Identification and characterization of a novel NAC transcription factor gene from triticale (x Triticosecale Wittmack)

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Abstract

The NAM, ATAF, and CUC (NAC) family of transcription factors plays several important roles in plants, helping to regulate plant growth, development, senescence, and the response to biotic and abiotic stressors. NAC proteins also act as molecular switches, modulating hormonal responses to stress.A novel coding sequence (1059 bp) was cloned from hexaploid triticale. The putative protein encoded by this sequence (352 amino acids) was more than 95% similar to the amino acid sequence of a NAC protein from Aegilopsis tauschii (goatgrass; XP020161331), and phylogenetic analysis indicated that the novel gene formed a clade with goatgrass, Triticum turgidum , and barley. The novel protein contained a conserved nature actomyosin (NAM) domain (129 consecutive amino acids) between the 20 th and 148 th amino acids at the N-terminus and three transcriptional activation regions at the C-terminus. TwNAC01 was localized to the nucleus. Based on this evidence, the novel gene was identified as a triticale NAC gene and designated TwNAC01 (GenBank accession no. MG736919). After exposure to drought, Macrogol 6000 (PEG6000), NaCl, cold, methyl jasmonate (MeJA), and abscisic acid (ABA), TwNAC01 expression levels were greatest in triticale roots, followed by leaves and stems. Transgenic Arabidopsis thaliana overexpressing TwNAC01 had significantly lower leaf water loss rates and significantly longer roots than wild-type A. thaliana. Virus-induced silencing of the TwNAC01 gene in triticale delayed root development and decreased taproot length. Under drought stress, leaves of TwNAC01- silenced triticale had higher levels of malondialdehyde (MDA) and hydrogen peroxide (H 2 O 2) than the leaves of the wild type (WT), as well as lower relative water content (RWC), net photosynthetic rate, stomatal conductance, intercellular CO 2 concentration, and transpiration rate.Gene overexpression and silencing experiments suggested that TwNAC01 improves plant stress tolerance by increasing taproot length, regulating the water content of the plant leaves, reducing MAD and H 2 O 2 content, and adjusting respiration rate.

Introduction

Triticale (· *Triticosecale* Wittmack) is a new allopolyploid crop derived from the intergeneric hybridization of wheat (*Triticum*) and rye (*Secale*), followed by chromosome doubling (Zilinsky et al., 1974). This crop, which can be used as both a food crop and a forage crop, combines the high grain yield and good quality of wheat with the strong stress resistance of rye (Cao et al., 2011; Sun et al., 2002). Because triticale exhibits strong stress resistance in cultivation, the mining of its resistance genes is important for the molecular breeding of even more stress-resistant triticale varieties.

During growth and development, plants are often affected by a variety of natural adverse environmental factors, such as high temperatures, drought, salinity, and extreme weather. To survive despite these challenges, plants use series of defense mechanisms to resist and tolerate a variety of biotic and abiotic stresses (Cramer et al., 2010; Pinheiro et al., 2011). By binding to cis-acting elements in the target gene promoter, transcription factors act as molecular switches for gene expression, activate or inhibit the expression of target genes, regulate the expression of plant-related genes, and participate in the stress response (Puranik et al., 2012; Nakashima et al., 2012). Many transcription factor families are found in plants, including NAC, WRKY, DREB, and MYB transcription factors (Puranik et al., 2012). Of these, the plant-specific NAC transcription factor family has the most members (Kim et al., 2014; Perez-rodriguez et al.,

2010). Several studies have shown that NAC transcription factors play important roles in a variety of plant processes, including growth and development, leaf decay, hormone increase and decrease, and the regulation of the defense response to biotic and abiotic stresses (Purankik et al., 2012; Nakashima et al., 2012). For example, in *Arabidopsis thaliana*, overexpression of the *AtNAC2* gene improved lateral root elongation (He et al., 2005). In addition, Jensen et al. (2013) reported that *Arabidopsis* overexpressing ATAF1 showed obvious dwarfism and flowering delay; the endogenous ABA content of the transgenic *Arabidopsis* overexpressing ATAF1 was also significantly greater than that of the *Arabidopsis* wild-type (WT; a 6–8-fold increase), which improved the drought tolerance of the transgenic lines. Similarly, overexpression of the *OsNAC10* gene, which is specifically expressed in rice roots, increased the diameter of rice roots, improved rice tolerance of drought, and significantly increased rice yield under drought stress (Jeong et al., 2010). Also in rice, the overexpression of the *SNAC1* gene improved drought and salt resistance both in the field and in the greenhouse; transgenic plants overexpressing *SNAC1* also had lower water loss rates and were more sensitive to ABA than wild-type plants (Xiong et al., 2001). Overexpression of the wheat genes *TaNAC2* and *TaNAC67* in *A. thaliana* significantly improved salt, drought, and cold tolerance (Mao et al., 2012). Indeed, RT-PCR analysis showed that *TaNAC4* and *TaNAC8* were induced by phytohormones (e.g., salicylic acid, SA; methyl jasmonate, MeJA; and abscisic acid, ABA), pathogens (stripe rust), abiotic stressors (salinity and cold), and mechanical injury (Kang et al., 2010). Transgenic wheat overexpressing the *TaNAC69* gene had greater biomass and longer roots than the wild type, and thus had a better survival rate under salt and drought stress (Xue et al., 2011). These previous studies demonstrate that *NAC* genes can be induced by biotic and abiotic stresses under a variety of conditions, and thus are likely to play important roles in plant stress resistance. However, little is known about *NAC* genes in triticale. To address this knowledge gap, we aimed to identify triticale *NAC* genes, determine triticale *NAC* gene function, and provide candidate genes for the molecular breeding of more stress-resistant triticale varieties.

In this study, a novel *NAC* transcription factor gene, *TwNAC01*, was cloned from triticale. Gene expression pattern analysis demonstrated that *TwNAC01* was upregulated by exposure to drought, NaCl, PEG6000, cold (4°C), ABA, and MeJa. TwNAC01 enhanced tolerance to drought stress in transgenic *Arabidopsis* and increased taproot length. In *A. thaliana* overexpressing TwNAC01, the water loss rate in the leaves was significantly slower, the physiological indexes of stress resistance were significantly improved, and the taproots were significantly longer as compared to wild-type *A. thaliana*. Compared to the control (CK), triticale carrying the silenced *TwNAC01* gene had significantly shorter roots and fewer fibrous roots. The mutant triticale was more sensitive to drought than the control, and also differed from the control with respect to relative water content, hydrogen peroxide (H_2O_2) content, malondialdehyde (MDA) content, and photosynthetic indexes in the plant leaves.

