighter (Fig. 2E). The dry weight of 6-week-old plants from the *T. halophila* OX lines was reduced by 10.1% to 25.4% in the shoots and by 49.5% to 52.5% in the roots compared with those of the wild type (Fig. 2E). In contrast, the NR10 and NR11 plants had larger leaves and stronger roots (Fig. 2B). The biomass and growth rates were inversely related

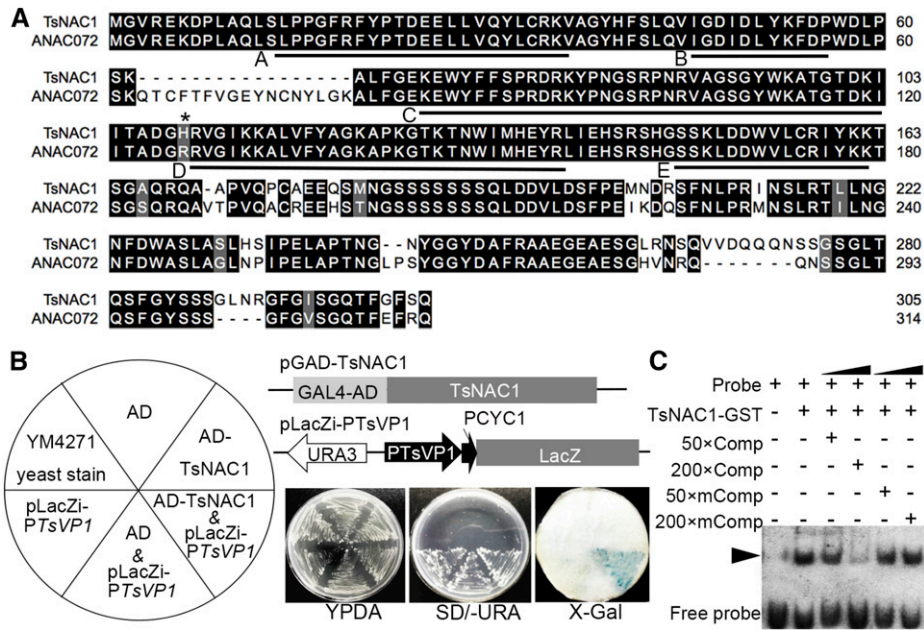


Figure 1. The 130-bp key region of the promoter of *TsVP1* is a direct binding target of TsNAC1. A, Multiple amino acid sequence alignment of TsNAC1 and its ortholog in Arabidopsis (RD26). Identical residues are shaded in black, and five subdomains (A-E) are indicated on the sequences. B, Transcriptional activity assay of TsNAC1 in yeast strain YM4271. *TsNAC1* was cloned and fused with the GAL4 AD. The promoter of *TsVP1* was cloned into pLacZi vector. Streaked transformants are shown on Yeast extract Peptone Dextrose medium with 0.003% Adenine hemisulfate (YPDA) and SD/-uracil medium. The activities of β -galactosidase were examined by X-gal staining. C, Electrophoretic mobility shifts assay (EMSA). Probes, TsNAC1-GST, competitors (130-bp unlabeled fragments), and mutated competitors at 50 \times and 200 \times molar excess were present (+) or absent (-) in each reaction. The protein-DNA complexes are marked by black arrowheads.

to the expression levels of *TsNAC1* (Fig. 2E). Taken together, these results led to the conclusion that TsNAC1 retarded plant growth.

Overexpressing *TsNAC1* Improves Abiotic Stress Resistance of Plants

MeJA-responsive and ABA-responsive cis-acting regulatory elements are observed in the promoter sequences of *TsNAC1* and *RD26*, and the *TsNAC1* promoter has an anaerobic induction element and certain cold- and dehydration-responsive elements, including the TGA element, ARE, and TC-rich repeats (Supplemental Table S1). To identify whether *TsNAC1* expression enhances plant tolerance to drought, high salinity, low temperatures, and reactive oxygen species (ROS) stress in transgenic *T. halophila*, the seeds from the transgenic lines were germinated in a medium of peat and vermiculite (at a 2:1 ratio) and the seedlings were grown for 6 weeks under normal conditions. Under the low-temperature stress (-4°C) treatment (Fig. 2D), the overexpressing plants had a higher survival rate after 48 h of treatment (Supplemental Fig. S3A). After 6 weeks of growth, plants were watered with 18% (w/v) PEG6000 solution for 1 week (Fig. 2D), and overexpressing plants exhibited higher tolerance to drought conditions than wild-type or RNAi transgenic plants (Supplemental Fig. S3B). To examine

salt resistance, the plants were thoroughly watered with an 800 mM NaCl solution and then watered with 10 mL of a 400 mM NaCl solution every 3 d (Fig. 2D). After 14 d of exposure, the transgenic RNAi and wild-type plants developed necrotic leaves and certain senescent leaves died, whereas the *TsNAC1* overexpression plants displayed the same characteristics as untreated plants (Supplemental Fig. S3C). Uniform seedlings of transgenic and wild-type plants were sprayed with 0.1 mL of 3 mM paraquat per pot. After a 36-h treatment, the leaves of OX lines all showed greater resistance and higher survival probabilities than the wild-type or NR plants (Supplemental Fig. S3D). These results suggested that overexpressing *TsNAC1* significantly improved plant abiotic resistance.

Exploration of the Target Genes of TsNAC1

To investigate how the overexpression of TsNAC1 retarded plant vegetative development, the downstream target genes of TsNAC1 were identified by ChIP-Seq using overexpressing lines OX7 and OX10 and the TsNAC1 polyclonal antibody, the specificity of which was tested by western blot, with Columbia-0 (Col-0) and OXA leaves as materials and with the β -Actin antibody as the contrast (Supplemental Fig. S4). Each sequencing read was mapped to the *T. halophila* genome

