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Research article

# Overexpression of the wheat NAC transcription factor *TaSNAC4-3A* gene confers drought tolerance in transgenic *Arabidopsis*

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ABSTRACT

NAC transcription factors (TFs) play critical roles in plant abiotic stress responses. However, information on the roles of NAC TFs is limited in wheat (*Triticum aestivum* L.). In this study, we isolated three wheat *TaSNAC4* homeologous genes, *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D*, and characterized the function of *TaSNAC4-3A* in plant drought tolerance. *TaSNAC4* is highly expressed in seedling leaves, and expression is induced by various abiotic stresses. Transient expression and transactivation assays showed that TaSNAC4-3A is localized to the nucleus, and the C-terminal region has transcriptional activation activity. Overexpression of *TaSNAC4-3A* in *Arabidopsis* led to stimulated germination and root growth when exposed to salt and osmotic stresses, and drought stress tolerance was significantly increased in the *TaSNAC4-3A* transgenic lines. When compared to the control plants, the transgenic lines overexpressing *TaSNAC4-3A* exhibited reduced stomatal aperture size under drought stress, and therefore had lower water loss rates. In addition, the overexpression of *TaSNAC4-3A* led to abscisic acid (ABA) hypersensitivity at the root elongation and seed germination stages. Further transcriptomic analysis demonstrated that there was a significant up-regulation of stress responsive genes in the *TaSNAC4-3A* transgenic lines. Our findings have revealed the important role of *TaSNAC4-3A* in plant drought tolerance.

## 1. Introduction

Over the past several decades, drought has become a severe environmental problem due to global climate change, resulting in a 13.7% average loss in worldwide cereal production (Pennisi 2008; Lesk et al., 2016). Drought tolerance involves many factors, making it a particularly complex phenotype in plants. At present, hundreds of genes that control key processes in the response to drought stress have been identified including signal perception, signal transduction, and the transcriptional regulation of genes necessary for drought tolerance. This includes genes for protein kinases and transcription factors (TFs), such as members of the bZIP, NAC, and AP2/ERF TF families (Nakashima et al., 2012; Hu and Xiong, 2014). TFs also activate a range of stress-related genes that function in the regulation of diverse physiological and biochemical responses to drought stress (Hu and Xiong, 2014; Gahlaut et al., 2016). All of this makes TFs worthy of further study for the genetic improvement of abiotic stress tolerance in crops.

NAC (NAM, ATAF1/2, and CUC2) proteins comprise one of the largest plant-specific TF families, and these proteins contribute to a range of regulatory and developmental processes. The NAC family is characterized by a conserved N-terminal DNA-binding domain (Ooka et al., 2003). NAC TFs play a variety of roles in regulating various abiotic stresses, and have been studied in a number of species of plants, such as *Arabidopsis*, rice, wheat, and soybean. The ectopic expression of *ANAC055*, *ANAC019*, or *ANAC072/RD26* is associated with increased tolerance to both drought and salt stress (Tran et al., 2004). ANAC016 positively regulates responses to drought stress and can improve drought tolerance by suppressing the expression of *ABA-RESPONSIVE ELEMENT-BINDING PROTEIN 1* (*AREB1*), which negatively regulates the ABA signaling pathway and is associated with stress responses (Sakuraba et al., 2015). ANAC096 can also positively regulate the drought stress response after it was found that it activates, along with several bZIP TFs, a subset of genes that are susceptible to stress by way of the ABA-dependent pathway (Xu et al., 2013). A certain subset of NAC TFs

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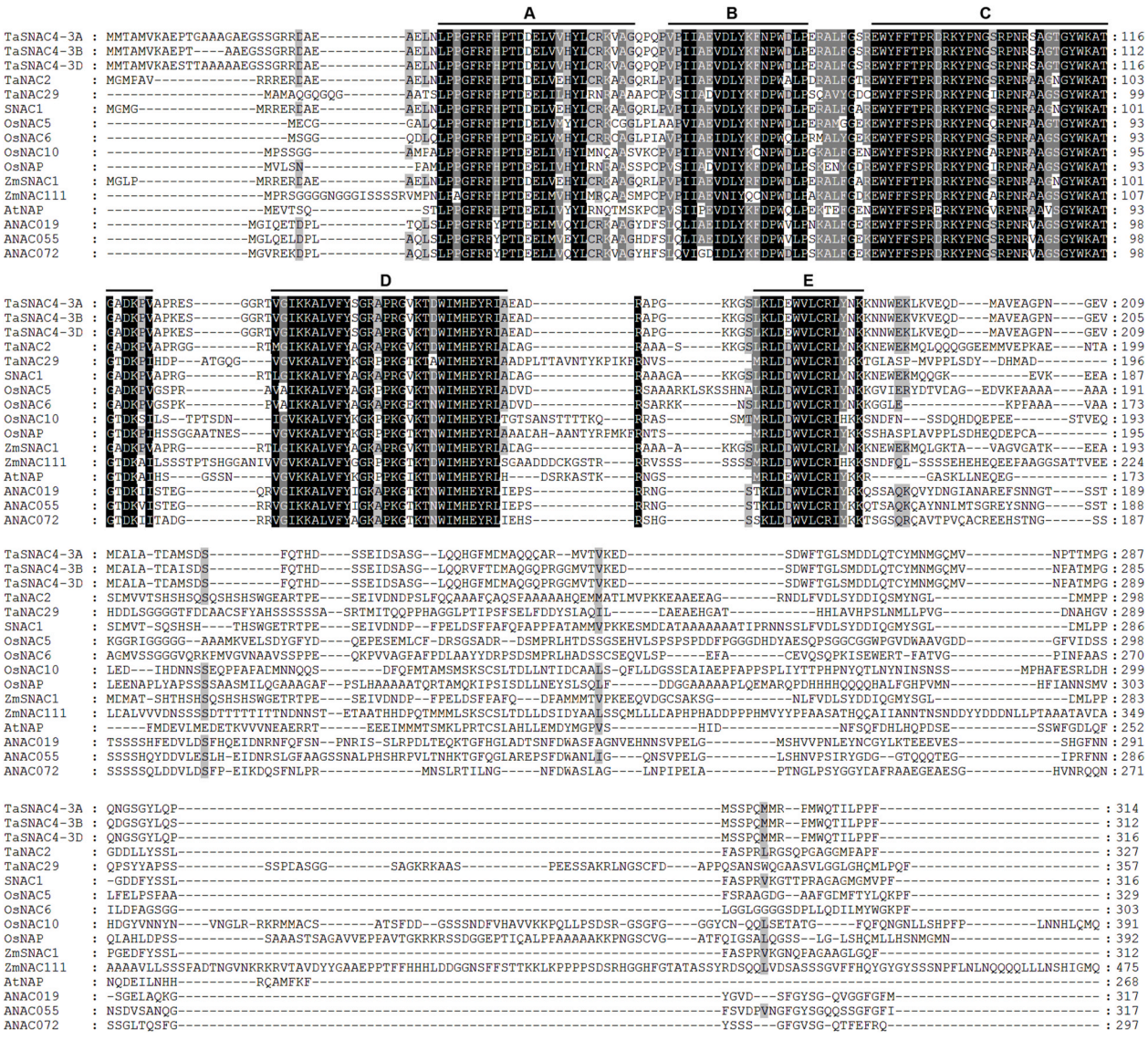


**Fig. 1. Sequence alignment of NAC domains from *TaSNAC4* proteins and NAC protein family members from other plant species.** Identical amino acids are shaded in black and similar amino acids are shaded in gray. The locations of the five highly conserved amino acid motifs (A–E) are indicated by black lines.

has been found to be involved in different stress responses that are related to abiotic factors in rice. Drought and salt tolerance have been shown to be significantly enhanced by the overexpression of *SNAC3*, *OsNAC2*, *OsNAC045*, *OsNAC10*, *OsNAC063*, *SNAC2/OsNAC6*, *OsNAC5*, or *SANC1/OsNAC9* in transgenic rice (Hu et al., 2006, 2008; Nakashima et al., 2007; Yokotani et al., 2009; Zheng et al., 2009; Jeong et al., 2010, 2013; Redillas et al., 2012; Fang et al., 2015; Shen et al., 2017). A few transgenic lines have developed increased drought tolerance. Under moderate to severe drought conditions, some transgenic lines had maintained or even increased grain yield (Hu et al., 2006; Jeong et al., 2010, 2013; Redillas et al., 2012). In wheat, The NAC TFs TaNAC2, TaNAC67, and TaNAC29 function in the responses to abiotic stress, and they improved tolerance to low temperature, high salinity, and drought stress when overexpressed in transgenic *Arabidopsis* (Mao et al., 2012, 2014; Huang et al., 2015). The expression of *GmNAC20* and *GmNAC11* can respond to various hormone treatments and abiotic stresses in soybean. In transgenic *Arabidopsis*, overexpression of *GmNAC20* increased tolerance to high salinity and low temperature, while overexpression of *GmNAC11* increased salt tolerance (Hao et al., 2011). Therefore, NAC TFs play important roles in plant responses to different abiotic stresses.