Materials And Methods

Plant materials

Seeds produced by the hexaploid triticale variety Xinxiaoheimai 3 were selected and provided by the Wheat Crop Research Institute of Shihezi University (Xinjiang, China). After cleaning and disinfection, the triticale seeds were planted in the drought-stress plot at the Experimental Station of the Agricultural College of

Shihezi University (Xinjiang, China). To eliminate the effects of natural rainfall, we build a rain shelter over the drought-stress plot before the flowering stage. Plants flowering at the same time were selected during the flowering stage. The control area was irrigated normally. In the drought stress area, irrigation was stopped after the triticale plants reached the heading stage. Both control and experimental plants were visually assessed daily for signs of drought stress. Proline, MDA, electrical conductivity, and chlorophyll levels in the leaves of both sets of plants were also assessed daily as described previously (Chen et al., 2002). During drought stress, proline and MDA levels increased significantly, while soil moisture content levels decreased significantly (Figure S1). When physical indicators of drought stress were observed, the roots, stems, flag leaves, and young grains of both the experimental and control triticale plants were collected.

Full-length 5’- and 3’-RACE

RNA was extracted from the triticale leaves using Hipure HP Plant RNA Mini Kits (Magen). With these RNA sequences as templates, we performed reverse transcriptase PCR (RT-PCR) to synthesize cDNA sequences using SMARTScribe genome Reverse Transcriptase (TaKaRa, China). Synthesized cDNA was stored at −20°C until use. Based on the RNA sequence of Unigene c51971 (708 bp), which was obtained via RNA-Seq sequencing, we designed specific primers for 3’- and 5’-RACE using SMARTer RACE kits (Clonetech; Schedule 1). The RACE procedure was as follows: 94°C for 2 min, 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 35 min, and 16°C for 10 min. The PCR products were recovered and purified using 1.0% agarose gel electrophoresis. The purified PCR products were ligated to the pMD19-T vector (TaKaRa, China) and transformed into TOPO10 cells (TIANGEN, China). Positive clones were identified and sequenced. The open reading frame (ORF) of the full-length cDNA sequence was obtained by splicing the sequencing results using an on-line tool (CAP3;http://doua.prabi.fr/software/cap3) (Shang et al., 2018) and National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI-BLAST). Sequence alignment analysis preliminarily identified the sequence as a triticale NAC gene and predicted the positions of the start and stop codons. We then designed a primer pair to amplify the full-length gene (Schedule 1); this gene was preliminarily designated *TwNAC01*.

Analysis of the triticale *TwNAC01* sequence

We used the NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/) to identify sequences homologous to the ORF and coding sequences of *TwNAC01.* We identified conserved structures in the *TwNAC01* gene using Smart (http://smart.embl-heidelberg.de/). We analyzed the physical and chemical properties of the predicted TwNAC01 protein, as well as its hydrophobicity, using the ExPASy server (https://web.expasy.org). Multi-alignment of TwNAC01 and other NAC proteins in different species was conducted on DNAMAN. We predicted the subcellular location of the protein using Protcomp and TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP). The relevant sequences were aligned using MEGA (version 10.0) (Shang et al., 2018) and MegAlign (DNAStar).

Real-time fluorescence quantitative PCR (qPCR)

The plants to be used for qRT-PCR were cultured at 25°C with a normal watering regime, under a 12 h light/12 h dark cycle in an artificial climate box. When the seedlings had two leaves and one main shoot, they were transplanted in hydroponic boxes. Three hydroponic boxes were allocated to each treatment; each box had 12 holes, and each hole had 5 seedlings. After transplantation, seedlings were allowed to acclimate for 5 days. After acclimation, one set of boxes was transferred to a cold room (4°C). Other boxes were treated with one of the following: 1 L of 20% PEG6000, 200 mM NaCl, 100 μM MeJA, 100 μM ABA. In all treatments, plant roots were soaked and leaves were sprayed with the same solution. Roots and leaves were collected after 0, 1, 3, 6, 12, and 24 h of treatment. After collection, the materials were frozen in liquid nitrogen and then transferred to a freezer at − 80°C.

RNA was extracted from the triticale materials collected from each of the six groups [cold (4°C), drought, 20% PEG6000, 200 mM NaCl, 100 μM MeJA, and 100 μM ABA using Hipure HP Plant RNA Mini Kits (Magen, China), and cDNA was synthesized using 5XAll-In-One RT MasterMix (abm, Canada) with specific primers (RT-PCR primer pair; Schedule 1). The wheat actin gene was used as internal reference for real-time qPCR (Schedule 1). qPCR was performed using SuperReal PreMixPlus (SYBR Green) kits (Tiangen, China). Each 10 μL qPCR volume contained 6 μL 2· SuperReal PreMixPlus, 0.25 μL forward primer, 0.25 μL reverse primer, 1 μL cDNA template, and sufficient ddH_2O to make 10 μL. qPCR amplifications were performed using a Roche Light-Cycler 480R with the following cycling conditions: pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 61°C for 30 s, and amplification at 72°C for 30 s. All reactions were performed in triplicate, and relative gene expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001).

Sub-cellular localization of the TwNAC01 protein

The coding sequence of the *TwNAC01* gene was cloned into the plant subcellular expression vector pCAMBIA1301S Enhanced Green Fluorescent Protein (EGFP; GenBank accession no. E17099). Insertion primers containing BamHI-XbaI restriction sites (Schedule 1) were designed and ligated using a ClonExpressII one-step cloning kit (TaKaRa, Dalian, China). After verification via sequencing, the recombinant plasmid and the empty pCAMBIA1301S (EGFP) vector were transformed into *Agrobacterium tumefaciens* GV3101 (TaKaRa, China) (Mao et al., 2014). *A. tumefaciens* carrying the recombinant plasmid or the empty vector (TaKaRa, China) were cultured on Luria-Bertani (LB) medium containing Kan + and Rif+. When the OD600 of the bacterial solution was 0.5–0.6, the bacterial solution was collected and re-suspended in infection buffer [10 mM $MgCl_2$, 10 mM fatty acid methyl ester sulfonate (MES), 150 μM surfactant-AS, pH 5.7)].