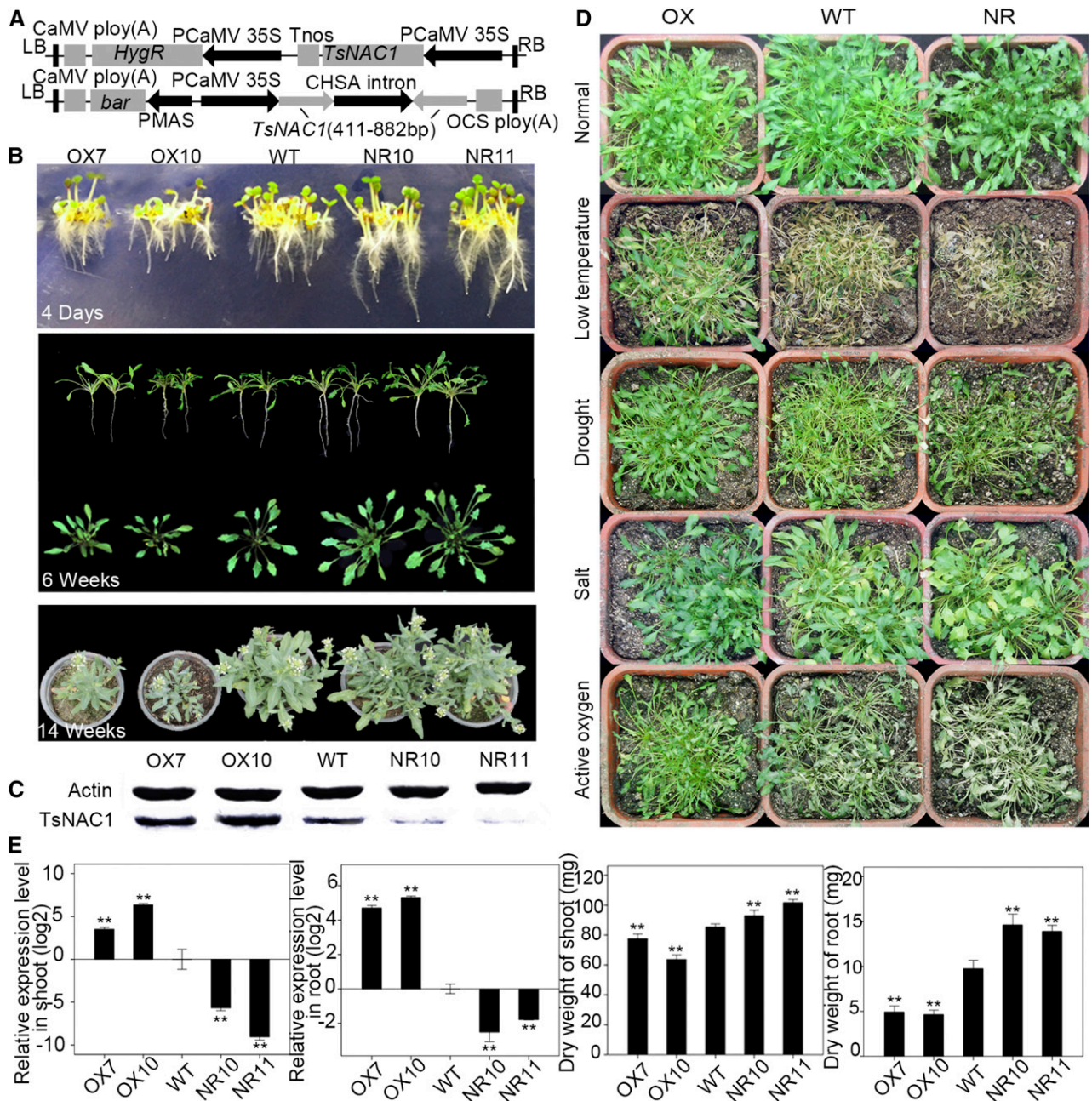


Figure 2. TsNAC1 regulated the growth of *T. halophila*. **A**, Diagrams of the plant overexpression vector and RNAi vector. **B**, Phenotypes of TsNAC1 transgenic plants. Shown are seedlings grown on Murashige and Skoog-agar for 4 d after sowing and plants grown in soil for 6 weeks and 14 weeks (after vernalization treatment for 5 weeks). **C**, Western-blot results of transgenic lines and the wild type (WT). **D**, Phenotypes of OX, wild-type, and NR *T. halophila* plants after 48 h of treatment at -4°C with 3,500 lx, after 1 week of drought stress with an 18% PEG6000 solution, after keeping the 600 mM NaCl concentration of culture medium for 2 weeks, and after 36 h of active oxygen stress with 0.1 mL of 3 mM paraquat solution for every pot. **E**, *TsNAC1* expression levels in OX7, OX10, NR10, NR11, and the wild type (the expression was normalized with Actin), and the dry weight of shoots and roots. Bars represent means \pm SD, with three biological replicates in the experiment and five plants for each repeat. Significant differences by Student's *t* test are indicated with asterisks: **, $P < 0.01$.

(<http://thellungiella.org/>) to identify its position using the ultrafast Bowtie aligner (Langmead et al., 2009). The ChIP-Seq data included 14.2 million mapped reads, with 7.8-fold of genome coverage on average. After excluding the peaks in intergenic regions, MACS software (Zhang

et al., 2008) detected 1,868 and 2,454 genes associated with a gene model with $P < 0.05$ for each of the ChIP-Seq data sets. A total of 892 genes were highly enriched in both sets, and 22.16% of these gene peaks were located in the promoter region (a fragment from $-2,000$ bp to

ATG; Fig. 3A). Additionally, except for the peaks in the intergenic region, the read distribution was close to the transcription start site at -200 to $+100$ bp (Fig. 3B). TsNAC1 is an upstream regulator of *TsVP1*. The ChIP-Seq signals of *TP1G13990* (*TsVP1*) and *TP2G25870* (*Tub2*), which were used as the positive and negative controls, respectively, were visualized on the genome browser (Fig. 3C). Based on ChIP-Seq, motifs around the binding peaks located at the promoter regions and a short and ungapped CA(T/A)G sequence were investigated (Fig. 3D). The ChIP samples were quantified via qPCR using primers specific to the promoters of *TsVP1* and *Tub2*. As shown in Figure 3E, the promoter region of *TsVP1* was effectively enriched, and *TsVP1* was expressed at higher levels in OX lines than in the NR lines. These data demonstrated that TsNAC1 targeted the promoter of *TsVP1* and that the ChIP-Seq results were valid.

To further explore the biological processes regulated by TsNAC1, the binding target genes were classified into Gene Ontology (GO) groups. The GO analysis results for 892 coenriched genes showed a significant and coincident enrichment for biological regulation (GO:0065007), developmental processes (GO:0032502), and responses to stimuli (GO:0050896) compared with the input sample data (Fig. 3F). The next hierarchical classification of these genes primarily involved biological processes, including cell growth (GO:0016049), embryonic development ending in seed dormancy (GO:0009793), and response to salt stress (GO:0009651; Fig. 3F). Genes with fold enrichment greater than 20 (normalized by the input sample) and peaks located in the promoter region of genes from these three groups were selected to test for the expression levels in transgenic plants, including the levels of *TsVP1*. Seven genes were up-regulated [\log_2 (OX/NR) > 2] and two genes were down-regulated [\log_2 (OX/NR) < -1] by TsNAC1 (Table I). The promoter regions for these nine genes were recombined into a pLacZi vector for one-hybrid yeast system assays of the YM4271 strain. X-gal staining showed that these regions were targeted by TsNAC1 in yeast (Fig. 4A).

TsNAC1-Regulated Genes with the CA(T/A)G Element in the Promoter

Motif discovery with the DNA fragments identified via ChIP-Seq around the binding peaks was performed using MEME-ChIP (Bailey et al., 2015), and 197 DNA sequences (length between 67 and 434, average length of 151.1) located at the promoter regions and a short and ungapped CA(T/A)G motif with $E < 8.5e-018$ were investigated (Fig. 3D). The distribution of the motif was visualized using a histogram that showed the percentage occurrence of the CA(T/A)G motif (x axis) plotted against the \log_2 fold enrichment of immunoprecipitation samples compared with the input sample (y axis), with the color of each square mapped to the number of indicated peak motifs (Fig. 3D). In general, peaks had one or more motifs at $\log_2 > 0$, and the proportion of peaks containing at least one motif increased along with the \log_2 fold enrichment of TsNAC1 binding.

It had been demonstrated that *TsNAC1* could recognize the 130-bp key region of the promoter of *TsVP1* in vivo and vitro. To identify the specific binding site, a serious 5' deletion fragment was amplified by PCR from -667 to -388 (280 bp). M1 was the full length of 280 bp (Supplemental Data S2), M2 was 10 bp shorter than M1, and by such analogy, M14 lost the full length of the 130-bp key fragment. We incubated the TsNAC1-GST with both a moderately labeled 130-bp key fragment and excess unlabeled 5' deletion fragments, and the results of the gel retardation assay validated that the combination between TsNAC1 and M13/M14 was weaker than M1 to M12, and the 20-bp fragment GAATATACCATGGA-TAAGCA was the core recognition sequence (Supplemental Fig. S5B). The promoter of *TsVP1* was able to be recognized by TsNAC1, with two CA(T/A)G motifs (-545 and -400 to ATG) in this region (Supplemental Table S3). To identify the specific binding site via EMSA, TsNAC1 was bound to these sites, and when excess competitors (unlabeled sequences) were added, the binding signals decreased remarkably while the mutant competitors (unlabeled mutated sequences) could not (Supplemental Fig. S5A). The one-hybrid yeast system results using series substitutions as reporters indicated that CA(T/A)G is the core DNA-binding motif of TsNAC1 (Supplemental Fig. S5C). These results suggested that TsNAC1 could recognize CA(T/A)G and, thus, regulate the downstream network.