Common wheat (*Triticum aestivum*; 2n = 6x = 42; AABBDD) is an important cereal crop that is cultivated worldwide, especially in arid and semiarid regions (Jia et al., 2013; Ling et al., 2013). There are two allopolyploidization events that occurred during the evolution of hexaploid wheat. The first event was the hybridization of *T. urartu* (A genome donor, AA; 2n = 14) with *Aegilops speltoides* (B genome donor, SS; 2n = 14) to produce the allotetraploid species *T. turgidum* (AABB). The second event was the hybridization of the AABB donor with *A. tauschii* (D genome donor, DD; 2n = 14) to produce the allohexaploid wheat species *Triticum aestivum* (AABBDD) (Feldman et al., 2005; Marcussen et al., 2014). *T. aestivum* has a large (>17 Gb) and complex genome which makes genomic studies difficult, but due to wheat's global importance, extensive research has been performed to sequence and annotate its genome (Brenchley et al., 2012; Jia et al., 2013; Ling et al., 2013, 2018; Choulet et al., 2014; Luo et al., 2017; Zhao et al., 2017). More recent efforts have sequenced isolated chromosome arms and constructed a reference sequence of the hexaploid wheat genome (IWGSC 2018). This detailed genome sequence of the cultivar 'Chinese Spring' allowed Guérin et al. (2019) to identify 488 NAC family members in common wheat, subsequently finding that expression of many of these *NAC* genes is affected by different abiotic stresses. However, when compared with *Arabidopsis* and rice, there are fewer detailed studies of

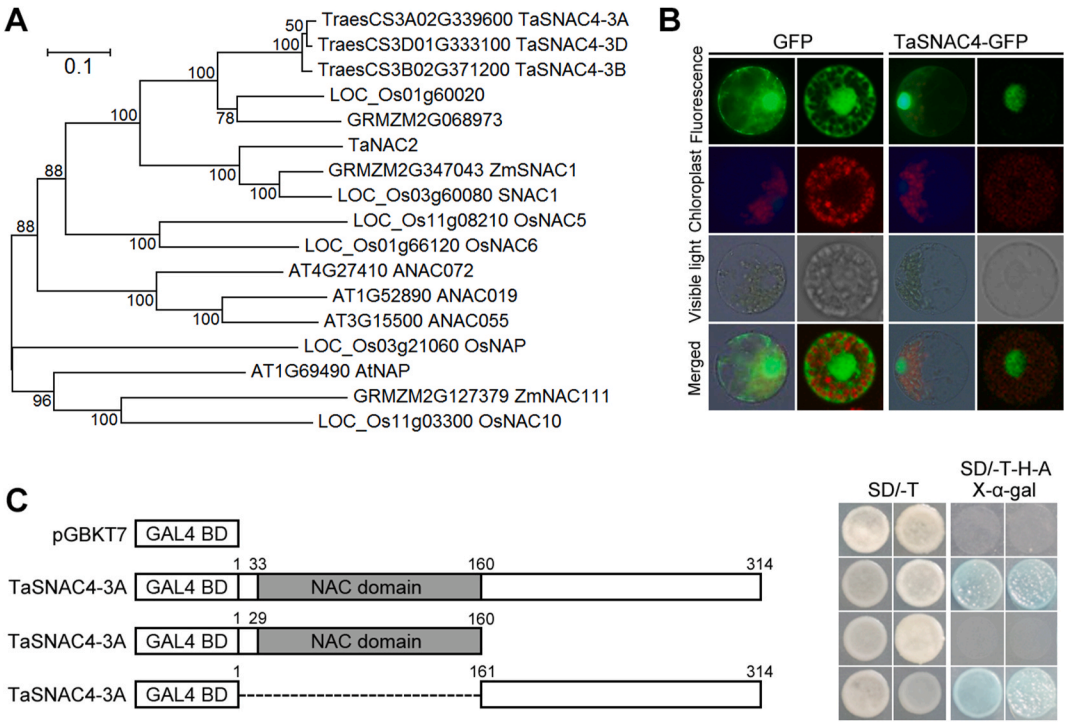


**Fig. 2. Phylogeny, subcellular localization, and transactivation activity of the TaSNAC4 homeologous proteins. (A)** Phylogenetic relationships between the three TaSNAC4 homeologous proteins and typical stress-responsive NAC proteins. The multiple sequence alignment was performed using ClustalW, and the phylogenetic tree was constructed using the neighbor-joining method as implemented in MEGA6.0. The numbers at each node are the percentage of 1000 bootstrap replicates that give the same branch and indicate the branch confidence. **(B)** Subcellular localization of TaSNAC4-3A. The right panels show the localization of GFP-TaSNAC4-3A in wheat protoplasts in a transient assay, while the left panels show the localization of GFP as a control. **(C)** Transactivation assay of the truncated TaSNAC4-3A proteins. The full-length *TaSNAC4-3A* gene and different regions were fused in frame with the GAL4 DNA-binding domain and then expressed in yeast strain AH109. The transformed yeast cells were plated and grown on control plates (SD/-Trp) or selective plates (SD/-Trp-His-Ade + X-α-gal). Photos were taken 3–6 days after inoculation, and the plates were cultured at 30 °C.

the NAC TF family in common wheat.

In this study, we screened and characterized three *TaSNAC4* homeologous genes, *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* from common wheat, and investigated the subcellular localization and transcriptional activation properties of the TaSNAC4-3A protein. Using quantitative real-time PCR (qRT-PCR), the expression patterns of *TaSNAC4* were analyzed in response to mannitol, salt, drought, and exogenous ABA treatments. We also measured the phenotypic and physiological characteristics of transgenic *Arabidopsis* plants overexpressing *TaSNAC4-3A* under drought, osmotic, and salt stress conditions, as well as the transcriptomic changes in transgenic *Arabidopsis* plants expressing *TaSNAC4-3A*. This study contributes to an increased understanding of the structure and function of *TaSNAC4*.

## 2. Materials and methods

### 2.1. Plant materials and abiotic stress treatments

The wheat *cv*. Chinese Spring was used to analyze the expression of *TaSNAC4*. The seeds were surface sterilized with 75% ethanol for 3 min and then washed in sterile deionized water. They were then allowed to germinate for three days on wet filter paper at 25 °C. We then placed the germinated seeds in a nutrient solution (0.75 mM $K_2SO_4$, 0.1 mM KCl, 0.25 mM $KH_2PO_4$, 0.65 mM $MgSO_4$, 0.1 mM EDTA-Fe, 2.0 mM $Ca(NO_3)_2$, 1.0 mM $MnSO_4$, 1.0 mM $ZnSO_4$, 0.1 mM $CuSO_4$, 0.005 mM $(NH_4)_6Mo_7O_{24}$) for hydroponic cultivation in a growth chamber at 16 °C under a 16-h light/8-h dark photoperiod. At the three-leaf stage, the seedlings were separately subjected to four different treatments that included high salinity, osmotic stress, drought, and ABA. The seedlings were cultured in solutions containing 20% PEG to simulate drought conditions, 100 mmol/L NaCl for high salt conditions, 100 mmol/L mannitol for osmotic stress, and 100 μmol/L ABA for the ABA treatment. We collected the leaves and roots from a minimum of three seedlings at 0, 1, 3, 6, 12, and 24 h of treatment. We used field-grown wheat plants (*cv*. Chinese Spring, from Northwest A&F University, China) to measure the organ-specific expression patterns of *TaSNAC4* in the roots, stems, and leaves of seedlings at the five-leaf stage, the young spike at the early booting stage, the spike and the flag leaf at the heading stage, and the grain at 5 and 10 DPA (days past anthesis). We collected the tissue samples from at least five different plants, three replicates each, and immediately froze the samples in liquid nitrogen at −80 °C prior to RNA extraction.

### 2.2. RNA extraction and quantitative RT-PCR

We isolated and then purified total RNA using the Total RNA Rapid Extraction Kit for Polysaccharides Polyphenol Plant (BioTeke) according to the manufacturer's instructions. We treated the purified RNA with RNase-free DNase I (TaKaRa, China) to remove contaminating genomic DNA. We used recombinant M-MLV reverse transcriptase (Promega, USA) to synthesize first-strand cDNA using 1 μg of total RNA as template. We then used an ABI7300 Thermo-cycler (Applied Biosystems, USA) to perform qRT-PCR in optical 96-well plates. Each amplification reaction was performed in a 10 μl volume that contained 1 μl diluted cDNA, 5 μl SYBR Premix Ex Taq II (TaKaRa), and 200 nM gene-specific primers. The amplification conditions were as follows: an initial denaturation for 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. The exact amplicon for each pair of primers was confirmed using a melting curve analysis. For internal gene expression controls, we used the *Actin8* (At1g49240) gene for stress-responsive genes in