Subcellular location was visualized in tobacco leaves. Tobacco seeds were planted in an artificial climate box and cultured at 23°C, with 60% relative humidity and a 16 h light/8 h dark cycle, for 3 weeks prior to vector inoculation. The cultured *A. tumefaciens* solution was then injected into tobacco leaves with 5 mL needleless sterile syringe, and tobacco seedlings cultured in darkness for 36 hours. Tobacco leaves exhibiting normal growth after inoculation were selected for examination. The area of each selected leaf around the infection site was excised. Enhanced Green Fluorescent Protein (EGFP) fluorescence signals in the tobacco leaves were observed using a Fluo-View confocal microscope (FV300; Olympus, Japan).

Generation of transgenic *Arabidopsis* overexpressing *TwNAC01*

To obtain transgenic *Arabidopsis* plants, the coding sequence of TwNAC01 containing the termination codon was amplified by RT-PCR and cloned into the KpnI and XbaI restriction sites of the pCAMBIA1300-35S vector (Clontech, TaKaRa, China) under the control of the 35S promoter of the cauliflower mosaic virus (CMV). The primers containing the KpnI and XbaI restriction sites are listed in Additional file 2: Table S1. The recombinant vector pCAMBIA1300-35S-TwNAC01 and the empty vector pCAMBIA1300-35S-VC were introduced into *A. tumefaciens* strain GV3101 (TaKaRa, China). Finally, transgenic *Arabidopsis* plants were generated using the *A. tumefaciens*-mediated floral dipping method (Clough and Bent et al., 1998). To generate homozygous progeny, T1 and T2 seeds were selected on kanamycin (50 mg/L) plates. T3 transgenic *A. thaliana* and wild-type *A. thaliana* plants were watered once at the rosette stage and then subjected to drought stress for 25 days. After 25 days of drought stress, RNA was extracted from the leaves and roots of both transgenic and wild-type *A. thaliana* using Hipure HP Plant RNA Mini Kits (Magen, China). *TwNAC01* gene expression levels were then measured using semi-quantitative analysis with gene-specific primers [Table S1; please see the section “Real-time fluorescence quantitative PCR (qPCR)” for details]. Representative lines overexpressing *TwNAC01* were used for further analysis.

Drought resistance of transgenic *A. thaliana* overexpressing *TwNAC01*

We then measured various stress-related physiological indexes in 35-day-old transgenic *A. thaliana* overexpressing *TwNAC01*, mock transformed *A. thaliana* (transformed with the empty vector), and wild-type *A. thaliana.* Leaf relative water content (RWC) was determined following the methods of Flexas et al. (2006), leaf electrical conductivity was determined following Chen Aikui et al. (2010), leaf MDA content was determined following Chen and Wang et al. (2002), and leaf H_2O_2 content was determined using an H_2O_2 measurement kit (China Nanjing Jiancheng Science and Technology Co., Ltd). We also determined the rate of water loss in the leaves. Five rosette leaves from each group of *A. thaliana* plants (wild-type, empty vector, and the three *TwNAC01*-overexpression lines) were collected, transferred to filter paper, and placed in a constant temperature incubator at 25°C. Leaves were weighed every hour for 8 h and photographed after 2 h, 5 h, and 8 h. Water loss was judged based weight loss and on the degree of leaf curl. Water loss rate measurements were replicated six times. After growing for 55 days, plants of all *A. thaliana* lines were carefully removed from the nutrient soil and washed. The length of the main root system of each plant was measured.

Virus-induced gene silencing (VIGS) of the triticale *TwNAC01* gene.

Specific primers were designed for the PCR amplification of silencing fragments based on the 3'-UTR region of *TwNAC01* gene (Schedule 1), and the barley stripe mosaic virus (BSMV) vector was constructed using ligation-independent cloning (LIC) as previously described (Lee et al., 2015). The BSMV-*γb* vector was digested with the ApaI restriction enzyme and the vector skeleton was recovered. The PCR fragments were treated with T4 DNA polymerase, and the BSMV-rb vectors were digested with the ApaI restriction enzyme. A final concentration of 5 mM deoxythymidine triphosphate (dTTP) was added to the carrier reaction system, and the system was allowed to react for 30 min at room temperature. After the completion of the reaction,

the system was heated to 75°C for 10 min to inactivate T4 DNA polymerase. The treated fragments (200 ng) and the carrier (20 ng) were mixed, heated to 66°C for 2 min, and then cooled slowly to room temperature. We transformed 10 μl of the mixed product into *Escherichia coli* using the heat shock method. Positive clones were screened using colony PCR and verified via sequencing. The positive clones were shaken, and the plasmids were extracted for follow-up experiments. The extracted viral vector plasmids were transferred into *A. tumefaciens* GV3101 for triticale inoculation.

Stress resistance of triticale after *TwNAC01* gene silencing.

A. tumefaciens carrying BSMV-phytoene desaturase BSMV::asTaPDS constructs (BSMV-*PDS*) induce photobleaching or yellow-orange coloration in the silenced tissue due to depletion of enzymes involved in biosynthesis of carotenoid pigments or chlorophyll, respectively (Lee et al., 2015). Thus, these constructs may be used as positive controls for gene silencing. Triticale plants were inoculated with BSMV-*PDS*, BSMV-γb, or BSMV-*TwNAC01* for about a week (two to three leaves were treated per plant). After an additional two weeks of growth, white stripes began to appear on the leaves due to the action of the indicator gene. At this point, samples of the leaves were taken and stored at − 80°C. Total RNA was extracted from these samples for quantitative reverse transcription PCR (qRT-PCR). The RWC of the leaves was determined following the methods of Flexas et al. (2006), MDA content in the leaves was determined following Chen et al. (Chen et al., 2002), and H_2O_2 content in the leaves was determined using an H_2O_2 measurement kit (Nanjing Jiancheng Science and Technology Co., Ltd). Stomatal conductance, net photosynthesis rate, transpiration rate, and intercellular CO_2 concentration were measured using a LI-6400 portable photosynthesis meter (Li-cor).

Statistical analysis

Microsoft Excel was used for data analysis and Origin and One-way ANOVA was conducted on SPSS Statistics 22.0 software to assess the significant differences. The data were analyzed using Student's t tests; we considered $P < 0.05$ statistically significant.