Multiple Cell Expansion Regulators Are Targeted by TsNAC1

LIGHT-DEPENDENT SHORT HYPOCOTYLS1 (*LSH1*) and *UDP-XYLOSYLTRANSFERASE2* (*TXT2*) exerted positive regulation on cell expansion by acting as transcription factors (Iyer and Aravind, 2012) or maintaining the structure of cell walls (Wang et al., 2014). *MULTICOPY SUPPRESSOR OF IRA1 4* (*MSI4*) negatively regulated the vegetative biomass by silencing the expression of *FLOWERING LOCUS C* (*FLC*; Morel et al., 2009). The ChIP-Seq and ChIP-PCR analyses (Fig. 4B) indicated that these loci were the TsNAC1 target genes. Quantitative RT-PCR results indicated that the expression of *MSI4* was up-regulated but that of *TXT2* and *LSH1* was down-regulated (Table I) by TsNAC1 in the OX lines. Taken together, these data suggest that the role of *LSH1*, *TXT2*, and *MSI4* in retarding cell expansion is directly regulated by TsNAC1. *TsVP1*, which was shown previously to play an important role in cell expansion, was a target gene of TsNAC1 and was up-regulated in the OX lines but down-regulated in the NR lines.

TsNAC1 Plays a Crucial Role in Plant Tolerance to Salt Stress

An analysis of the TsNAC1 ChIP-Seq data identified a number of positive regulators of salt stress tolerance, including *TsVP1*, *HOMEBOX12* (*HB12*), and *MYB HYPOCOTYL ELONGATION-RELATED* (*MYBH*), as candidate TsNAC1 target genes. TsNAC1 has been shown

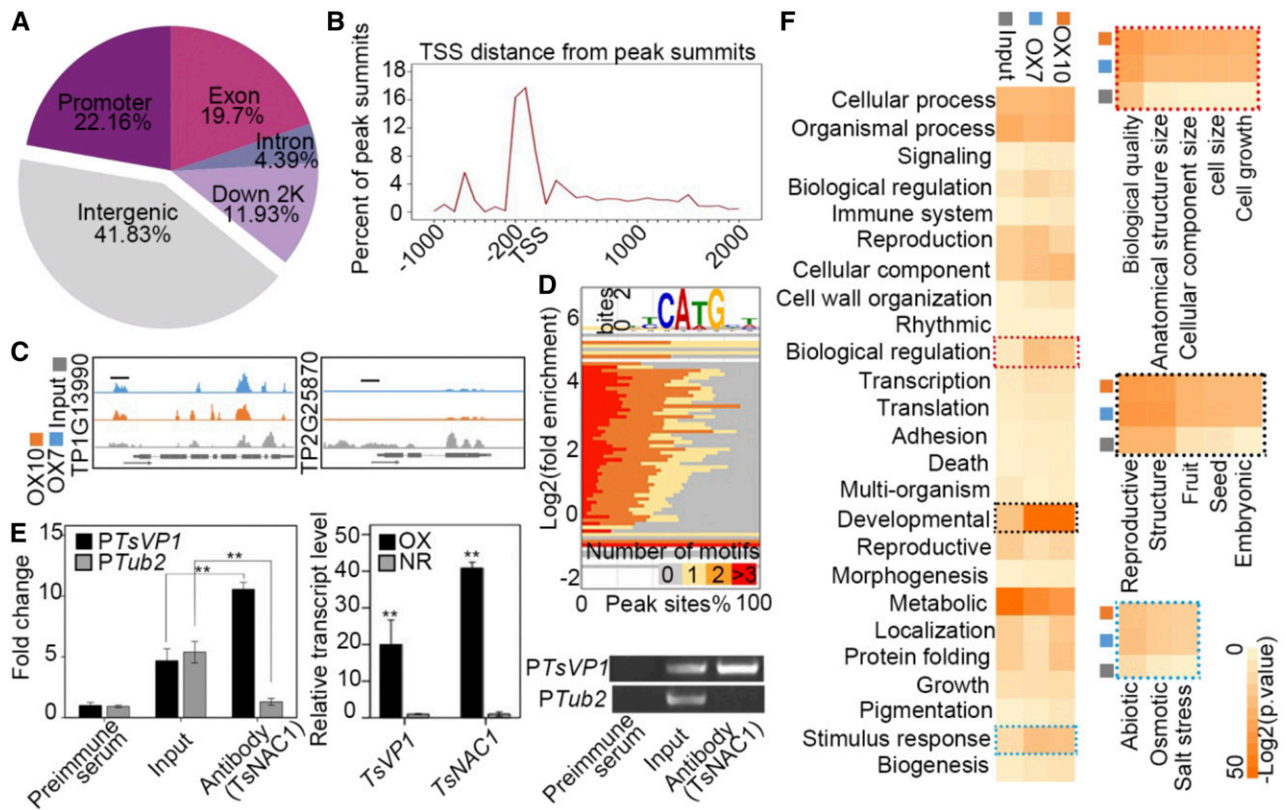


Figure 3. ChIP-Seq assay of TsNAC1. A, Distribution of TsNAC1 transcription factor-binding sites. B, Identified peak distance from transcription start sites (TSS) for TsNAC1. The peaks were highly enriched from -200 to $+100$ bp to the transcription start sites. C, ChIP-Seq signals of TP1G13990 (*TsVP1*) and TP2G25870 (*Tub2*) on the genome browser. The tag counts were normalized in each bin according to the total number of reads. Short black lines mark regions used for the ChIP-quantitative PCR (qPCR) assay. D, Binding situation of TsNAC1 quantified by the percentage occurrence of the CA(T/A)G motif (x axis) plotted against the \log_2 fold enrichment of immunoprecipitation samples compared with the input sample (y axis), with the color of each square mapped to the number of indicated peak motifs. E, Anti-TsNAC1 ChIP-qPCR validation and transcript detection of *TsVP1* and *TsNAC1*. Samples immunized by preimmune serum were used as the negative control in the ChIP-qPCR assay. Bars represent means \pm SD, with three biological replicates in the experiment and five plants for each repeat. Significant differences by Student's t test are indicated with asterisks: **, $P < 0.01$. F, GO analysis results of the input sample data and data for two immunoprecipitation samples (OX7 and OX10). The dotted rectangles indicate significant and consistent GO enrichment and the hierarchical classification results of biological regulation, developmental processes, and responses to stimuli.

to bind to the 130-bp sequence of the *TsVP1* promoter in vivo and in vitro. Two peaks were identified at 450 to 600 bp (29.76 relative fold enrichment) and 300 to 450 bp (14.29 relative fold enrichment) upstream of the ATG of *TsVP1* (Fig. 3C). Binding of TsNAC1 to two other genes also was identified by the one-hybrid yeast assay (Fig. 4A) and by ChIP-PCR assay (Fig. 4B). The expression levels of *TsVP1*, *HB12*, and *MYBH* were all up-regulated by TsNAC1 (Table I). The results confirmed that TsNAC1 positively regulates the expression of *TsVP1*, *HB12*, and *MYBH* in plant salt stress tolerance.

TsNAC1 Shows Higher Transcriptional Activation Activity Than RD26

Two one-hybrid yeast systems were constructed to test for differences between the binding domain and AD of RD26 and TsNAC1. First, open reading frames

were cloned by translational fusion with the yeast GAL4 AD, and the *TsVP1* promoter was linked to pLacZi or pHis2.1 as reporter. The cotransformed YM4271 strains were cultured on SD/-His, and β -galactosidase activity was examined to verify their DNA-binding ability. Although TsNAC1 and RD26 were able to bind to the *TsVP1* promoter, X-gal staining demonstrated that the binding ability of RD26 was slightly weaker than that of TsNAC1 (Fig. 5A).