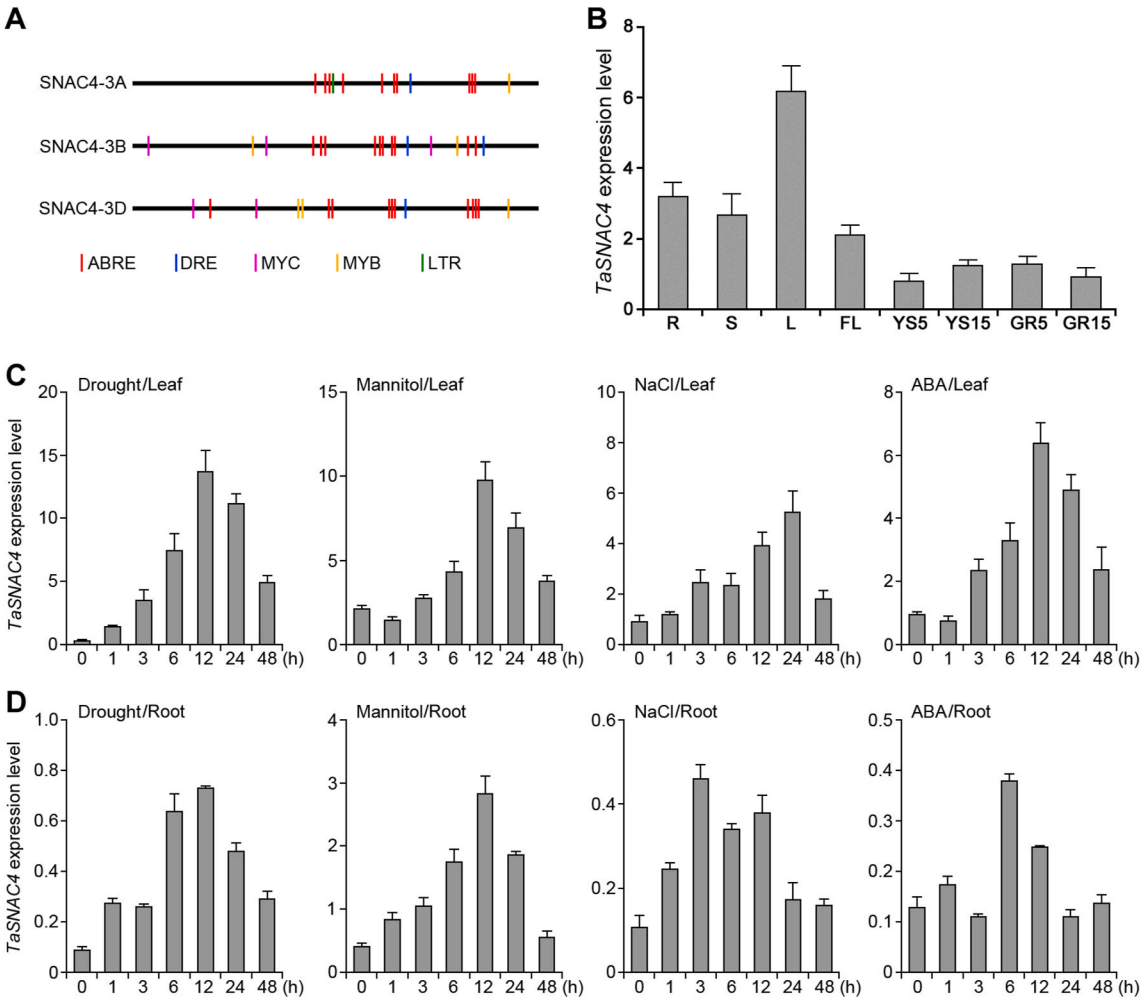


**Fig. 3. Expression profiles of *TaSNAC4* in wheat. (A)** Distribution of several stress-related *cis*-elements in the promoter regions (~1.5 kb upstream) of the *TaSNAC4* homeologous genes. ABRE, ABA-responsive element; DRE, dehydration-responsive element; MBS, MYB binding site involved in drought-inducibility; LTRE, low temperature responsive element; MYBRS, MYB recognition site; MYCRS, MYC recognition site. **(B)** Expression of the *TaSNAC4* genes in six different tissues at various wheat developmental stages. R, S, and L, seedling root, stem, and leaf sampled at the three-leaf stage, respectively; FL, flag leaf at the heading stage; YS5, young spike at the early booting stage; YS15, spike at the heading stage; GR5 and GR15, the grain at 5 and 15 days post-anthesis, respectively. **(C)** Relative gene expression levels of *TaSNAC4* genes in wheat leaves under drought, salt, mannitol, and ABA treatments. **(D)** Relative gene expression levels of the *TaSNAC4* genes in wheat roots under drought, salt, mannitol, and ABA treatments. The *TaActin* gene, which is expressed under a wide range of conditions, was used as the internal control for expression normalization. Both leaf and root tissues were collected at 0, 1, 3, 6, 12, 24, and 48 h of drought treatment. Data represent the mean ± SD of three replicates.

*Arabidopsis* and *TaActin* (*TraesCS1A01G274400*) for analysis of the expression of *TaSNAC4* in wheat. We calculated the relative gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), and estimated the variation in expression from the three different replicate samples. The names and sequences of the primer pairs for the qRT-PCR analyses are given in Table S1.

### 2.3. Multiple sequence alignments and phylogenetic analysis

We performed a phylogenetic analysis on the full-length protein sequences of the TaSNAC4 homeologs and NAC TF proteins, characterized by their functions, from various species of plants. The phylogenetic tree was created using MEGA (v6.0) software by the Neighbor-Joining (NJ) algorithm with 1000 bootstrap re-samplings. Multiple sequence alignments were performed using ClustalW software, and were manually edited using BioEdit (v7.1).

### 2.4. Transcriptional activation assay of TaSNAC4-3A

To analyze the transactivation of TaSNAC4-3A in yeast cells, we used bait vectors to transform the yeast strain AH109 (Clontech). Three pairs of primers (Table S1) were used to separately amplify the full-length cDNA, the N-terminal region, and the C-terminal region, and the PCR products were cloned to the GAL4 binding domain vector pGBKT7, as directed by the manufacturer (Clontech). For a negative control, we used the empty pGBKT7 vector. We adjusted the yeast cell concentrations to an $OD_{600}$ of 0.1, and plated them on SD/-Trp and SD/-Trp-His-Ade + X-α-gal media to compare the survival rates. We then incubated the plates for 3–6 days at 30 °C, after which they were photographed.

### 2.5. Subcellular localization of the TaSNAC4-3A-GFP fusion protein

Using specific primer pairs (Table S1), we amplified the full-length cDNA sequence of *TaSNAC4-3A* by PCR, and cloned it into the binary vector pCAMV35S::GFP between the *Xba*I and *BamH*I sites in order to identify the subcellular localization of the TaSNAC4-3A protein. We identified the positive clones by DNA sequencing, and the construct was introduced into wheat mesophyll protoplasts as previously described (Yoo et al., 2007). We observed the GFP fluorescence using a confocal microscope (Olympus, FluoViewTM FV300, Japan). The promoter

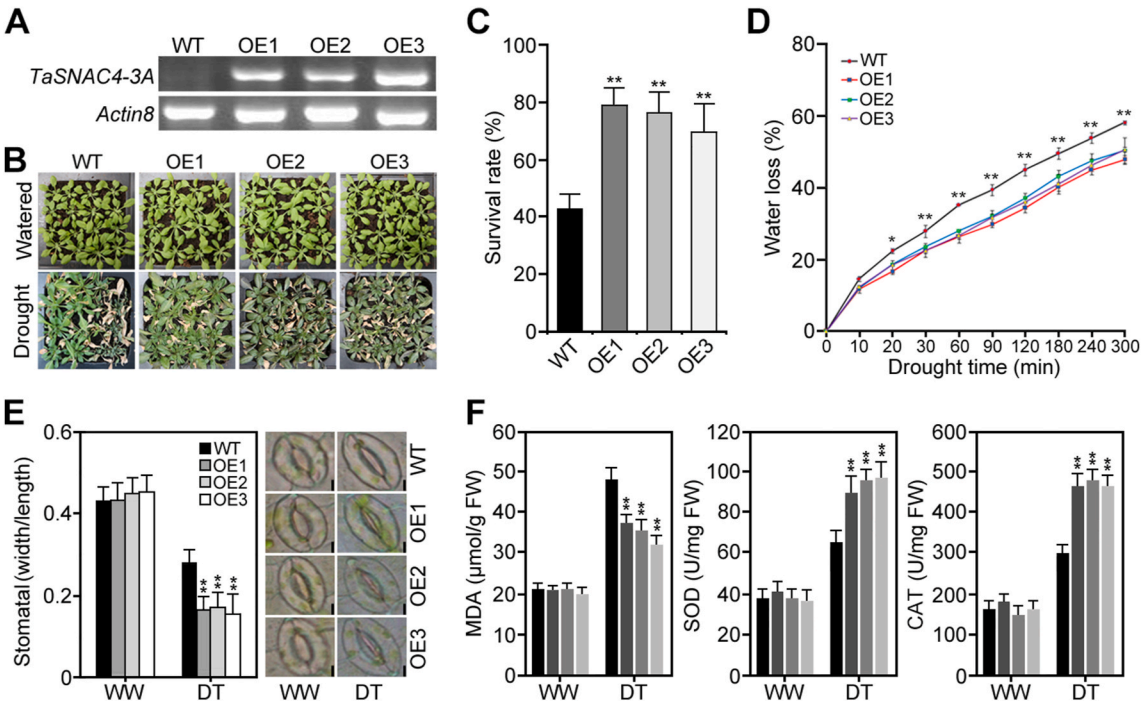


**Fig. 4. Drought stress tolerance of *TaSNAC4-3A* overexpressing transgenic *Arabidopsis* plants. (A)** RT-PCR identification of the *TaSNAC4-3A* overexpressing *Arabidopsis* lines. **(B)** Performance of transgenic *Arabidopsis* plants overexpressing *TaSNAC4-3A* under drought stress. **(C)** Statistical analysis of survival rates after the drought-stress treatment. The average survival rates and standard errors were calculated based on data obtained from four independent experiments. Each experiment comprised 48 plants for each line. **(D)** Water loss from detached rosettes of the control and transgenic *TaSNAC4-3A* overexpressing plants. Values are means from eight plants for each of three independent experiments. **(E)** Stomatal aperture width/length ratios calculated from plants after 10 days under dehydration conditions. Values are means ± SD (n = 40) in the assays. WW, well-watered; DT, drought. **(F)** MDA contents and SOD and CAT activities in control and *TaSNAC4-3A* overexpression plants before and after drought stress. Values represent mean ± SD of three replicates. Asterisks indicate significant differences from the control based on Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

regions (~1500 bp upstream of the start codon) of the *TaSNAC4* homeologs were used to search for stress-related *cis*-acting elements as previously reported (Yamaguchi-Shinozaki and Shinozaki, 2005).

### 2.6. Generation of transgenic Arabidopsis plants overexpressing TaSNAC4-3A

The full-length opening reading frame of *TaSNAC4-3A* was amplified from cDNA of wheat *cv*. Chinese Spring using gene-specific primers (Table S1). It was then cloned into the pGreen vector that carries the cauliflower mosaic virus (CaMV) *35S* promoter between the *Not*I and *Xho*I sites. We transformed the recombinant vector (*35S::TaSNAC4-3A*) into *Agrobacterium tumefaciens*, and the recombinant strain was used for transformation of *Arabidopsis thaliana* ecotype Columbia by the floral dip method (Clough and Bent, 1998). We confirmed three homozygous transgenic lines (OE1, OE2, and OE3) using qRT-PCR and used them in the experiments.

### 2.7. Germination and green cotyledons assay

In order to analyze the germination rate, we sowed 100 surface-sterilized seeds on solid 0.5 × MS (Murashige Skoog) salts medium supplemented separately with 150 mM mannitol, 150 mM NaCl, and 1 μM ABA. The seeds were first vernalized at 4 °C in the dark for three days and then incubated at 22 °C with a 16-h/8-h light/dark cycle. Seed germination rates were calculated as cotyledon greening. For the experiment studying seedling root length, 5-day-old seedlings were cultivated on solid 0.5 × MS medium supplemented separately with 1 μM ABA, 100 mM mannitol, or 100 mM NaCl. After 14 days of vertical culture, the primary root lengths were measured in each treatment.