Result

The full-length *TwNAC01* gene

Using primers designed based on the sequence of Unigene c51971 (GSP-R/F; Schedule 1), we amplified an intermediate sequence from the extracted triticale RNA (Figure S2a) that was 502 bp long (Figure S2b). Based on this intermediate sequence, we used 5'-RACE to amplify the 224 bp 5' sequence (Figure S2c) and 3'-RACE to amplify the 557 bp 3' sequence (Figure S2d). Splicing of the 3' and 5' sequences based on the intermediate sequence (Unigene c51971), yielded a fill-length ORF of 1059 bp. Using specific primers designed based on this sequence, we successfully amplified the gene (Figure S2e). Transformation of this gene fragment into *E. coli* via the pMD19-T vector confirmed the expression of a 1059 bp sequence (Figure S2f). The predicted amino acid sequence of the gene was 352 bp long and had more than 95% homology with the NAC amino acid sequences from barley, wheat, and other plants. We thus inferred that the cloned

gene was a triticale NAC gene, which we designated *TwNAC01*. This gene has been submitted to GenBank (accession number MG736919).

Gene sequence analysis

A NJ phylogenetic tree based on these highly similar sequences showed that the triticale TwNAC01 protein formed a clade with NAC proteins from Aegilops (XP-020161331), Triticum houdeum (KAE8777325), and Hordeum vulgare (CBZ41151) (Fig. 1a). In particular, the amino acid sequence of TwNAC01 was more than 95% similar to the wheat (T. aestivum) protein TaNAC20 (KY461026.1) and the Aegilops tauschii protein AtNAC92 (XM020305742.2). The predicted TwNAC01 protein sequence was highly homologous to NAC sequences from barley, wheat, goatgrass, and durum wheat. Sequence alignment revealed that TwNAC01 shared 97.3% and 83.8% similarity with that of XP020161331 and KAE87777325,TwNAC01 also belonged to the NAM subgroup(Fig. 1b) .The predicted TwNAC01 sequence contained a conserved NAM-superfamily domain composed of 129 consecutive amino acids at the N-terminus (between ammino acid 20 and 148) and three transcriptional activation domains at the C-terminus (Fig. 1c).

Subcellular localization in the nucleus

Localization prediction analysis indicated that the TwNAC01 protein was not located in the chloroplasts or mitochondria. This protein was unlikely to be a chloroplast transport peptide, mitochondrial transport peptide, or signal peptide. The target protein was found in the ”_”,(in other organelles) secretory pathway. These results, in conjunction with the Protcomp analysis (see section “Analysis of the triticale TwNAC01 sequence”), indicated that predicted protein was located in the nucleus. The shear site was consistent with the predicted upper transmembrane region and signal peptide (Table 1) That is, the shear site was 62 amino acids long with a maximum of 0.113, and the comprehensive splicing site was 62 amino acids with a maximum value of 0.107 for the signal peptide to show: NO (Table 1). In the control group, the EGFP localization signal was dispersed throughout the cell, with the strongest signals originating primarily from the cell membrane and nucleus (Fig. 2). In the treatment group, the EGFP protein signal was restricted to the nucleus (Fig. 2). This suggested that the fusion protein was located in the nucleus, as was predicted by our bioinformatics analysis.

Table 1
Subcellular localization scores.

[a] Position	cTP	mTP	SP	Other	Loc	RC	TPlen
Positioning score	0.09	0.104	0.171	0.887		2	
[b] Position	Nucleus	Cell membrane	Extracellular	Mitochondria	Chloroplast	Bubble	
Positioning score	8.26	1.01	0.04	0.01	0.11	0.48	

[a] cTP, chloroplast transit peptide; mTP, mitochondrial transit peptide; SP, signal peptide; RC, reliability level; Tplen, Other organelles and [b]scores were obtained online using the Target P1.1 Server (http://www.cbs.dtu.dk/services/TargetP/).

Analysis of *TwNAC01* gene expression in triticale under stress

The *TwNAC01* gene was significantly upregulated ($P < 0.05$) in triticale roots and grains after drought treatment as compared to the control; *TwNAC01* was also upregulated with respect to the control in the stem and leaf tissues, but these differences were not significant (Fig. 3a). This suggested that the triticale *TwNAC01* gene was upregulated in response to drought stress, with the strongest upregulation found in the grain, followed by the root, leaf, and stem.

After 24 h of salinity, MejA, and ABA stress, *TwNAC01* was significantly upregulated in the roots as compared to the leaves; in response to these stressors, *TwNAC01* expression levels appeared to increase over time (Fig. 3e, 3f). After 1 h and 24 h of NaCl treatment, *TwNAC01* expression was significantly more upregulated in the roots than in the leaves (Fig. 3b). In contrast, after 24 h of cold stress and PEG6000-induced dehydration, *TwNAC01* was significantly upregulated in the leaves as compared to the roots (although *TwNAC01* was significantly upregulated in the roots as compared to the leaves after 12 h of PEG6000 treatment; Fig. 3d, 3c). This might suggest that the strength of the cold and drought response in the roots increased over time, and that the leaves are more sensitive to cold than the roots. After ABA treatment, *TwNAC01* gene expression levels in roots were consistently significantly higher than those in leaves at the same time point (Fig. 3f). Thus, it was likely that the *TwNAC01* gene played a role in the stress response of the triticale roots and leaves.

Confirmation of transgenic *A. thaliana* lines overexpressing *TwNAC01*

PCR analysis of *E. coli* transformed with the pCAMBIA1300-35s overexpression vector recovered 1059 bp band, indicating that transformation had been successful and that the target gene was connected to the vector (Figure S3a). After restriction endonuclease digestion, the vector skeleton (10 kb) and a band slightly longer than 1000 bp were obtained (Figure S3b), indicating that the recombinant expression plasmid containing the target gene had been successfully constructed. The *A. tumefaciens* solution containing the expression vector plasmid was also analyzed using PCR, and a 1059 bp band was recovered (Figure S3c). This indicated that the recombinant plasmid had been successfully introduced into *A. tumefaciens*. After

the plasmid was introduced into *A. thaliana* inflorescences using the floral dip method, three T_0 plants expressing *TwNAC01* were identified via PCR and 1/2 MS Kan + medium. From these T_0 lines, three T_3 lines were generated that were confirmed to express *TwNAC01*.