RD26 was reported as having a repression domain (122–141) that suppresses the AD (Hao et al., 2010). The full-length RD26 (1–314), a C-terminal AD (181–314), and the AD with the repression domain (122–314) were each recombined into a pBD vector and then transformed in the YRG-2 strain. The strains containing only the AD (181–314) grew well on the selection SD/-His medium and showed high β -galactosidase activity, and the other two strains were stunted on SD/-His medium

Table 1. Candidate target genes of *TsNAC1* identified by ChIP-Seq

| TGI No. ^a | AGI No. ^b | Motif ^c (Y/N) | Alias ^d | Enrichment Fold ^e | Relative Expression Level ^f |
|----------------------|----------------------|--------------------------|--------------------|------------------------------|--|
| Tp3g32900 | AT2G19520 | Y | MSI4 | 35.71 | 3.93 |
| Tp2g20180 | AT5G28490 | Y | LSH1 | 25.51 | -2.18 |
| Tp6g02410 | AT4G02500 | Y | TXT2 | 31.06 | -1.92 |
| Tp1g10290 | AT1G11680 | Y | CYP51 | 59.52 | 3.46 |
| Tp2g04490 | AT1G60170 | Y | PRP31 | 44.64 | 3.01 |
| Tp1g21950 | AT1G26830 | Y | CUL3 | 41.21 | 2.96 |
| Tp1g13990 | AT1G15690 | Y | TsVP1 | 29.76 | 3.43 |
| Tp5g01460 | AT3G61890 | Y | HB12 | 39.68 | 2.57 |
| Tp2g12110 | AT5G47390 | Y | MYBH | 29.76 | 2.18 |

^aTGI, *T. halophila* Genome Initiative. ^bAGI, the ortholog of *T. halophila* in the Arabidopsis Genome Initiative. ^cMotif, whether with CAT(A)G in the promoter region of target genes in *T. halophila*. ^dAlias, the ortholog of target genes' short names in Arabidopsis, except for TsVP1, which has been reported. ^eEnrichment fold, which was normalized by the input sample data. ^fRelative expression level, log₂ (relative expression of the target gene in OXA lines against that in wild-type plants, normalized with the expression level of Tub2), referenced to the results of real-time RT-PCR.

and showed much lower β -galactosidase activity. The same *TsNAC1* deletion strains (1–305, 105–305, and 164–305) also were examined following training on SD/-His medium with an ONPG assay, and the region from amino acid 105 to 163 of *TsNAC1* was found to suppress the transcriptional activation of AD (164–305; Fig. 5B).

The 164 to 305 region of *TsNAC1* exerted limited higher transcriptional activity compared with the 181 to 314 region of RD26, although the β -galactosidase activity of the strains with the *TsNAC1* 105 to 305 region was twice as great as that of the strains with the RD26 122 to 314 region. These results suggest that the repression ability of the *TsNAC1* 105 to 163 region was lower than that of the RD26 122 to 180 region. Alignments with ClustalW indicated that the presence of Arg at RD26-126 was the only difference between the two protein sequences (Fig. 1A). A point mutation fragment was constructed in pBD, and the results of the transcriptional activity assay supported our hypothesis that the mutation from Arg to His weakened the repression ability (Fig. 5B). Overall, significant differences were not observed in the binding ability of RD26 and the *TsNAC1* target to the promoter of *TsVP1*, although the transcript activation activity was much lower than that of *TsNAC1* in yeast.

Referenced to the results of ChIP-Seq and the one-hybrid yeast system, seven genes (including *TsVP1*) were up-targeted by *TsNAC1*. And except for *MSI4*, the promoters of these genes contained the target motifs of RD26. To demonstrate whether *TsNAC1* has higher transcript activation activity in vivo, the promoter regions of these seven genes were connected into pGreenII 0800-Luc (with *PMSI4:Luc* as the negative control) and cotransformed with 35S:*TsNAC1* or 35S:RD26 into the protoplasts of *T. halophila* (Fig. 4C). Injection of the transformed *Agrobacterium tumefaciens* GV3101 pGreenII 0800-*PtsVP1:Luc* and 35S:*TsNAC1* into the leaves of tobacco in pairs also showed higher luciferase signal than that of 35S:RD26 (Fig. 4D). All these results demonstrated that *TsNAC1* had higher transcript activity than RD26 in vivo.

DISCUSSION

Abiotic stress, such as high-salt stress, has a considerable impact on the quality and yield of agricultural products. Thus, the mechanisms of the abiotic stress response must be investigated at the molecular level. *T. halophila* is a halophytic plant with excellent salt and drought resistance; therefore, it is an interesting system in which to study the mechanisms of the abiotic stress response. Here, we explored the abiotic stress resistance of *TsNAC1* overexpression lines to identify target genes for further investigation.

TsNAC1 Participates in Plant Responses to Multiple Abiotic Stressors

Previous studies of Arabidopsis showed that RD26 is an important regulator of abiotic stress that is involved in the ABA-dependent dehydrate stress-signaling pathway (Fujita et al., 2004; Tran et al., 2004). *TsNAC1* is highly expressed in mature tissues (Supplemental Fig. S1A), which is similar to the expression pattern of RD26 (Schmid et al., 2005). However, the expression of *TsNAC1* was induced by salt stress, drought, and ABA treatments as well as by low temperatures, ROS stress, and MeJA treatments (Supplemental Fig. S1B), which is inconsistent with the findings for RD26. The promoter sequences of both *TsNAC1* and RD26 have light-responsive, MeJA-responsive, and ABA-responsive cis-acting regulatory elements. However, the *TsNAC1* promoter also had auxin-responsive, anaerobically induced, and cold- and dehydration-responsive elements (Supplemental Table S1), and these findings are consistent with the expression pattern (Supplemental Fig. S1B). Moreover, the overexpression lines of *T. halophila* showed slower growth and better survivability after exposure to low temperature, drought stress, high-salt stress, and ROS stress, whereas RNAi lines showed more rapid growth and were sensitive to abiotic stresses. Several regulator genes involved in the response to abiotic stress were positively regulated by *TsNAC1*. For example, the expression of DIACYLGLYCEROL KINASE2, ASPARTATE AMINOTRANSFERASE5 (AAT3), and

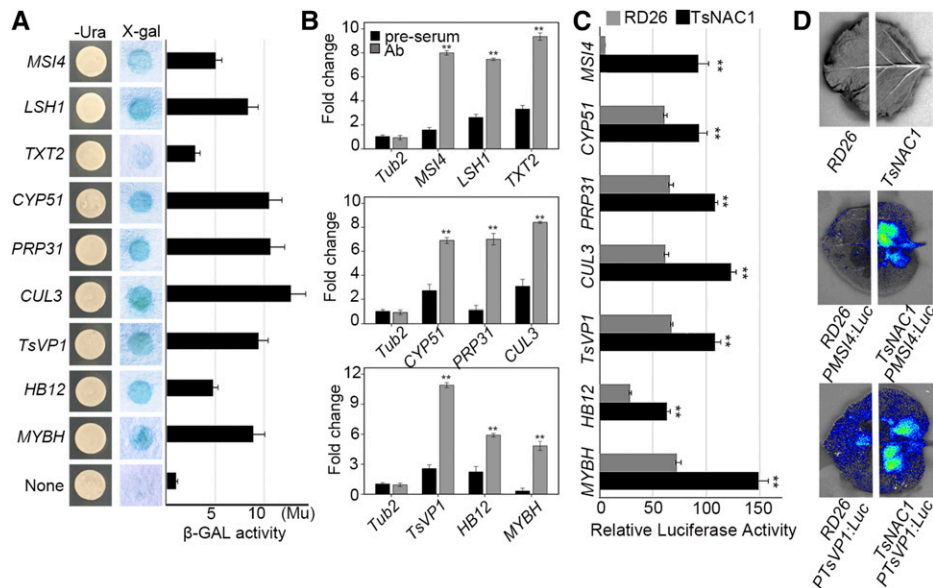


Figure 4. Validation of candidate downstream genes. A, Downstream target promoter fragments were connected into pLacZi and transformed into the pAD-GAL4-TsNAC1 YM4271 strain. β -Galactosidase (β -GAL) activities were validated by X-gal staining. β -Galactosidase activity was measured with *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate. B, Binding of TsNAC1 to the promoter of downstream genes as determined by CHIP-PCR. Six-week-old wild-type rosette leaves were used as materials and immunoprecipitated with TsNAC1 antibody and protein A agarose beads. Negative control reactions were performed in parallel using preimmune serum. β -Galactosidase units (Mu) were determined as follows: $1,000 \times OD_{574}/(t \times V \times OD_{600})$, where *t* is elapsed incubation time (min) and *V* is volume of culture (mL). C and D, Transient expression of the *P35S:TsNAC1* or *P35S:RD26* construct with promoter:Luc reporter constructs in *T. halophila* protoplasts (C) and tobacco leaves (D). Representative bioluminescence images are shown. Bars in B and C represent means \pm SD, with three biological replicates in the experiment. Significant differences by Student's *t* test are indicated with asterisks: **, $P < 0.01$.

CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR1 is induced by low temperature to improve plant freezing tolerance (Lee et al., 2005; Goulas et al., 2006; Doherty et al., 2009), and their expression was increased in the OX lines (Supplemental Table S2). ACONITASE3, POLLEN-PISTIL INCOMPATIBILITY2, and NAD-DEPENDENT MALIC ENZYME2 (NAD-ME2) are induced by oxidative stress and protect plants by increasing the stability in the molar ratio of metal ions (Moeder et al., 2007; Tan et al., 2010), and the expression of these genes was up-regulated by TsNAC1, as shown by the real-time RT-PCR results for the OX lines against the wild-type plants (Supplemental Table S2). These data suggest that TsNAC1 also plays a role in the control of ROS levels, which indicates that the expression level of TsNAC1 was positively correlated with the resistance to abiotic stresses of the plant and that TsNAC1 is a crucial regulator for multiple abiotic stress responses in *T. halophila*.

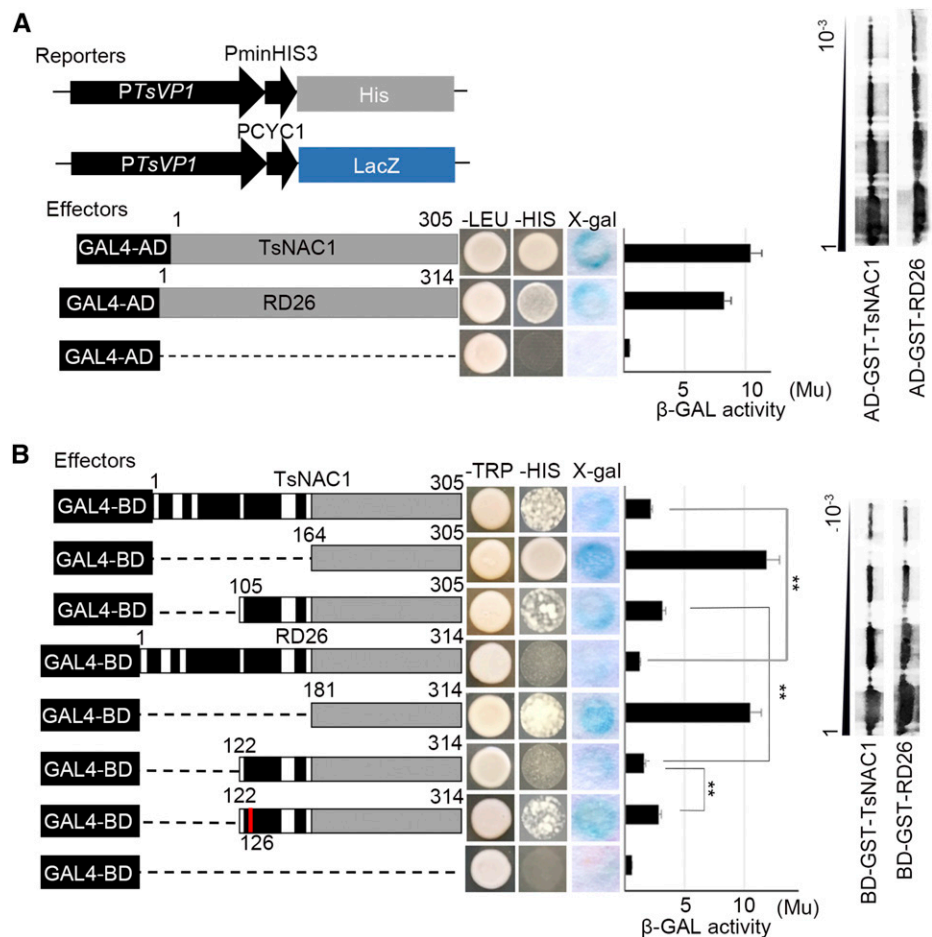
TsNAC1 Involvement in the Regulation of Cell Ion Transport

TsNAC1 has been shown to play an important role in salt stress. Moreover, TsNAC1 targets the promoter regions of *TsVP1*, *HB12*, and *MYBH*, and the expression levels of these genes were reduced in the NR lines. The homologous analysis identified two H^+ -PPases in

T. halophila, including *TsVP1*, which is a crucial proton transporter protein. Utilizing the energy from the hydrolysis of inorganic pyrophosphate, *TsVP1* cooperates with H^+ -ATPase (Silva and Gerós, 2009) to transfer H^+ into a vacuole or the extracellular environment to improve salinity and drought stress tolerance (Gao et al., 2006; Bao et al., 2009; Kumar et al., 2014). Prior studies have shown that *AVP1* could not respond to salt stress and was not positively regulated by RD26 (Sun et al., 2010). These differences between TsNAC1 and RD26 contribute to differences in salt tolerance between Arabidopsis and *T. halophila*.

HB12 is in the ABA-induced homeodomain-Leu zipper subfamily I, and it specifically functions in Na^+ ion homeostasis by regulating the expression of *PMR2A* (an Na^+/Li^+ translocating P-type ATPase) in yeast and responds to salt and drought stresses (Shin et al., 2004; Valdés et al., 2012). *AtHB-12*, the ortholog of *HB12*, is the target gene of *ANAC019* (Hickman et al., 2013), and its T-DNA insertion mutant exhibits longer inflorescence stems via the negative regulation of the expression of GA 20-oxidase (Son et al., 2010). In addition to *HB12*, the expression of *MYBH*, another transcription factor involved in salt and drought stresses, also is controlled by TsNAC1. *MYBH* in Arabidopsis responds to salt and drought stresses (Rasheed et al., 2016) and also plays a crucial role in auxin accumulation by up-regulating the expression

Figure 5. Assays of transcription factor activity in the yeast system. **A**, Binding activity of TsNAC1 and RD26 to the promoter of *TsVP1*. TsNAC1 and RD26 were cloned and fused with the yeast GAL4 AD. The promoter of *TsVP1* was linked into pHis2.1 and pLacZi vectors as reporters. **B**, Truncation analysis of the transcriptional activation activity of TsNAC1 and RD26. Numbers on the top indicate the positions of amino acids. Subdomains A to E of the NAC DNA-binding domains are colored in black, the ADs are colored in gray, and the mutation of the amino acid is colored in red. The C-terminal AD (164–305 and 181–314), the AD with the repression domain (105–305 and 122–314), and the full-length transcription factor domain (1–305 and 1–314) were cloned and fused with the yeast GAL4 binding domain (BD). Transformants were screened on SD/-Leu or SD/-Trp and SD/-His, and the β -galactosidase (β -GAL) activities were validated by X-gal staining and quantified by ONPG assay. Western blot was executed with the GST antibody. Bars represent means \pm SD, with three biological replicates in the experiment. Significant differences by Student's *t* test are indicated with asterisks: **, $P < 0.01$.