### 2.8. Drought tolerance assay

We transferred 7-day-old seedlings of the*35S::TaSNAC4-3A* transgenic lines and WT from MS medium to pots (16 plants per pot) containing 230 g of a 2:1 ratio of nutrient soil and vermiculite for the drought tolerance assays. We then exposed 21-day-old plants growing in a favorable water environment (a relative humidity of 60%, a photoperiod of 16-h/8-h light/dark, and a temperature of 22 °C) to drought stress. Water was withheld from the plants for about 14 days, at which point they were watered again and allowed to recover. The number of surviving plants was recorded six days later. We compared at least 48 plants from each transgenic line with the WT plants in each test. The data used for statistical analyses were obtained from four independent experiments, and we used Student's *t*-test to analyze the difference between the transgenic and WT plants.

### 2.9. Water loss measurement

Detached leaves drawing from 3-week-old *35S::TaSNAC4-3A* transgenic and wild-type plants were used to measure the water loss rates. They were weighed immediately (fresh weight, FW) once detached and then left on the laboratory bench at 22–24 °C with a relative humidity of 40–45%, and the desiccated weights were recorded at predetermined times. We then oven-dried the plants at 80 °C for 24 h to obtain a constant dry weight (DW). The water loss rate was considered to be the percentage of the initial fresh weight and the weight at each weighing. We weighed three replicates for each line. We then peeled the leaf epidermis off both the WT and transgenic plants to examine the stomatal apertures, and this was done both before and after the drought stress treatment using an Olympus IX71 inverted microscope (Olympus Corporation, Tokyo, Japan). We calculated the stomatal length to width

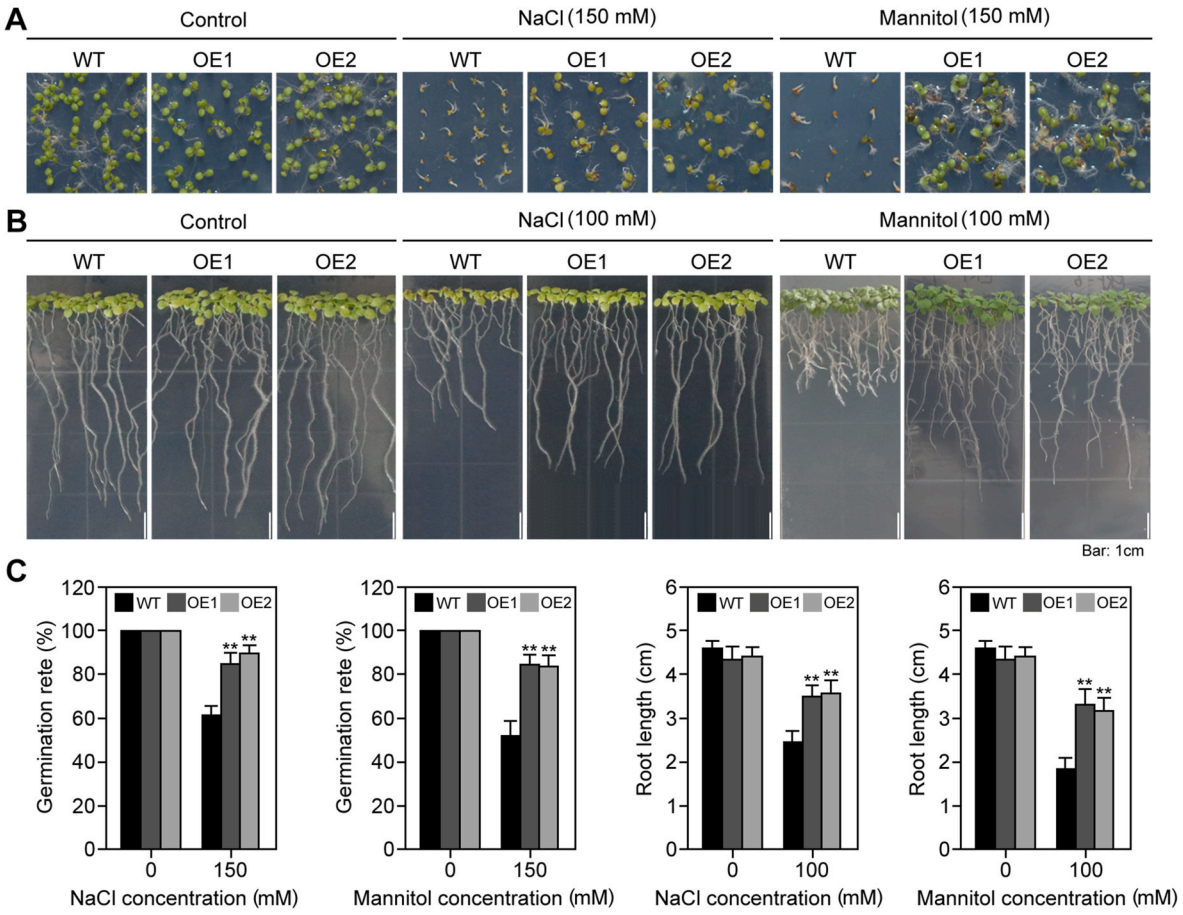


**Fig. 5. Comparison of germination rate and root length between *TaSNAC4-3A* overexpressing and control plants. (A)** Seed germination assay in the *TaSNAC4-3A* overexpression and control lines. The surface-sterilized *Arabidopsis* seeds were sown on 0.5 × MS solid medium supplemented with either 150 mM NaCl or 150 mM mannitol and incubated at 22 °C for seven days. **(B)** Root length assay in the *TaSNAC4-3A* overexpression and control lines. Five-day-old *Arabidopsis* seedlings were cultured vertically on 0.5 × MS solid medium supplemented with either 100 mM NaCl or 100 mM mannitol for 14 days. **(C)** Statistical analyses of germination rates and primary root lengths in the NaCl and mannitol treatments. Data were scored from three independent experiments. Values represent mean ± SD of three replicates. Asterisks indicate significant differences from the control based on Student's *t*-test (*$P < 0.05$; **$P < 0.01$).

ratio to obtain the stomatal aperture using IMAGE J software (v1.7). To analyze the difference between transgenic and wild-type plants, we used Student's *t*-test.

### 2.10. Antioxidant enzyme behavior and MDA measurement

For the WT and transgenic plants that underwent drought stress treatment, we measured the physiological characteristics related to antioxidation. We collected leaf samples from the transgenic and control plants under both normal and drought conditions and used detection kits (Solarbio) to measure the SOD and CAT activities as well as the MDA contents as directed by the manufacturer.

### 2.11. Transgenic Arabidopsis RNA-sequencing

Three-week-old *35S::TaSNAC4-3A* and WT *Arabidopsis* seedlings grown under normal conditions were harvested for RNA-sequencing. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Evaluation of the quality and quantity of the RNA was performed on an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). The extracted RNA samples were incubated with RNase-free DNase I (New England Biolabs) at 37 °C for 30 min to remove the residual DNA. Library preparation from the resulting DNA-free RNA was performed using the TruSeq paired-end mRNA-Seq kit, and RNA-seq libraries were sequenced on the Illumina Hiseq-2500 platform. The clean reads were mapped to the *Arabidopsis* reference genome (TAIR10). Differential gene expression was determined using the Tuxedo RNA-seq analysis pipeline (Trapnell et al., 2012, 2013). We performed an analysis of the enrichment of the gene ontology of biological pathways (GOBPs) using the DAVID software program (Huang et al., 2009). From this, we obtained *P*-values that categorized the significance of each GOBP, which are represented by the genes. We considered GOBPs with $P < 0.01$ to be enriched processes.

### 2.12. Statistical analyses

Each experiment was repeated at least three times. We presented and analyzed the data after assessing the mean ± standard deviation (SD) of the individual replicates. We used Student's *t*-test to analyze of statistical differences; $P < 0.05$ is considered to be statistically significant and $P < 0.01$ extremely significant.

### 2.13. Accession numbers

The RNA-seq raw reads have been deposited in the SRA under accession numbers SRR10120513-SRR10120515.