Root length and leaf water loss rate in transgenic *A. thaliana* overexpressing *TwNAC01*

After two hours of drying at 25°C, the leaves of wild-type *A. thaliana* and those of *A. thaliana* expressing the empty vector had curled slightly, while the leaves of the transgenic *A. thaliana* lines overexpressing *TwNAC01* (TwNAC01-1, TwNAC01-2, and TwNAC01-3) exhibited no obvious curling (Fig. 4a). After five hours of drying at 25°C, the leaves of the wild-type and empty-vector plants were obviously curled, while the leaves of the *TwNAC01*-overexpression lines were only slightly curled. After eight hours of dehydration, the leaves of the wild-type and empty-vector plants were noticeably withered and crumpled in appearance; although the leaves of the transgenic lines were also somewhat withered, the observed degree of dehydration was much less severe (Fig. 4a). Water loss rate in the wild-type, empty-vector, and transgenic plants increased significantly over time ($P < 0.05$) (Fig. 4b). After one hour of drying, water loss rates were similar across the three lines, and, as drying continued, water loss rates increased linearly in all lines (Fig. 4b). However, for most of the drying time, water loss rates in the transgenic lines were significantly ($P < 0.05$) lower than those in the wild-type and empty-vector lines (Fig. 5b). The three transgenic lines had significantly longer roots than the wild-type and empty-vector lines (differences of 1.5-fold and 1.2-fold, respectively; Fig. 5a, 5b).

Physiological indexes of stress resistance in *A. thaliana* overexpressing *TwNAC01*

Although the relative leaf water contents of the three transgenic *Arabidopsis* lines were slightly higher than those of empty-vector and wild-type lines, these differences were not significant (Fig. 6a). However, leaf electrical conductivity (reflecting electrolyte leakage and thus membrane damage) was significantly greater in the empty-vector and wild-type lines as compared to the transgenic lines (Fig. 6b). H_2O_2 and MDA levels were significantly greater in the leaves of the wild-type line as compared to all other lines; there were no significant differences in hydrogen peroxide or MDA levels between the empty-vector plants and any of the transgenic lines (Fig. 6c, 6d).

After drought stress, *TwNAC01* was upregulated in the roots and leaves of the transgenic plants as compared to the empty-vector and wild-type plants; in all three transgenic lines, *TwNAC01* gene expression was significantly greater in the roots than the leaves (Fig. 6e). On average, TwNAC01 gene expression levels in the transgenic A. thaliana lines were 8-fold and 38-fold greater than the empty-vector and wild-type plants, respectively. Thus, in response to drought stress, TwNAC01 was upregulated in transgenic A. thaliana overexpressing TwNAC01 as compared to mock-transformed and wild-type A. thaliana.

Expression of *TWNAC01* after VIGS in triticale under drought stress

A 327 bp sequence was amplified using the VIGS primers (Figure S4a, Fig. 5). After transformation of the amplified sequence into *E. coli*, positive clones were identified via PCR amplification (Figure S4b, S4c). After drought stress, *TwNAC01* gene expression levels in the control plants were significantly greater than those

in the *TwNAC01*-silenced plants (BSMV-TwNAC01). In contrast, drought stress significantly upregulated *TwNAC01* in the empty-vector (BSMV-*γb*) and indicator-gene (BSMV-*PDS*) plants as compared to the control plant. This indicated that drought stress upregulated the triticale *TwNAC01* gene; after *TwNAC01* gene silencing, *TwNAC01* was downregulated, even under drought conditions (Fig. 7).

Phenotypic implications of *TwNAC01* gene silencing in triticale

About two weeks after inoculation with the BSMV vectors, the indicator-gene (BSMV-*PDS*) plants began to exhibit symptoms of stripe mosaic virus. At 20 days after inoculation, large areas of the leaves of these plants were bleached, while the leaves of empty-vector (BSMV-*γb*) and *TwNAC01*-silenced (BSMV-TwNAC01) plants showed slight bleaching (Fig. 8a). In general, the growth potential of each of the inoculated groups (BSMV-γb, BSMV-PDS, and BSMV-TwNAC01) was weaker than that of the control group (Fig. 8b, 8c). The mean RWC of the leaves of BSMV-TwNAC01 was significantly lower than that of the leaves of the CK (Fig. 8d). The roots of the TwNAC01-silenced plants were significantly shorter than those of the control, empty-vector, and indicator-gene plants (Fig. 8e, 8f). This suggested that the TwNAC01 gene substantially affects triticale root growth, and that TwNAC01 gene silencing inhibits triticale root development. This showed that silencing the *TwNAC01* gene significantly reduced triticale growth.

Physiological indexes of drought stress and photosynthesis in *TwNAC01*-silenced triticale

After drought stress, levels of H2O2 and MDA in the leaves of BSMV-TwNAC01 plants were significantly higher than those in the leaves of uninfected control plants (Fig. 9a, 9c), while RWC was significantly lower (Fig. 9b). This demonstrated that the triticale leaves were more stressed by drought when the TwNAC01 gene was silenced. That is, the stress-resistance ability of the plant decreased after TwNAC01 gene silencing. This suggested that TwNAC01 played an important role in triticale stress resistance.Net photosynthetic rate, stomatal conductance to H_2O, intracellular CO_2 level, and transpiration rate were significantly lower in the BSMV-TwNAC01 leaves as compared to the control (Fig. 9d–g); net photosynthetic rate and intracellular CO2 level were significantly lower than the control in the BSMV-γb and BSMV-PDS leaves (Fig. 9d, 9f), while transpiration rate was significantly lower than the control in the BSMV-PDS leaves (Fig. 9g). Net photosynthetic rate (Fig. 9d), stomatal conductance (Fig. 9e), intercellular CO_2 concentration (Fig. 9f), and transpiration rate (Fig. 9g) of control, BSMV-γb, BSMV-PDS, and BSMV-TwNAC01 triticale leaves decreased gradually as drought stress increased. Thus, TwNAC01 gene silencing significantly affected photosynthesis-related indexes in the leaves of BSMV-infected plants subjected to drought stress, again demonstrating that TwNAC01 silencing weakened the stress resistance of triticale.