of *PHYTOCHROME-INTERACTING FACTOR4* (Kwon et al., 2013). *MYB* families, such *MYB2*, *MYB21*, *MYB108*, *MYB112*, *MYB116*, etc., are the target genes of *RD26* (Hickman et al., 2013), and they are involved in salt and dehydration tolerance (Yang et al., 2012; Wang et al., 2015). The *RD26* target motif also occurs in the promoters of the *MYBH* and *HB12* genes in *Arabidopsis*, which suggests that these promoters are involved in the salt stress response. Previous studies of rice showed that the K^+ TRANSPORTER (*OsHKT1*) reduces Na^+ accumulation as a response to salt stress and showed that it is the downstream gene of *OsMYBc* (Zhang et al., 2015). Mutant *hkt1-1* in *Arabidopsis* is sensitive to $NaCl$ and exhibits greater root growth than the wild type. The *OsHKT1* ortholog in *T. halophila* is induced dramatically by salt stress (Ali et al., 2013), which suggests that *MYBH* might be involved in the transport of K^+ by regulating the expression of *HKT1*. These results show that *TsNAC1* improves the salt and drought tolerance of plants by directly or indirectly regulating the transport of ions to maintain the proton gradient across the cell membrane and also provides energy for secondary active transport, such as the transport of auxin, which is consistent with observations in *Arabidopsis* (Li et al., 2005; Yang et al., 2014).

TsNAC1 Retards Growth by Restricting Cell Expansion and Organ Size

The homozygotes of the T-DNA insertion mutants of *RD26* (N1, SALK_072276; N2, SALK_083756) were highly sensitive to high-salt conditions (C.L., Q. Sun, B. Li, Z.L., Z.P., and J.Z., unpublished data), although significant differences in organ size were not observed compared with *Col-0* (Fujita et al., 2004). A recent study indicated that transgenic *35S:RD26* plants displayed a stunted growth phenotype (Ye et al., 2017). When *TsNAC1* derived by the cauliflower mosaic virus 35S promoter was introduced into N1 and N2, the plants showed a smaller organ size than *Col-0* but bigger organs than the *TsNAC1* transgenic plants derived from *Col-0*, and the lines also had significantly improved salt tolerance (unpublished data). We calculated the cell size of vascular tissue in the root maturation zone 5 d after sowing, and the cell size of the NR lines was increased by 20% and that of the OX lines was reduced by 27.5% compared with the wild type (Supplemental Fig. S6).

In *Arabidopsis*, *AVP1* participates in auxin transport and accumulates in the root (Li et al., 2005). And *TsVP1*, which is the ortholog of *AVP1* in *T. halophila*, is up-regulated in *TsNAC1* overexpression lines and also

regulates plant growth. Moreover, the overexpression of *AVP1* increases the shoot biomass of plants (Li et al., 2005; Schilling et al., 2014), and the overexpression of *TsVP1* in tobacco, cotton, and maize also enhances plant biomass (Gao et al., 2006; Li et al., 2008; Lv et al., 2009). Thus, crucial regulators must retard growth and reduce organ size in TsNAC1 overexpression lines. Plants from TsNAC1 overexpression lines showed slower growth and smaller organs than plants that overexpressed *TsVP1*. This result suggests that the effect of *TsVP1* on growth in TsNAC1 overexpression lines was overwhelmed by the other target genes regulated by TsNAC1.

The organ size of plants is related to cellular processes, such as cell proliferation and expansion (Horváth et al., 2006; Du et al., 2014; Cheng et al., 2016). The expression of *MSI4* was up-regulated in TsNAC1 OX lines, and Arabidopsis *MSI4* mutants showed a severalfold increase in vegetative biomass and a 1.3-fold expansion in cell size (Morel et al., 2009). These results suggest that *MSI4* is a negative regulator of cell expansion. The ortholog of *MSI4* in Arabidopsis has been shown to associate with *HISTONE DEACETYLASE6* and *LYSINE SPECIFIC DEMETHYLASE1* to repress *FLC* (Ausín et al., 2004; Yu et al., 2016), and *flc-3* mutants flower later than wild-type plants (Shindo et al., 2006) and present phenotypes similar to that of OX lines (Supplemental Fig. S7). In addition to the up-regulation of *MSI4*, two positive regulators of cell expansion, *LSH1* and *TXT2*, are negatively regulated by TsNAC1. ALOG family proteins act as the key developmental regulators of shoot organs (Takeda et al., 2011) and seeds (Zhao et al., 2004). *LSH1* functions with several other transcription factors to promote unidimensional cell enlargement (Zhao et al., 2004; Iyer and Aravind, 2012). The development of primary cell walls plays a crucial role in plant growth (Cosgrove, 2005) and morphogenesis (Schopfer, 2006). Xyloglucan in the primary cell wall of dicotyledonous plants is a restraining factor on wall extensibility (Keegstra et al., 1973; Hayashi, 2003; Park and Cosgrove, 2015). The biosynthesis of xyloglucan is catalyzed by the ortholog of *TXT2*, a GT34 family member (Vuttipongchaikij et al., 2012; Zabolina et al., 2012), in Arabidopsis (Cavalier et al., 2008). Ye et al. (2017) discovered that *TCH4* and *EXPL2* were down-regulated in *35S:RD26* plants to retard plant growth. RT-PCR results indicated that *TXT2*, which is located in the same subfamily with *TCH4*, was suppressed 1.92-fold [log (OX/wild type)] (Table I), but *EXPA6*, which is located in the same subfamily with *EXPL2* and with a 6.19 relative fold enrichment in its promoter region, was suppressed less than 0.23-fold (data not shown) in OX lines. Overall, variations in the expression levels of *MSI4*, *LSH1*, and *TXT2* induced by TsNAC1 lead to the retardation of plant growth by retarding cell expansion.

TsNAC1 Regulates Embryonic Development

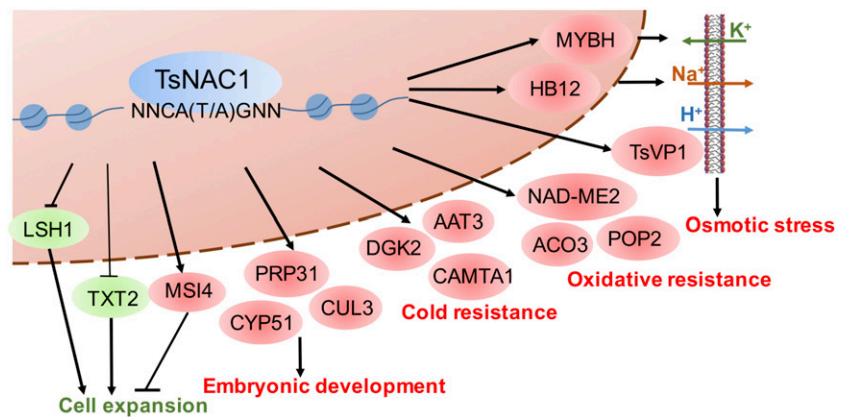
Although the OX transgenic lines had smaller organ size, the fruit set was not lower than that of the

wild-type and NR lines. Several positive regulators involved in embryo development are regulated by TsNAC1. Campesterol is the precursor in the synthesis of brassinosteroids (Hartmann, 1998) and affects postembryonic growth in higher plants (Clouse and Sasse, 1998). *CYP51* is involved in the biosynthesis of campesterol, and *cyp51* mutants are always embryo lethal (O'Brien et al., 2005) or seedling lethal (Kim et al., 2005) via the generation of ROS and ethylene (Kim et al., 2010). In the OX lines, *CYP51* exhibits up-regulated expression, which could enhance the biosynthesis of campesterol, which is beneficial for embryonic development. One member of the Cullin family, *CUL3*, associates with RING-FINGER PROTEIN and BROAD COMPLEX domain proteins and works as an E3 ligase (Figuroa et al., 2005), which affects embryo pattern formation at the heart stage (Thomann et al., 2005). In *T. halophila*, the expression of *CUL3* is up-regulated by TsNAC1 overexpression, which could regulate embryonic development. The ortholog of *PRP31* in Arabidopsis encodes a splicing factor and interacts with *STABILIZED1* and *ZINC-FINGER AND OCRE DOMAIN-CONTAINING PROTEIN1* to regulate embryonic development by regulating the expression of *RNA HELICASE22* (Kanai et al., 2013) and *TRIGALACTOSYLDIACYLGLYCEROL1* (Xu et al., 2005) under abiotic stress treatments, such as cold stress (Du et al., 2015). In the *T. halophila* OX lines, the expression of *PRP31* was up-regulated by TsNAC1 overexpression, which suggests that *PRP31* or TsNAC1 plays a role in abiotic stress resistance by improving embryonic development.