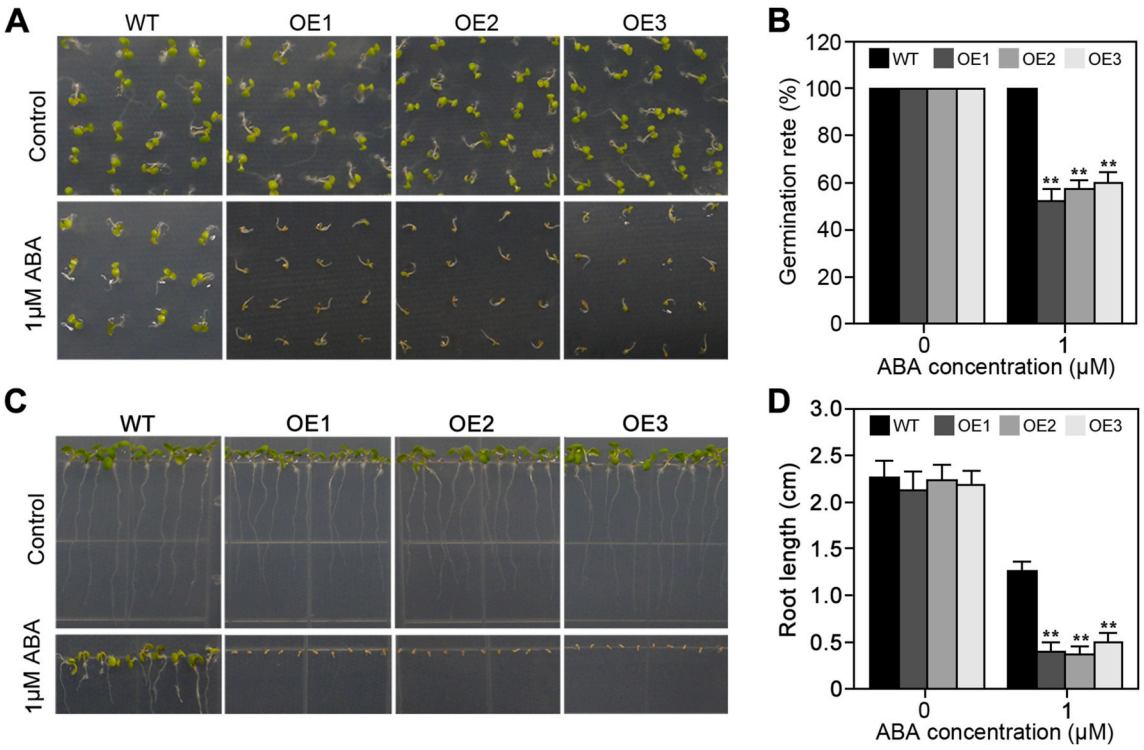


**Fig. 6. Response of *TaSNAC4-3A* overexpressing plants to exogenous ABA. (A)** Seed germination assay in *TaSNAC4-3A* overexpressing and control plants. The surface-sterilized *Arabidopsis* seeds were sown on 0.5 × MS solid medium supplemented with 0 or 1 μM ABA and incubated at 22 °C for seven days. **(B)** Germination rates of seeds of the *Arabidopsis* control and the *TaSNAC4-3A* overexpression lines under ABA treatment. **(C)** Root length assay of the *TaSNAC4-3A* overexpressing and control plants. Five-day-old seedlings were cultured vertically on 0.5 × MS solid medium supplemented with 0 and 1 μM ABA for seven days. **(D)** Quantification of primary root length in seedlings of *Arabidopsis* control plants and *TaSNAC4-3A* overexpressing lines under ABA treatment. Values represent the mean ± SD of three replicates. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) between transgenic lines and the control based on Student's *t*-test.

## 3. Results

### 3.1. *TaSNAC4* homeologous genes encode NAC domain-containing proteins

Three *TaSNAC4* homeologous genes *TaSNAC4-3A* (*TraesCS3A02G339600*), *TaSNAC4-3B* (*TraesCS3B02G371200*), and *TaSNAC4-3D* (*TraesCS3D02G333100*) were identified in our previous study (Mao et al., 2020). In this study, we cloned three full-length genomic DNA sequences of the *TaSNAC4* homeologous genes from wheat *cv*. Chinese Spring using gene-specific primers. We then used these full-length genomic DNA sequences to clone the coding regions (CDSs) of the three *TaSNAC4* homeologous genes. Sequence analysis showed that *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* share the same gene structures, with three exons and two introns. The *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* ORFs are 945 bp, 939 bp, and 951 bp in length, encoding predicted proteins of 314, 312, and 316 amino acids, respectively (Figure S1). *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* have 95–97% homology at the nucleotide level and 94–97% homology at the amino acid level.

Analysis of the amino acid sequences revealed that the three *TaSNAC4* homeologous proteins have theoretical molecular weights of 34.82–35.13 kDa, while analysis of the conserved domains showed that a highly conserved NAC domain that consists of five subdomains, A-E, is present in the N-terminal regions (amino acids 33–160 for TaSNAC4-3A, 29–156 for TaSNAC4-3B, and 33–160 for TaSNAC4-3D). The C-terminal regions showed no significant similarity to other members of the NAC family (Fig. 1). Further phylogenetic analyses indicated that TaSNAC4-3A, TaSNAC4-3B, and TaSNAC4-3D show affinity with the stress-responsive NAC (SNAC) subfamilies (Nakashima et al., 2012) and are most closely related to the rice LOC_Os01g60020 and maize GRMZM2G068973 proteins (Fig. 2A).

### 3.2. *TaSNAC4-3A* is a nucleus-localized transcriptional activator

In order to test the subcellular localization of TaSNAC4-3A, we constructed an expression cassette in which the GFP protein is fused in-frame with TaSNAC4-3A. This fusion protein was then transiently expressed in wheat protoplasts, and the pCAMV35S::GFP empty vector was used as a control. We examined the transfected wheat protoplasts using fluorescence microscopy, and found that the TaSNAC4-3A-GFP fusion protein is exclusively localized to the nucleus of the protoplasts, whereas the control GFP protein was distributed uniformly throughout the cell (Fig. 2B). This result confirmed that TaSNAC4-3A is a nuclear-localized protein.

In order to examine whether the TaSNAC4-3A protein possesses any activity relating to transactivation, we separately fused different parts of TaSNAC4-3A with the GAL4 DNA-binding domain in the pGBKT7 vector, and these constructs were separately transformed into the yeast strain AH109. For each construct, the transformants grew wells on selective SD medium lacking tryptophan (SD/-Trp). Yet on SD medium without tryptophan, histidine, and adenine (SD/-Trp-His-Ade), only transformants carrying the full-length TaSNAC4-3A protein or the C-terminal domain were able to grow. Transformants carrying the N-terminal domain or the pGBKT7 vector did not grow on SD/-Trp-His-Ade medium. The transformed yeast cells carrying the full-length TaSNAC4-3A and its C-terminal domain turned blue in the presence of X-α-gal. This indicates a significant level of *β*-galactosidase activity (Fig. 2C). Based on these findings, we believe that TaSNAC4-3A is a nuclear-localized transcriptional activator, and that its transactivation domain is located in the C terminus.

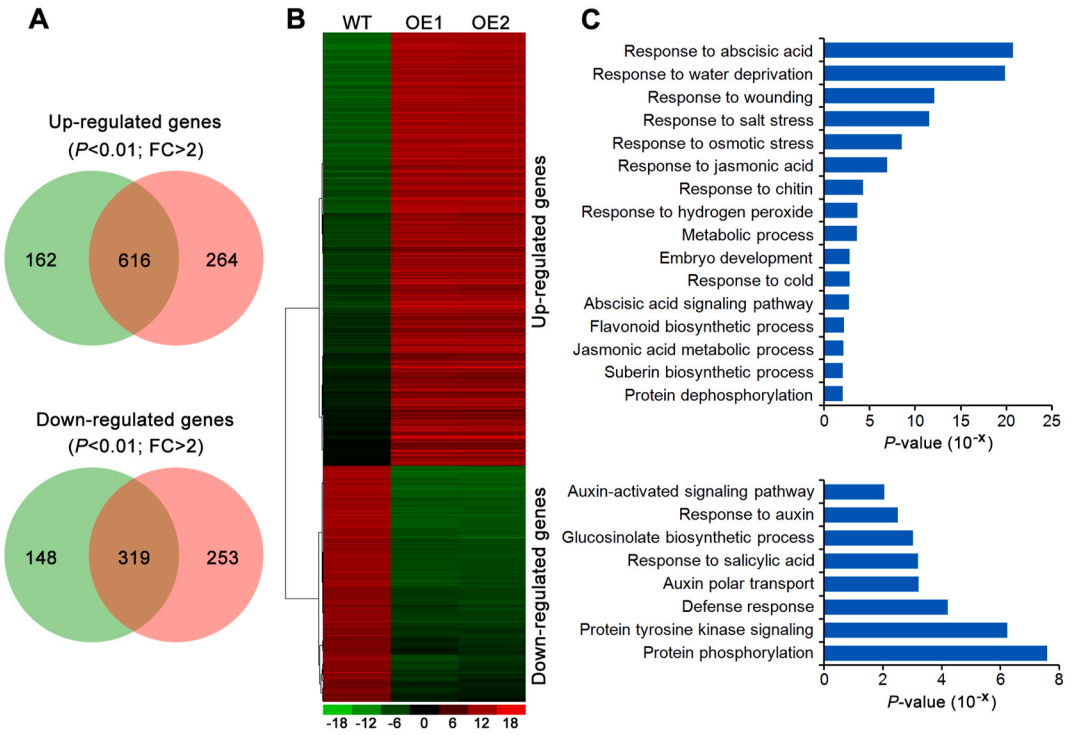


**Fig. 7. Transcriptomic analysis of *TaSNAC4-3A* overexpressing transgenic *Arabidopsis* plants under normal conditions. (A)** Venn diagrams of up- or down-regulated genes in plants of *TaSNAC4-3A* overexpressing lines OE1 and OE2 relative to control plants using a significance cutoff of $P < 0.01$ and a fold-change (FC) > 2. **(B)** Hierarchical clustering of differentially expressed genes in the *35S:TaSNAC4-3A* overexpression lines relative to the control plants. The indicated scale is the $\log_2$ value of the normalized gene expression. **(C)** Biological pathway enrichment analysis of up- or down-regulated genes in the *35S:TaSNAC4-3A* overexpression lines.

### 3.3. *TaSNAC4* expression is induced by abiotic stresses

To examine the expression pattern of *TaSNAC4*, we first scanned the promoter region (~1500 bp upstream of the start codon) of *TaSNAC4* homeologs to search for stress-related *cis*-acting elements. We identified a number of *cis*-acting elements in the promoter regions of the *TaSNAC4* homeologs, including DREs (Dehydration-responsive elements), ABREs (ABA-responsive elements), MBS (MYB binding site involved in drought-inducibility), MYCRSs (MYC recognition sites), MYBRSs (MYB recognition sites), and LTREs (low temperature responsive elements) (Fig. 3A). Following this, we examined the expression of the *TaSNAC4* genes in different organs of wheat plants under normal growth conditions. The cDNA sequences of *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* are highly conserved; therefore, the PCR primers were designed to amplify the homeologous alleles at a conserved locus. Our results show that the expression levels of *TaSNAC4* genes are high in the seedling roots, stems, and leaves, with the highest expression levels found in the leaves. In contrast, *TaSNAC4* genes are expressed at very low levels in the spikes and grains (Fig. 3B). We also examined the expression patterns of *TaSNAC4* genes in the roots and leaves of seedlings at the 3-leaf stage under various abiotic stresses, and found that the *TaSNAC4* genes were upregulated under all abiotic stress conditions, particularly in the leaves (Fig. 3C and D). After treating seedlings with 20% PEG6000 to mimic drought stress, the *TaSNAC4* genes transcript levels gradually increased, peaking at 12 h, after which a decrease was observed. Similar results were observed when plants were grown in the presence of mannitol, ABA or NaCl (Fig. 3C). These results indicate that expression of *TaSNAC4* genes responds to multiple stresses, but has a particularly strong response to drought stress. Therefore, we focused on the role of *TaSNAC4* genes in drought tolerance in this study.