Discussion

NAC transcription factors are considered the most important family of transcription factors in plants; these transcription factors play various important roles in the stress response, as well as in the regulation of plant growth and development (Xu et al., 2015). Stress-related NAC transcription factors have been well examined in wheat, rice, and *Leymus triticoides*, but relatively few studies have explored stress-related NAC transcription factors in allohexaploid triticale. Here, we used RACE RNA-seq and RT-PCR to clone the first

putative *NAC* gene from heterohexaploid triticale. This putative *NAC* gene had more than 95% similarity with known NAC genes from other crops, including common wheat, goatgrass, and durum wheat. The molecular formula of the encoded protein was predicted to be $C_{1722}H_{2642}N_{464}O_{522}S_{19}$, with a predicted molecular weight of 38805.86 M_r, and a theoretical isoelectric point of 5.44. The total number of positive/negative charge residues predicted in this protein were 46/37, and the atomic composition was C_{1722}, H_{2642}, N_{464}, O_{522}, and S_{19}, The extinction coefficient of the predicted protein was 46,996, and its absorbance at a wavelength of 280 nm was 1.211 L/(g·cm). The total average hydrophobic coefficient of the predicted protein was − 0.494, indicating that the putative protein was hydrophilic. These findings were consistent with our phylogenetic analysis. In addition, the predicted protein sequence of the putative *NAC* gene included a conserved NAM domain between the 20th and 148th amino acids at the N-terminus, as well as three transcriptional activation regions at the C-terminus. We thus concluded that the putative gene was indeed an *NAC* gene. This gene was designated *TwNAC01.*

Our results showed that *TwNAC01* was upregulated by a variety of abiotic stressors and signal molecules, including salinity, drought, PEG6000, and ABA. Several studies have shown that about 20–25% of plant *NAC* genes respond to stress treatments and participate in stress alleviation (Puranik et al., 2012; Nuruzzaman et al., 2010; Fang et al., 2008). In addition, *A. thaliana* overexpressing the wheat *NAC* genes *TaNAC2* and *TaNAC67* exhibited significantly improved tolerance of drought, salinity, and cold as compared to the wild-type (Mao et al., 2014). Similarly, the overexpression of wheat *NAC* genes (*TaNAC2a, TANAC4a, TaNAC6, TaNAC7, TaNAC13*, and *TaNTL5*) in tobacco significantly improved drought tolerance (Tang et al., 2012). Previous studies have also shown that *NAC* gene expression increases in response to drought stress (Wu et al., 2009; Chen et al., 2014; Nakashima et al., 2007). Consistent with this, *TwNAC01* expression was significantly upregulated in the drought-stressed plant roots and young grains as compared to the unstressed controls. This suggested that *TwNAC01* expression is induced by drought stress, and that this gene plays an important role in the response to drought in triticale.*NAC* genes have also been shown to be upregulated in aging plant tissues, by treatment with plant signal molecules (e.g., ABA, ethephon, JA, and SA) (Jensen et al., 2010; Tang et al., 2012; Bu et al., 2008; Xia et al., 2010), and by exposure to ethylene and methyl jasmonate (Scharrenberg et al., 2003). Here, the upregulation of *TwNAC01* in response to ABA and various abiotic stressors indicated that the abiotic stress process may be regulated by the ABA hormone in triticale.The upregulation of *TwNAC01* in response to stress was stronger in the roots as compared to the leaves, consistent with a previous study, which showed that *TaNAC4* was more strongly upregulated in wheat roots as compared to leaves and stems (Xian et al., 2010). This suggested that *TwNAC01* will be overexpressed in triticale roots first in response to stress, in order to promote the development of plant roots and reduce the damage caused by adverse conditions.

The mechanisms by which *NAC* transcription factors alleviate drought stress in plants have been well studied. For example, the overexpression of the *NAC* gene *ATAF1* in *A. thaliana* decreased transpiration rate and increased drought tolerance (Christiansen et al., 2011). Similarly, rice overexpressing *OsNAP* had lower water loss rates during vegetative growth, increased sensitivity to exogenous ABA, and improved tolerance of salt, drought, and low temperature stress (Negi et al., 2018). Also in rice, NAC transcription factors regulate the expression of *OsSRO1c* genes, which are primarily expressed in guard cells; the overexpression

of *OsSRO1c* increases H_2O_2 accumulation in guard cells and reduces the number of completely open stomata, thus reducing water loss via transpiration (You et al., 2013). Finally, the *SNAC3* gene in rice targets a ROS-scavenging gene, and *SNAC3* overexpression upregulates this target gene (Fang et al., 2015). These previous results suggest that, when plants are under stress, the *NAC* gene can reduce transpiration rate by promoting plant root elongation, improving H_2O_2 and MAD accumulation, and increasing leaf water content. Ultimately, these factors enhance plant adaptability to adversity. Here, *TwNAC01* expression was successfully silenced in triticale BSMV-*TwNAC01* plants: *TwNAC01* expression levels in the triticale BSMV-*TwNAC01* plants were significantly lower than those in the control, BSMV-*γb*, and BSMV-*PDS* plants. In general, the growth potential of the *TwNAC01*-silenced plants was significantly lower than that of the other lines. In particular, the *TwNAC01*-silenced plants had shorter roots and reduced water content as compared to the other plants. Under drought conditions, markers of physiological stress (i.e., MDA and H_2O_2 levels) were significantly increased in *TwNAC01*-silenced plants as compared to the controls, while relative water content and markers of photosynthetic activity (net photosynthetic rate, stomatal conductance, transpiration rate, and intercellular CO_2 concentration) were significantly reduced. The observed changes in these physiological indexes suggested that triticale growth and stress resistance were substantially impaired by *TwNAC01* silencing.

In Conclusion, The overexpression of the triticale *TwNAC01* gene in *A. thaliana* improved the drought resistance of *A. thaliana* by increasing the water retention capacity of the leaves, reducing cellular membrane damage, decreasing the production of ROS in the leaves, and promoting root elongation. In *TwNAC01*-silenced triticale, leaf relative water content and the root length were significantly decreased as compared to the control, while leaf H_2O_2 and MDA levels were significantly increased. Leaf net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were also significantly lower in the *TwNAC01*-silenced plants as compared to the control. These results indicated that *TwNAC01* silencing decreased the drought resistance of triticale, suggesting that the *TwNAC01* gene plays an important role in the response of triticale to drought stress.

Declarations

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.

Author Contributions

GK planned and designed the research and wrote the main manuscript text; MW performed most of the experiments and data acquisition; LR, XW, HG, SY and YL helped with experiments and data analysis; MW participated in figure preparation and manuscript organization. All the authors agreed on the contents of the paper and declare no conflicts of interest.