Stress Tolerance Coupled to Growth Retardation

Prior studies showed that plants or tissues with lesser cell size contribute to plant abiotic stress tolerance, such as moisture resistance (Cutler et al., 1977; Bradford and Hsiao, 1982). Under abiotic stress, one way to improve the tolerance of plants is to construct a new ion homeostasis via active transport, such as improving the salt stress tolerance of plants by overexpressing H^+ -PPase *AVP1* (Li et al., 2005). In this study, TsNAC1 improved the salt stress tolerance of plants by enhancing the expression of *TsVP1*, *HB12*, *MYBH*, etc., to regulate the transformation of ion, and it retarded plant growth via restricting cell expansion by affecting the expression level of some plant growth genes, such as *MSI4*, *LSH1*, and *TXT2* (Fig. 6). With the advantage of lesser volume, smaller cells wasted less energy on the active transportation of ion to maintain osmotic pressure, and more energy could be used to power metabolic reactions to improve the abiotic stress resistance of plants. Recently, Ye et al. (2017) reported that *RD26* mediates cross talk between drought and brassinosteroid (BR) signaling. When overexpressed, the *BES1* target gene *RD26* can inhibit BR-regulated growth. Global gene expression studies suggest that *RD26* can act antagonistically to BR to regulate the expression of a subset of *BES1*-regulated genes, thereby inhibiting BR function. In this study, we found that the overexpressed TsNAC1 increased the expression of *CYP51* that took part in the biosynthesis

Figure 6. Proposed model for the regulation of plant development and stress responses by TsNAC1. TsNAC1 functions as an upstream regulator of plant osmotic stress (regulates ion transportation), oxidative stress, and cold stress responses and coordinates both vegetative growth (retards cell expansion) and reproductive growth.



of campesterol, the precursor of BR, but the nexus of TsNAC1 function and BR signaling should mandate further investigations. It is concluded that TsNAC1 acts as an upstream regulator of plant abiotic stress resistance and vegetative growth functions directly on the promoters of *MYBH*, *HB12*, *TsVP1*, *NAD-ME2*, *AAT3*, *PRP31*, *CYP51*, *MSI4*, *LSH1*, *TXT2*, etc., upon increased or reduced expression and coordinates both abiotic stress resistance and growth retardation by reducing cell expansion and growth rate.

Comparison between RD26 and TsNAC1

The binding activity to the promoter, the candidate motif, and the downstream genes of TsNAC1 and RD26 were compared in this study. As shown in Figure 5, a higher transcriptional activation activity of TsNAC1 was found compared with RD26. In this study, the CA(T/A)G sequence was verified as the core DNA-binding motif for TsNAC1 (Supplemental Fig. S5), while RD26 recognizes and binds to regions containing a CACG consensus sequence (Tran et al., 2004). TsNAC1 preferred CATG to CAAG. The *TsVP1* promoter sequence (−667 to −138 bp) used for the binding activity assay contained both of these motifs, which explains why the binding activity assay showed a similar level. A comparison of promoter sequences of major TsNAC1-targeted genes in *T. halophila* and their ortholog genes in *Arabidopsis* (Supplemental Table S3) showed that the distribution of motifs was different between species. Compared with the RNA sequencing results with the RD26 OX lines as materials from Ye et al. (2017), in the 892 downstream target candidate genes of TsNAC1, 135 genes were up-regulated in RD26 OX lines, and the main biological process was enriched in response to salt stress (including the orthologs of *HB12* and *MYBH*) and the regulation of transcription (Table I; Supplemental Fig. S8). A total of 258 genes were down-regulated and enriched in the plant developmental process (including the orthologs of *LSH1*, *TXT2*, *TCH4*, and *EXPA6*, which play roles in restricting cell expansion) and nitrogen metabolism (Table I; Supplemental Fig. S8). It was worth noting that the expression of *AVP1* is not regulated in RD26 OX

lines. All these findings were consistent with the RT-PCR results of our study (Table I). The other 499 genes were enriched in postembryonic development, ion transport, response to cold, and oxidative stress. These findings may provide evidence to clarify the similarities and differences between the OX lines of *TsNAC1* and *RD26* in plant development and stress resistance.

The promoter and the coding sequence regions of *AVP1* and *TsVP1* have approximately 75% similarity. Five RD26-targeted motifs are observed in the *AVP1* promoter, whereas three TsNAC1-targeted motifs are observed in the *TsVP1* promoter (Supplemental Table S3). Both motifs and promoters are differential, which might have caused the discrepancy in response to salt stress. In the *T. halophila* OX lines, the TsNAC1 target genes presented altered expression levels, with *MSI4*, *TsVP1*, etc., showing up-regulated expression and *LSH1* and *TXT2* showing down-regulated expression. These data suggest that there may be different regulation patterns of TsNAC1 based on the distribution of the target motifs. Additional work is required to determine whether polymorphisms of the regulatory mechanisms occur. TsNAC1 appears to play similar but not identical roles in *T. halophila* and *Arabidopsis* for the regulation of plant growth and responses to abiotic stressors.

CONCLUSION

Plants from TsNAC1 overexpression lines showed higher survival rates under abiotic stress and grew slowly under normal conditions. The ChIP-Seq data offer evidence that the overexpression of TsNAC1 up-regulates the genes involved in the response to abiotic stresses and ion transport, thereby improving stress resistance, and negatively regulates the genes involved in plant vegetative growth, thereby reducing cell expansion (Fig. 6). TsNAC1 regulates target genes with the CA(T/A)G consensus sequence, which differs from the results for RD26. In the model extremophile plant *T. halophila*, TsNAC1 is a crucial upstream regulator for abiotic stress resistance and cell expansion.

MATERIALS AND METHODS

One-Hybrid Yeast Screening of *Thellungiella halophila* cDNA Libraries

Six-week-old *T. halophila* wild plants, exposed to a 12-h high-salt stress treatment, were used to construct the cDNA library. The extraction of mRNA was performed with the Oligotex mRNA Mini Kit (Qiagen). Long-distance PCR (LD-PCR) was executed by the PCR cDNA Synthesis Kit (SMART). All the fragments were linked to the vector pGADT7-AD and transformed into yeast strain Y187 (Invitrogen) following the Yeast Protocols Handbook (Clontech). The transformed strains were cultured on SD/-Leu medium. Yeast plasmid was extracted and amplified with the T7 Sequencing Primer and the 3' AD Sequencing Primer on plasmid; most fragments were from 700 to 2,000 bp. Considering the stability of the specific binding between the protein and the DNA sequence, 3× tandem-repeated 530-bp DNA fragments of the *TsVP1* promoter (−667 to −138 bp) were cloned into pHis2.1 as bait. The bait vector was transformed into the cDNA library and cultured on SD/-His/-Trp with 5 mM 3-aminotriazole. The plasmid of the surviving strains was extracted and amplified with the T7 Sequencing Primer and the 3' AD Sequencing Primer. The amplicons were transformed into *Escherichia coli* and sequenced at BioSune.