### 3.4. *TaSNAC4-3A* overexpression confers tolerance to drought stress

Salt, mannitol, and drought all induced the expression of the *TaSNAC4* genes. This indicates that *TaSNAC4* genes could be involved in stress tolerance. In order to better understand how *TaSNAC4* genes function in plant abiotic stress tolerance, we generated transgenic *Arabidopsis* plants expressing the coding sequence of *TaSNAC4-3A* cloned from wheat *cv*. Chinese Spring. We then selected three independent transgenic lines in which *TaSNAC4-3A* was highly expressed to analyze their response to drought stress (Fig. 4A) by comparing drought tolerance in transgenic and control plants transformed with the empty vector. We grew the control and *TaSNAC4-3A*-expressing transgenic plants for three weeks in soil before withholding water for 14 days. Compared to the control *Arabidopsis* plants, the transgenic plants displayed significantly higher drought tolerance, and the overexpression of *TaSNAC4-3A* did not affect plant growth under well-watered conditions (Fig. 4B). While the rate of survival of the control was approximately 37%, 65–80% of the transgenic plants survived the experiments where water was withheld (Fig. 4C). In order to better understand the drought sensitivity of the *TaSNAC4-3A* transgenic plants, we measured the natural water loss rate and the stomatal apertures of the leaves in three-week-old soil-grown control and *TaSNAC4-3A* transgenic plants. Analysis of the relative water content indicated that as drought conditions intensified, the rate of water loss was lower in the transgenic lines than it was in the control plants, indicating that they tolerated drought stress better (Fig. 4D). The stomatal apertures in the control plants and the transgenic lines all changed significantly after 10 days of drought stress. The stomatal apertures in the transgenic plants decreased from ~0.44 to ~0.18. This was significantly smaller than the decrease observed in the control plants (from ~0.43 to ~ 0.27) (Fig. 4E). Under normal conditions, no significant differences were observed in the malondialdehyde (MDA) content and the activities of superoxide dismutase (SOD) and catalase (CAT) between the transgenic and control plants (Fig. 4F).

**Table 1**
List of upregulated genes in transgenic *Arabidopsis* plants expressing *35S:TaS-NAC4-3A* (FC > 2.0, $P < 0.01$) that are involved in abiotic stress and the ABA response.

| Gene ID | Gene symbol | FC | *p*-value | Description |
|---|---|---|---|---|
| AT3G60140 | DIN2 | 15.82 | 9.60E-12 | Glycosyl hydrolase superfamily protein |
| AT5G59310 | LTP4 | 14.73 | 1.49E-06 | Lipid transfer protein 4 |
| AT2G35300 | LEA18 | 13.32 | 2.75E-11 | Late embryogenesis abundant protein |
| AT3G02480 | ABR | 13.12 | 6.37E-06 | Late embryogenesis abundant protein (LEA) family protein |
| AT5G52300 | LTI65 | 12.30 | 1.72E-08 | CAP160 protein |
| AT1G02205 | CER1 | 12.19 | 2.91E-09 | Fatty acid hydroxylase superfamily |
| AT1G43160 | RAP2.6 | 12.14 | 5.53E-07 | Related to AP2.6 |
| AT5G51760 | AHG1 | 10.52 | 6.99E-10 | Protein phosphatase 2C family protein |
| AT5G59320 | LTP3 | 10.49 | 2.72E-04 | Lipid transfer protein 3 |
| AT2G47770 | TSPO | 10.31 | 1.94E-06 | Outer membrane tryptophan-rich sensory protein |
| AT5G64750 | ABR1 | 10.29 | 2.33E-09 | Integrase-type DNA-binding superfamily protein |
| AT5G22500 | FAR1 | 10.08 | 2.52E-09 | Fatty acid reductase 1 |
| AT1G16850 | – | 9.77 | 1.20E-08 | Transmembrane protein |
| AT3G50970 | LTI30 | 9.17 | 1.59E-05 | Dehydrin family protein |
| AT1G03220 | – | 8.91 | 5.82E-07 | Eukaryotic aspartyl protease family protein |
| AT5G06760 | LEA4-5 | 8.37 | 2.55E-05 | Late Embryogenesis Abundant 4-5 |
| AT3G48360 | BT2 | 8.32 | 5.92E-04 | BTB and TAZ domain protein 2 |
| AT4G19810 | ChiC | 8.21 | 3.29E-07 | Glycosyl hydrolase family protein |
| AT5G59220 | HAI1 | 8.12 | 5.24E-04 | PP2C protein |
| AT3G05640 | EGR1 | 7.97 | 1.93E-07 | Protein phosphatase 2C family protein |
| AT2G40170 | GEA6 | 7.79 | 3.16E-05 | Stress induced protein |
| AT1G58340 | ZF14 | 7.71 | 2.97E-07 | MATE efflux family protein |
| AT1G32560 | LEA4-1 | 7.62 | 3.24E-04 | Late embryogenesis abundant protein |
| AT3G05880 | RCI2A | 7.48 | 4.51E-06 | Low temperature and salt responsive protein family |
| AT2G21490 | LEA | 7.34 | 6.65E-05 | Dehydrin LEA |
| AT3G06490 | MYB108 | 7.31 | 2.50E-05 | Myb domain protein 108 |
| AT4G05100 | MYB74 | 7.16 | 1.52E-06 | Myb domain protein 74 |
| AT2G36270 | ABI5 | 7.02 | 2.01E-06 | bZIP transcription factor |
| AT4G27410 | RD26 | 7.02 | 1.57E-04 | NAC domain transcriptional regulator superfamily protein |
| AT3G14440 | NCED3 | 7.02 | 2.91E-04 | Nine-cis-epoxycarotenoid dioxygenase 3 |
| AT3G15500 | NAC3 | 6.85 | 1.05E-05 | NAC domain containing protein 3 |
| AT5G54230 | MYB49 | 6.78 | 4.30E-06 | Myb domain protein 49 |
| AT1G69600 | ZFHD1 | 6.70 | 3.18E-06 | Zinc finger homeodomain 1 |
| AT2G38760 | ANNAT3 | 6.69 | 1.41E-04 | Annexin 3 |
| AT5G66400 | RAB18 | 6.67 | 1.55E-03 | Dehydrin family protein |
| AT5G49450 | bZIP1 | 6.66 | 1.46E-06 | Basic leucine-zipper 1 |
| AT2G20880 | ERF53 | 6.60 | 2.97E-06 | Integrase-type DNA-binding superfamily protein |
| AT5G02020 | SIS | 6.57 | 1.32E-05 | E3 ubiquitin-protein ligase RLIM-like protein |
| AT1G47510 | 5PTASE11 | 6.50 | 2.98E-06 | Inositol polyphosphate 5-phosphatase 11 |
| AT4G40010 | SNRK2.7 | 6.45 | 1.04E-05 | SNF1-related protein kinase 2.7 |
| AT3G28210 | PMZ | 6.43 | 1.66E-05 | Zinc finger (AN1-like) family protein |
| AT1G08920 | ESL1 | 6.40 | 8.40E-05 | ERD (early response to dehydration) six-like 1 |
| AT1G77120 | ADH1 | 6.16 | 2.55E-05 | Alcohol dehydrogenase 1 |
| AT3G28600 | – | 6.12 | 2.45E-04 | Nucleoside triphosphate hydrolases superfamily protein |
| AT4G21440 | MYB102 | 5.76 | 1.71E-05 | MYB-like 102 |
| AT1G13740 | AFP2 | 5.70 | 1.51E-05 | ABI five binding protein 2 |
| AT2G40340 | DREB2C | 5.69 | 2.08E-05 | Integrase-type DNA-binding superfamily protein |
| AT3G02140 | TMAC2 | 5.46 | 1.21E-04 | AFP2 (ABI five-binding protein 2) family protein |
| AT1G30100 | NCED5 | 5.43 | 1.80E-03 | Nine-cis-epoxycarotenoid dioxygenase 5 |
| AT5G67480 | BT4 | 5.42 | 1.13E-04 | BTB and TAZ domain protein 4 |
| AT1G05510 | OBAP1A | 5.41 | 2.57E-03 | Naphthalene 1,2-dioxygenase subunit alpha |
| AT5G05410 | DREB2A | 5.20 | 1.68E-04 | DRE-binding protein 2A |
| AT5G24030 | SLAH3 | 5.18 | 7.31E-05 | SLAC1 homologue 3 |
| AT5G13330 | Rap2.6L | 5.17 | 6.10E-05 | Related to AP2.6 |
| AT2G15970 | COR413 | 5.11 | 2.85E-03 | Cold regulated 413 plasma membrane 1 |
| AT3G05890 | RCI2B | 5.04 | 6.66E-03 | Low temperature and salt responsive protein family |
| AT5G39610 | NAC6 | 5.01 | 2.43E-03 | NAC domain containing protein 6 |
| AT5G07440 | GDH2 | 4.88 | 9.20E-04 | Glutamate dehydrogenase 2 |
| AT1G61340 | FBS1 | 4.85 | 1.41E-04 | F-box family protein |
| AT4G02280 | SUS3 | 4.83 | 3.34E-04 | Sucrose synthase 3 |
| AT5G62520 | SRO5 | 4.72 | 1.87E-04 | Similar to RCD one 5 |
| AT3G11020 | DREB2B | 4.69 | 1.44E-03 | DRE/CRT-binding protein 2B |
| AT4G15910 | DI21 | 4.55 | 2.56E-04 | Drought-induced 21 |
| AT2G03760 | SOT12 | 4.46 | 1.32E-03 | Sulfotransferase 12 |
| AT4G38410 | – | 4.43 | 3.58E-04 | Dehydrin family protein |
| AT2G38530 | LTP2 | 4.25 | 7.75E-04 | Lipid transfer protein 2 |
| AT1G14170 | – | 4.22 | 5.93E-04 | RNA-binding KH domain-containing protein |
| AT3G19580 | ZF2 | 4.06 | 1.85E-03 | Zinc-finger protein 2 |
| AT2G19450 | TAG1 | 4.06 | 8.98E-04 | Membrane bound O-acyl transferase (MBOAT) family protein |
| AT3G25780 | AOC3 | 4.05 | 2.16E-03 | Allene oxide cyclase 3 |
| AT2G38340 | DREB19 | 4.04 | 1.44E-03 | Integrase-type DNA-binding superfamily protein |
| AT4G25480 | DREB1A | 4.01 | 8.13E-03 | Dehydration response element B1A |