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Figures

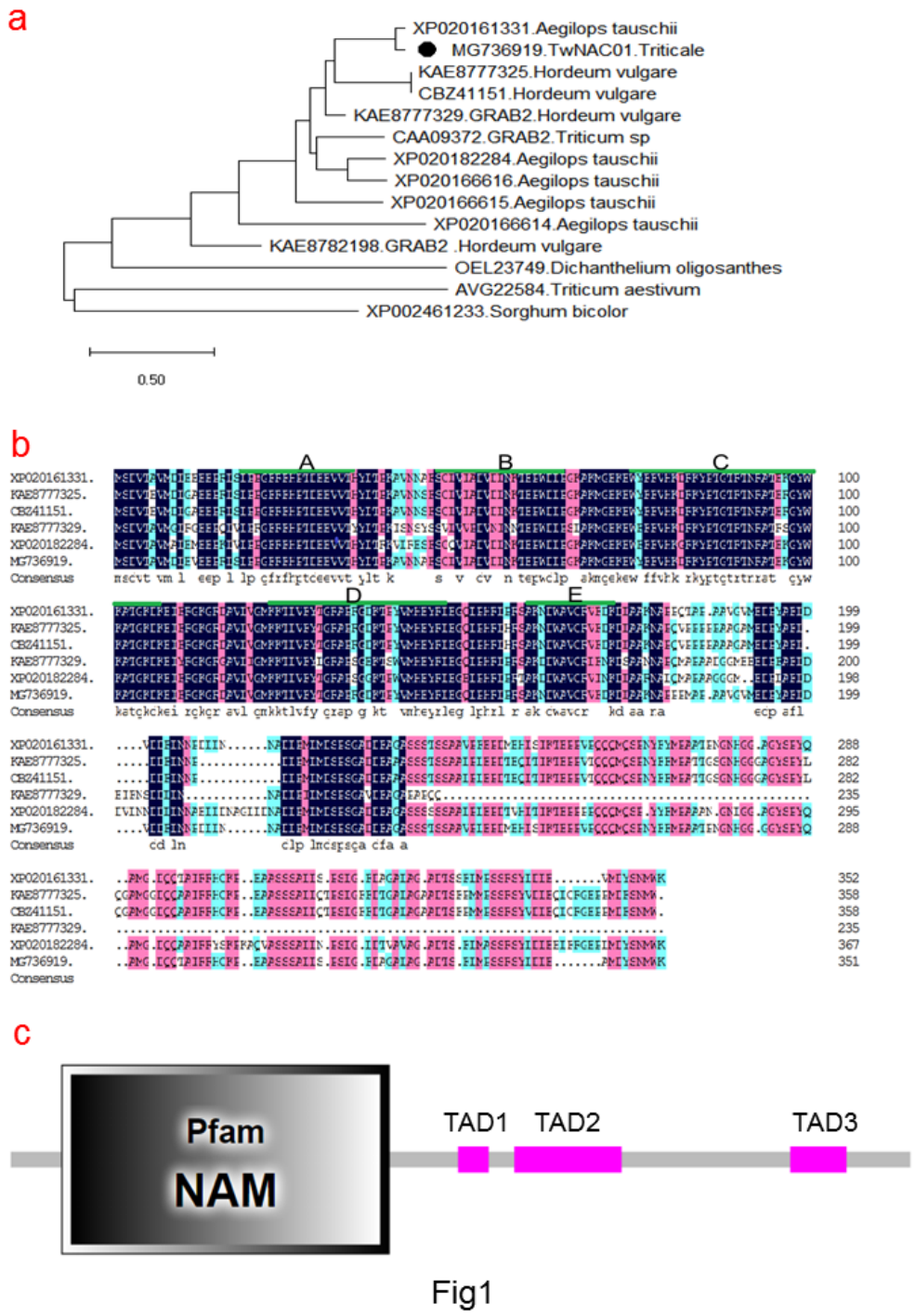

Fig1

Figure 1

Phylogenetic relationships among NAC proteins in plants, and the conserved NAM superfamily in TwNAC01. a. Neighbor-joining phylogenetic relationships between the TwNAC01 protein and NAC proteins in other plants. b. Amino acid sequence alignment analysis of the conserved homeodomain region of TwNAC01 with its closest homologs from barley, wheat, goatgrass, and durum wheat. c. The conserved NAM superfamily domain in the TwNAC01 protein. N, N-terminal NAC superfamily conserved domain; C, C-terminal transcriptional activation region; TAD, transcription activation domain.

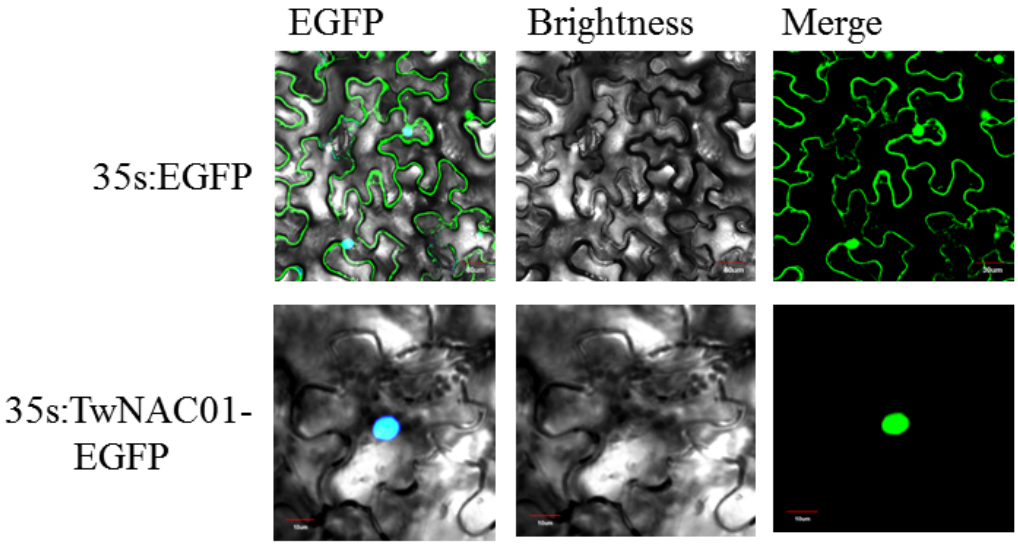

Fig 2

Figure 2

Subcellular localization of the TwNAC01 protein in the nuclei of tobacco cells.