Binding Active Assay of TsNAC1 and RD26 in the Yeast System

The coding sequences of *TsNAC1* and *RD26* were obtained by PCR, and the products were digested by *EcoRI/SmaI* and *BamHI/SacI*, then connected into the pGADT7-AD vector, which containing the GAL4 AD. The promoters of *TsVP1* and candidate target genes were cloned into the *EcoRI/KpnI* sites of pLacZi or the *EcoRI/SpeI* sites of pHis2.1. Plasmids were transformed in the yeast strain YM4271 (Invitrogen) in pairs. The β-galactosidase activities were examined by X-gal staining and measured as described in the Yeast Protocols Handbook (Clontech) using ONPG as the substrate.

Vector Construction and Transformation

The full-length cDNA of *TsNAC1* was ligated into the vector pCambia1300 that contains hygromycin as the selective marker. The specific fragment (418–882 bp) of *TsNAC1* was reorganized into the vector pFGC-5941 to interfere with the expression of *TsNAC1*, and *bar* was used as the selective marker. Vectors were introduced into *T. halophila* upon *Agrobacterium tumefaciens* GV3101-mediated transformation by the floral dip method. *T. halophila* seeds were planted in peat:vermiculite (2:1) and grown at 24°C under long-day conditions (16 h of light and 8 h of dark) with a relative humidity of 50% for 6 weeks. The plants were transformed to 2°C to 4°C for 5 weeks of vernalization and then put back to 24°C to enter the mature period.

Polyclonal Antibody of TsNAC1

First, the full-length *TsNAC1* was cloned and connected into pGEX-4T-1, and then the integrated plasmid was transformed into BL21 strain. The prokaryotic expression strain was cultivated, and the expression of protein NAC-GST was induced as described in “Molecular Cloning: A Laboratory Manual III” (Sambrook and Russel, 2001). Purification of the soluble NAC-GST protein was performed by the Chelating Sepharose Fast Flow (GE Healthcare). Preparation of the polyclonal antibody was accomplished by Abmart. The titer of the antibody was over 5×10^4 IU.

EMSA

Probes were generated using the DIG Gel Shift Kit (Roche). The mutated competitor of the 130-bp key region in the promoter of *TsVP1* was the sequence between PT7 and PT8 (−667 to −538 bp), which region was reported with no salt response (Sun et al., 2010) and sited beside the 130-bp key region.

Plant Performance under Different Treatments in the Greenhouse

T. halophila seeds were planted in peat:vermiculite (2:1) and grown at 24°C under long-day conditions (16 h of light and 8 h of dark) with a relative

humidity of 50%. Different treatments were performed after 6 weeks. Uniform seedlings of transgenic plants and the wild type were sprayed with 0.1 mL of 3 mmol L^{−1} paraquat for every pot to mimic the active oxygen stress treatment. In the low-temperature stress treatment experiments, transgenic plants were transferred into the other culturing room at −4°C and with greater than 3,500 lx light intensity for 48 h. These plants were watered with 50 mL of 18% PEG6000 solution for every pot to mimic the drought stress and with an additional 10 mL every 2 d. These transgenic lines were watered with an 800 mM NaCl solution and then watered with 10 mL of a 400 mM NaCl solution every 3 d to keep the 600 mM NaCl concentration of culture medium for 2 weeks. The hormone treatment was performed by spraying and watering the 100 μM ABA or 100 μM MeJA, 1 mL for each pot.

Plant RNA Extraction and Real-Time RT-PCR

Total RNA was extracted by Trizol (Takara) reagent. An amount of 500 ng of total RNA was used for inverse transcription with the Transcript First Strand cDNA Synthesis Kit (Takara). The cDNA was diluted 10 times and then used as the template for real-time RT-PCR. The RT-PCR system was as follows: SYBR Premix Ex Taq (2×), 5 μL; PCR Forward Primer (10 μM), 0.2 μL; PCR Reverse Primer (10 μM), 0.2 μL; cDNA template, 1 μL, with distilled deionized water added up to 10 μL. The PCR conditions were as follows: incubate for 3 min at 95°C, followed by a total of 40 cycles of 15 s at 95°C, 30 s at 58°C, and 30 s at 72°C.

Luciferase Assay

Promoter fragments in the pGreenII 0800:Luc vector were cotransformed with the pSoup helper plasmid into *A. tumefaciens* GV3101 and injected together with 35S:*TsNAC1* or 35S:*RD26* into tobacco (*Nicotiana tabacum*) leaves. The isolation of *T. halophila* protoplast and transformation were performed according to the protocol described previously (Yoo et al., 2007; Wu et al., 2009). Cells were harvested 18 h posttransfection, and luciferase activity (Luc/Rluc) was measured after cell lysis using the Double-Luciferase Reporter Assay Kit (TransDetect).

ChIP and ChIP-qPCR

The details of ChIP were as described by Kaufmann et al. (2010). The sequencing platform was Illumina HiSeq 4000. Aligner and parameter were Clean Parameter soap_mm_gz -p 4 -v 5 -s 35 -m 0 -x 600. Peak caller and parameter were Peak Calling Parameter macs14 -g 411831487 -p 1e-5 -w -space 50 -m 10,30. Independent lines OX7 and OX10 were used for the ChIP-qPCR assay, and all samples were diluted to 10 ng μL^{−1} and reacted with the following system: SYBR Premix Ex Taq (2×), 5 μL; PCR Forward Primer (10 μM), 0.2 μL; PCR Reverse Primer (10 μM), 0.2 μL; cDNA template, 1 μL, with distilled deionized water added up to 10 μL.

The primers used to amplify the enriched region of the target genes are listed in Supplemental Table S4. All the target genes of *TsNAC1* are listed in Supplemental Table S5. The raw data of ChIP-Seq were uploaded to the data-sharing platform figshare: https://figshare.com/projects/Exploration_of_the_target_genes_of_TsNAC1/25135

Transcript Activation Activity Assay

The C-terminal AD (164–305), the AD with the repression domain (105–305), and the full-length transcription factor domain (1–305) of *TsNAC1* were connected into the *EcoRI* and *PstI* sites of pGBKT7-BD. The C-terminal AD (181–314), the AD with the repression domain (122–314), the full-length transcription factor domain (1–314), and the point mutant (R126H) of *RD26* also were inserted and connected into the *BamHI* and *PstI* sites of pGBKT7-BD vector. Plasmids were transformed in the yeast reporter strain YRG-2 (Agilent Stratagene). The β-galactosidase activities were examined by X-gal staining and measured by ONPG assay.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *TsNAC1*(GenBank: AK352535.1).

Supplemental Data

The following supplemental materials are available.

- Supplemental Figure S1.** Evolutionary relationships of TsNAC1.
- Supplemental Figure S2.** Expression pattern analysis of *TsNAC1* in wild-type plants of *T. halophila*.
- Supplemental Figure S3.** Abiotic stress treatment comparison between the OX, wild-type, and NR lines.
- Supplemental Figure S4.** Validity of the TsNAC1 antibody.
- Supplemental Figure S5.** Determination of the core binding motif.
- Supplemental Figure S6.** Comparison of growth parameters in root 5 d after sowing.
- Supplemental Figure S7.** Differences of phenotype and flowering time among the different lines.
- Supplemental Figure S8.** Biological process GO analysis.
- Supplemental Table S1.** Different sites between the promoters of TsNAC1 and RD26.
- Supplemental Table S2.** Candidate target genes of TsNAC1 on other abiotic stresses.
- Supplemental Table S3.** Distribution of motifs in the promoters of target genes.
- Supplemental Table S4.** Primers used in this study.
- Supplemental Table S5.** Candidate target genes of TsNAC1 in *T. halophila*.
- Supplemental Data S1.** TsNAC1 peptide sequence.
- Supplemental Data S2.** Promoter sequence of TsVPI.

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