(*continued on next page*)

**Table 1** (*continued*)

| Gene ID | Gene symbol | FC | *p*-value | Description |
|---|---|---|---|---|
| AT1G27730 | STZ | 3.74 | 7.05E-03 | Salt tolerance zinc finger |
| AT3G50980 | XERO1 | 3.65 | 5.06E-03 | Dehydrin xero 1 |
| AT5G37500 | GORK | 3.63 | 6.97E-03 | Gated outwardly-rectifying K+ channel |
| AT3G02410 | ICME-LIKE2 | 3.63 | 5.49E-03 | Alpha/beta-Hydrolases superfamily protein |
| AT5G52050 | DTX50 | 3.54 | 2.98E-03 | MATE efflux family protein |
| AT5G38710 | PDH2 | 3.45 | 4.05E-03 | Methylenetetrahydrofolate reductase family protein |
| AT3G63210 | MARD1 | 3.12 | 8.20E-03 | Mediator of aba-regulated dormancy protein (DUF581) |
| AT3G46930 | RAF43 | 3.07 | 8.78E-03 | Protein kinase superfamily protein |
| AT2G16720 | MYB7 | 3.05 | 8.96E-03 | Myb domain protein 7 |

However, under drought stress, transgenic plants exhibited higher levels of SOD and CAT activities, as well as lower levels of MDA compared with the control (Fig. 4F). This indicates that overexpression of *TaSNAC4-3A* results in increased tolerance to drought stress in transgenic *Arabidopsis*.

### 3.5. TaSNAC4-3A overexpression enhances tolerance to salt and osmotic stresses

In order to investigate whether *TaSNAC4-3A* overexpression confers increased tolerance to other abiotic stresses, we examined how the control and transgenic plants grew on 0.5 × MS medium supplemented with varying concentrations of mannitol and NaCl. We found that the rate of germination was comparable between the two groups under normal conditions, with both approaching 100%. However, there were significant differences in the germination rates of seeds from the control plants and the overexpression lines under salt and osmotic stresses (Fig. 5A, C). The overexpression lines showed a 20–30% increase in the germination rate when compared to the control on medium containing 150 mM NaCl or 150 mM mannitol (Fig. 5A, C). We also analyzed the effects of *TaSNAC4-3A* overexpression on root elongation under salt and osmotic stresses. Under normal conditions, there are no significant differences in the length of the roots between the control and *TaSNAC4-3A* overexpressing plants (Fig. 5B, C). However, when treated with either 100 mM of mannitol or 100 mM of NaCl, the two overexpression lines displayed significantly longer root lengths than did the control (Fig. 5B, C). This shows that overexpression of *TaSNAC4-3A* alleviates the inhibitory effects of salt or osmotic stress on root elongation. Our results indicate that overexpression of *TaSNAC4-3A* in *Arabidopsis* can increase tolerance to salt and osmotic stresses.

### 3.6. TaSNAC4-3A overexpression confers hypersensitivity to exogenous ABA

The increased expression of *TaSNAC4* genes in response to ABA treatment suggests that *TaSNAC4* could be part of the ABA-dependent stress signaling pathway (Fig. 3C and D). We found over 10 ABRE elements in the promoter regions of the *TaSNAC4* homeologous genes (Fig. 3A). The ABRE *cis*-acting elements could be the cause of the stress signaling pathway mediated by ABA, which prompted us to consider whether overexpressing *TaSNAC4-3A* affects ABA sensitivity. Under normal conditions, we found the rate of germination of control seeds to be comparable to that of the *TaSNAC4-3A* transgenic lines. However, in the presence of 1 μM ABA, there was a significant reduction in the rate of germination of seeds of the transgenic lines when compared to the control (Fig. 6A, B). This indicates that seed germination of the *TaSNAC4-3A* transgenic lines was severely inhibited by exogenous ABA when compared to the control. We also examined the sensitivity of the *TaSNAC4-3A* transgenic lines to ABA at the post-germination stage. Root lengths of the transgenic lines and control plants did not differ significantly without exogenous ABA (Fig. 6C and D). When ABA was present, however, the root lengths of both the transgenic lines and the control were significantly reduced. We observed significant reductions in the transgenic lines compared to control plants, which suggests that exogenous ABA had a greater inhibitory effect on root elongation in the *TaSNAC4-3A* transgenic lines (Fig. 6C and D). Our results indicate that hypersensitivity to ABA is conferred at the germination and post-germination stages by the overexpression of *TaSNAC4-3A*.

### 3.7. TaSNAC4-3A overexpression activates the expression of stress-responsive genes

Overexpression of *TaSNAC4-3A* mediates an extreme tolerance response to abiotic stresses in *Arabidopsis*, and to better understand the regulatory network through which it operates we analyzed and compared the transcriptomes of the control and transgenic overexpression lines under normal growth conditions (Figure S2). We identified 616 and 319 genes (adjusted $P < 0.01$, fold-change $> 2$ or $< 0.5$) as up-regulated and down-regulated, respectively, when comparing transgenic lines to the empty-vector control (Fig. 7A and B; Table S2). A Gene Ontology (GO) analysis revealed that the biological pathways "response to salt stress", "response to drought conditions", "response to abscisic acid" and "response to osmotic stress" were the most affected in the up-regulated genes. Conversely, the biological pathways categorized as "protein phosphorylation", "defense response", "auxin polar transport", and "response to salicylic acid" were the most affected in the significantly down-regulated genes (Fig. 7C). We believe that these changes in the transcriptome could contribute to a reduction in water loss and rapid stomatal closure in transgenic *Arabidopsis* plants exposed to drought stress conditions.

Eighty-one genes related to abiotic stress and the ABA response were significantly upregulated (FC $> 2$; $P < 0.01$) in *TaSNAC4-3A* overexpressing *Arabidopsis* plants compared with the control (Table 1). We examined the expression patterns of six stress-responsive genes, including *AHG1*, *ABI5*, *SnRK2.7*, *LTI30*, *LEA*, and *NCED3* in both the *TaSNAC4-3A* overexpression lines and the control before and after the abiotic stress treatments using qRT-PCR (Fig. 8). Our results showed that under normal conditions, the mRNA levels of the marker genes related to stress in the overexpression lines were significantly higher than in the control. Transcription of these genes was significantly up-regulated in all genotypes when they were exposed to salt stress, ABA treatment, or drought conditions. Compared to the control, the six stress-related genes had significantly higher expression levels in the *TaSNAC4-3A* overexpression lines. These results indicate that in response to abiotic stress, expression of *TaSNAC4-3A* activates the expression of genes related to stress signaling in transgenic *Arabidopsis*.

## 4. Discussion

### 4.1. TaSNAC4 is a stress-responsive NAC transcriptional activator

Drought is a major environmental stress that severely impacts the productivity of wheat crops. This fact has prompted a search for genes involved in drought tolerance (Mohammadi, 2018). There have been a number of NAC family genes identified in common wheat (Guérin et al., 2019), but studies of the roles they play in drought stress tolerance has been limited. In this study, we isolated three *TaSNAC4* homeologous genes that are potentially involved in drought tolerance from the wheat cultivar 'Chinese Spring'. We mapped the three genes, named *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* to wheat chromosomes 3A, 3B, and 3D, respectively. Sequence analysis showed that the three TaSNAC4 homeologous proteins contain a typical NAC-conserved domain, that is divided into five subdomains, all located in the

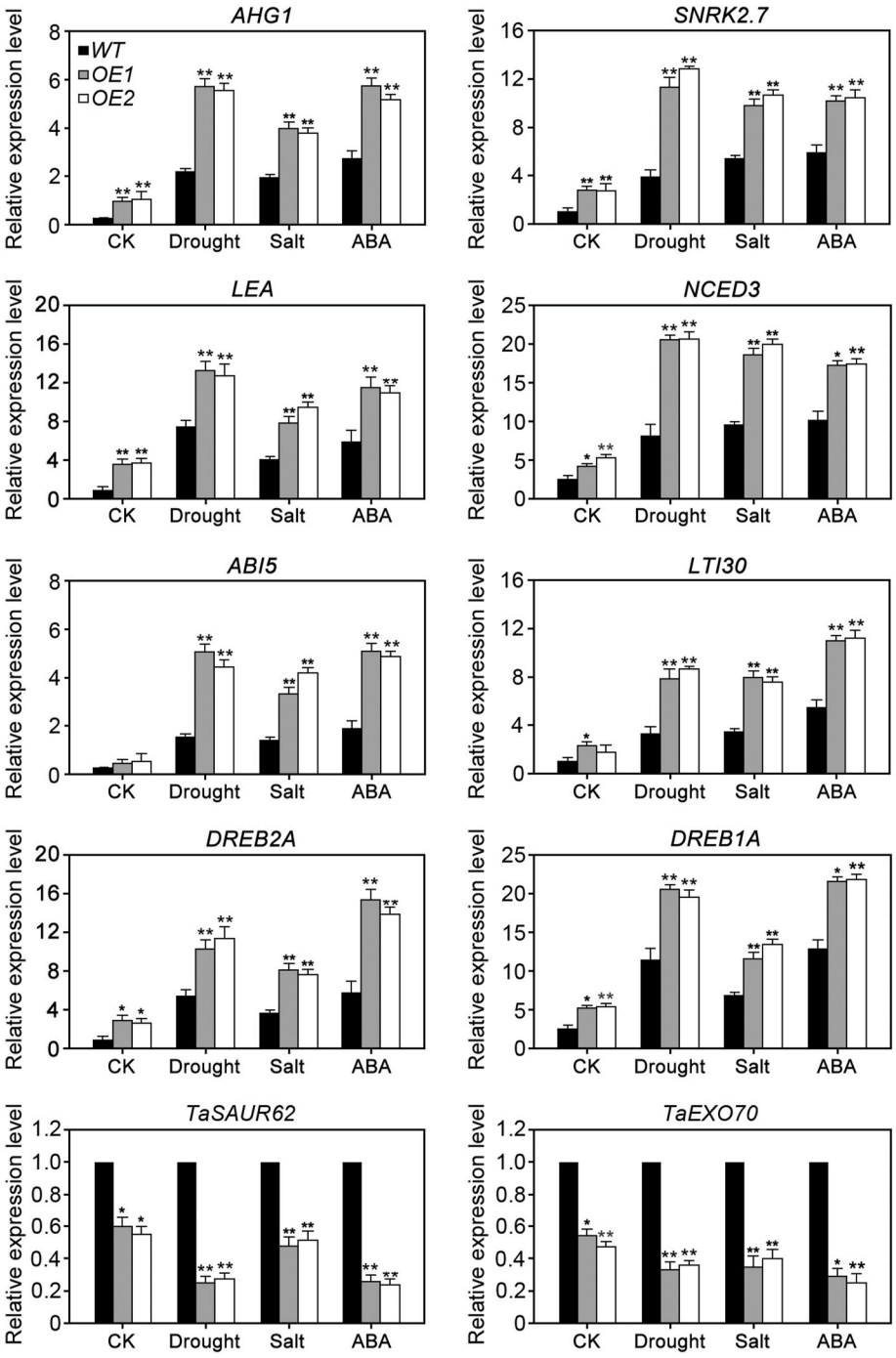


**Fig. 8. Expression levels of stress-responsive genes in control and *TaSNAC4-3A* over-expressing plants.** The 14-day-old seedlings grown on solid 0.5 × MS medium were subsequently submerged in 0.5 × MS medium supplemented with 15% PEG, 100 mM NaCl, or 1 μM ABA for seven days. The relative expression levels of stress-responsive genes were determined against expression of the *Actin8* gene. Values represent mean ± SD of three replicates. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$) between transgenic lines and the control based on Student's *t*-test.

N-terminal region (A-E, Fig. 1). Interestingly, we found that TaSNAC4-3B identified in our study has been reported in a previous study, where it was referred to as TaNAC4, and was characterized as having the features of nuclear localization, transactivation activity, and biotic and abiotic stress-induced expression (Xia et al., 2010). In our study, further assays showed that TaSNAC4-3A is localized to the nucleus and that it induced transactivation activity in a GAL4-containing reporter gene (Fig. 2B and C). These results are consistent with previous research (Mao et al., 2016). We found that the TaSNAC4 homeologous proteins are most closely related to the rice LOC_Os01g60020 and maize GRMZM2G068973 proteins, and also had sequence similarities to TaNAC2 (~71% sequence identity), ZmSNAC1 (~61% sequence identity), and SNAC1 (~68% sequence identity) (Fig. 2A). This indicates that the three *TaSNAC4* homeologs, *TaSNAC4-3A*, *TaSNAC4-3B*, and *TaSNAC4-3D* might be important candidate genes for increasing stress tolerance in wheat.

Stress tolerance in plants can be significantly increased by over-expression of genes related to the stress response (Hu et al., 2006; Mao et al., 2012; Lu et al., 2012). We also found that drought, salinity, mannitol, and exogenous ABA treatments all induced the expression of the *TaSNAC4* genes (Fig. 3C and D). After sequence analysis, we identified a number of *cis*-elements related to stress responses, such as ABRE, DRE, MBS, MYBRS, and MYCRS, in the promoter regions of the three *TaSNAC4* homeologous genes (Fig. 3A). These have all been shown to be involved in a number of stress responses, including ABA signaling. Along with our results, these analyses strongly suggest that TaSNAC4 is a NAC transcription activator, related to the stress response, and is a significant factor in the response to environmental stresses, especially drought

stress in wheat.

### 4.2. TaSNAC4-3A plays a positive role in and improves abiotic stress tolerance in plants

Overexpression or deletion mutations are the two best methods for studying factors related to NAC transcription in response to abiotic stresses. Therefore, we evaluated the stress tolerance in transgenic *Arabidopsis* plants overexpressing *TaSNAC4-3A*. We found that several physiological processes in the *TaSNAC4-3A* overexpression lines are involved in abiotic stress resistance. First, overexpression of *TaSNAC4-3A* increased salt and osmotic stress tolerance, which is supported by greater root length and higher rates of germination in the transgenic plants exposed to mannitol and NaCl treatments (Fig. 5). We found that *TaSNAC4-3A*, *OoNAC72*, and *CarNAC4* transgenic plants all exhibited increased salt and osmotic tolerance compared to the control plants. This was evidenced by both the physiological and morphological differences (Yu et al., 2016; Guan et al., 2019). Second, overexpression of *TaSNAC4-3A* in transgenic *Arabidopsis* significantly enhanced tolerance to drought conditions (Fig. 4A–C). Transgenic plants did not wilt as much as did the control plants under drought stress, and recovered to normal growth once they were re-watered. Changes in certain biochemical and physiological conditions provide evidence of the mechanisms involved in the increased abiotic stress tolerance of the transgenic *TaSNAC4-3A* overexpression lines. First, when compared to control plants, the overexpression lines had a much lower water loss rate after the detached leaves were assayed under drought conditions (Fig. 4D). This gave the overexpression lines an increased capacity to retain water, resulting in a significantly higher survival rate under conditions of drought stress. Previous studies have shown that the stomata can regulate water evaporation from leaves (Zhu 2002). In our study, the stomatal apertures in the *TaSNAC4-3A* overexpression lines were significantly smaller than in the control, which could lead to a lower rate of transpiration and evaporation in the leaves (Fig. 4E). Second, anti-oxidative enzyme activities were significantly increased in the *TaSNAC4-3A* overexpression lines (Fig. 4F). Abiotic stresses such as drought typically stimulate ROS production. This can cause damage related to oxidation of the plasma membrane, among other organelles (Apel and Hirt, 2004; Gill and Tuteja, 2010). The anti-oxidative enzymes that are primarily responsible for ROS scavenging in plants are POD, CAT, and SOD (Mittler 2002). In the *TaSNAC4-3A* overexpression lines, CAT and SOD activities were significantly higher than in the control plants (Fig. 4F), giving the transgenic plants a greater ability to regulate ROS. Third, the expression of genes related to stress responses, such as *AHG1*, *SnRK2.7*, *LEA*, *NCED3*, *ABI5*, and *LTI30* was significantly increased when *TaSNAC4-3A* was overexpressed (Table 1; Fig. 7; Fig. 8). These genes encode different types of proteins that mediate damage from osmosis or oxidative processes (Hu and Xiong, 2014; Qin et al., 2011), indicating that the ability of plants to deal with abiotic stresses is significantly enhanced by the increased transcription of these genes in the *TaSNAC4-3A* overexpression lines.

### 4.3. TaSNAC4-3A confers drought stress tolerance through an ABA-dependent pathway

ABA, which is a phytohormone, plays a role in the plant response to a range of abiotic stresses (Sah et al., 2016). NAC TFs regulate responses to abiotic stresses by either ABA-independent or ABA-dependent pathways (Fujita et al., 2004; Tran et al., 2004; Hu et al., 2006; Jensen et al., 2008; Hao et al., 2011; Fang et al., 2015). Our results demonstrate that *TaSNAC4-3A* is a positive regulator of drought stress tolerance, likely by way of a signaling pathway mediated by ABA. There is considerable support for this hypothesis. First, the *TaSNAC4-3A* promoter region contains 14 ABRE *cis*-acting elements. These could be responsible for ABA-mediated signaling transduction (Fig. 3A) which would indicate that treatment with ABA significantly increases *TaSNAC4* expression (Fig. 3C). Second, at the seed germination and root elongation stages, overexpression of *TaSNAC4-3A* induced ABA hypersensitivity. When exogenous ABA was present, the rate of germination was significantly lower in the *TaSNAC4-3A* overexpression lines than in the control (Fig. 6A and B). Compared with the control plants, exogenous ABA significantly inhibited root elongation in *TaSNAC4-3A* overexpression lines (Fig. 6C and D). ABA is a well-known regulator of stomatal closure, and contributes to a higher level of water retention during drought (Sah et al., 2016). Under drought conditions, the stomatal apertures of the *TaSNAC4-3A* overexpression lines was greatly reduced when compared with the control (Fig. 4E). Third, our transcriptome analysis demonstrated that the biological pathway responding to ABA was greatly enhanced among the upregulated genes in the *TaSNAC4-3A* overexpression lines (Fig. 7). Further analysis of gene expression showed that several ABA biosynthesis and signaling pathway genes, such as *NCED3* (Qin and Zeevaart, 1999), *AHG1* (Nakashima and Yamaguchi-Shinozaki, 2013), *SnRK2.7* (Mizoguchi et al., 2010), and *ABI5* (Nakashima and Yamaguchi-Shinozaki, 2013), were greatly upregulated in the *TaSNAC4-3A* overexpressing lines when compared with the control (Table 1; Fig. 8). Our data indicate that *TaSNAC4-3A* can indirectly or directly affect the expression of genes related to ABA biosynthesis. Increasing endogenous ABA levels eventually activates a succession of ABA signaling pathways, leading to an increased tolerance to abiotic stresses.

## 5. Conclusion

In conclusion, our results show that TaSNAC4-3A is a transcriptional activator that is localized to the nucleus and has a transactivation domain in the C-terminus. When *TaSNAC4-3A* was overexpressed in *Arabidopsis*, tolerance to salt and drought was improved due to the increased expression of genes related to abiotic stress responses. The *TaSNAC4-3A* overexpression lines also displayed hypersensitivity to ABA, which indicates that *TaSNAC4-3A* could be involved in the plant stress response through an ABA-dependent pathway. Our study suggests that *TaSNAC4-3A* is an important positive regulator of plant drought tolerance.

## Author contribution

H.M. and X.W. designed the research. F.M., B.C., F.L. and Y.Z. performed the experiments. H.M., X.W. and Z.K. wrote the manuscript with contributions from all authors.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2021.01.004.

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