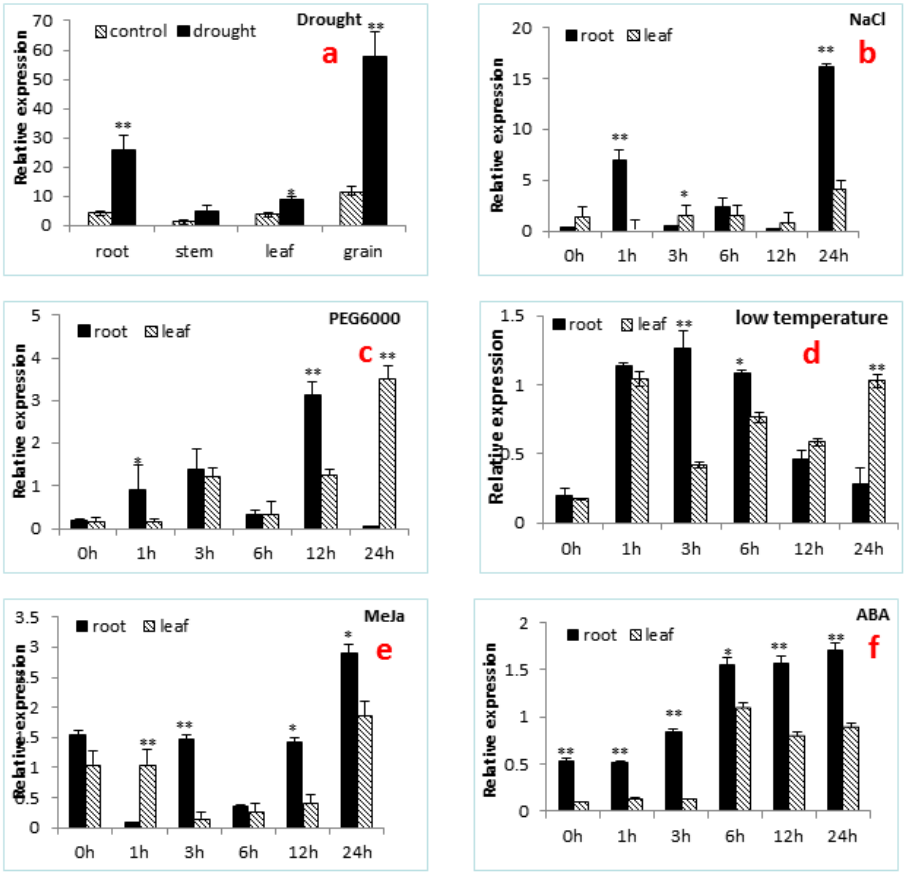

Fig 3

Figure 3

TwNAC01 gene expression patterns in triticale after stress treatments. Triticale plants were subjected to one of the following treatments: drought (A), 200 mM NaCl (B), 20% PEG6000 (C), cold (4°C) (D), 100 μM ABA (E), or 100 μM MeJA (F). Relative expression levels in the plant tissues were determined using qRT-PCR and the 2−ΔΔCT method. Transcript levels were normalized to the wheat actin genes (TaActin). Values shown are the means ± SE of three replicates of three independent samples. Asterisks indicate statistically significant differences between the root and the leaf (* $P < 0.05$; ** $P < 0.01$).

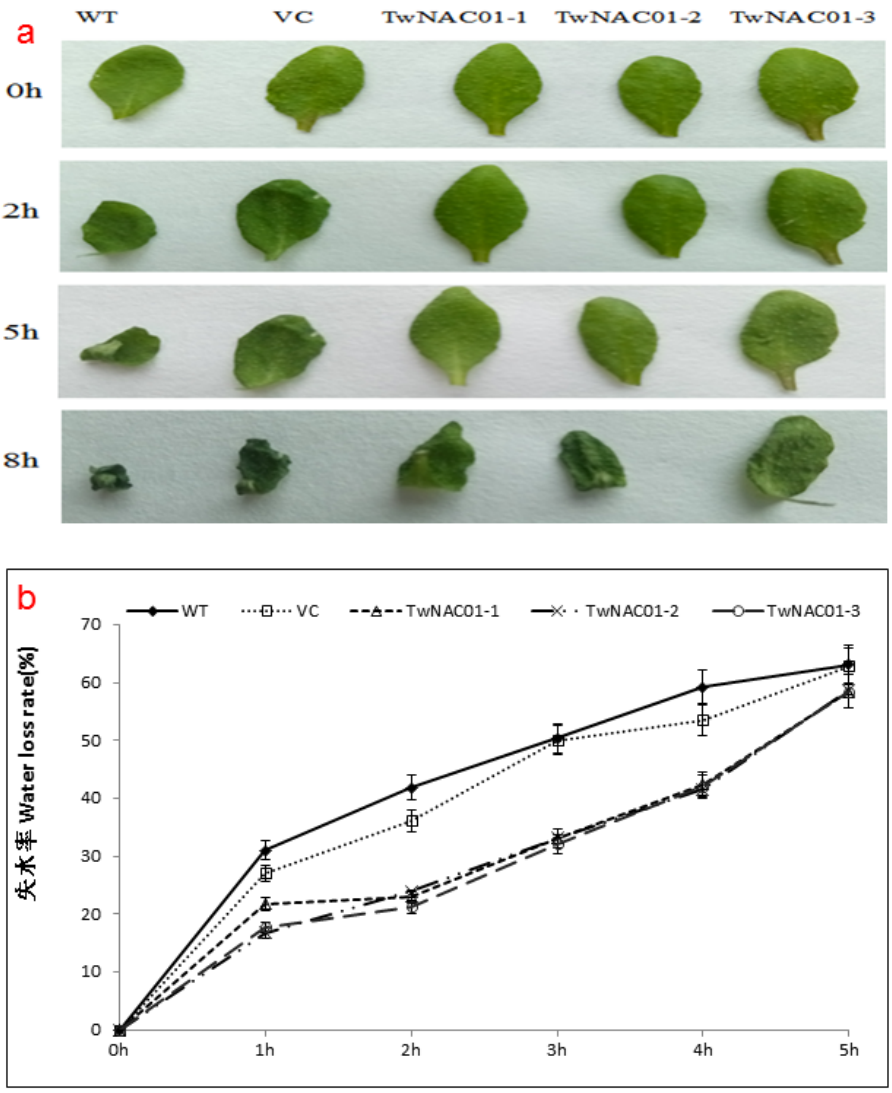

Fig 4

Figure 4

Three lines overexpressing TwNAC01 (TwNAC01-1, TwNAC01-2, and TwNAC01-3) exhibit reduced rates of water loss in the leaves. WT, VC (vector control). a. Leaves of various Arabidopsis lines after 0–8 hours of dehydration. b. Rates of moisture loss in the leaves of various Arabidopsis lines. Values are means ± SE of three replicates. Asterisks indicate statistically significant differences from the WT (**P< 0.01).

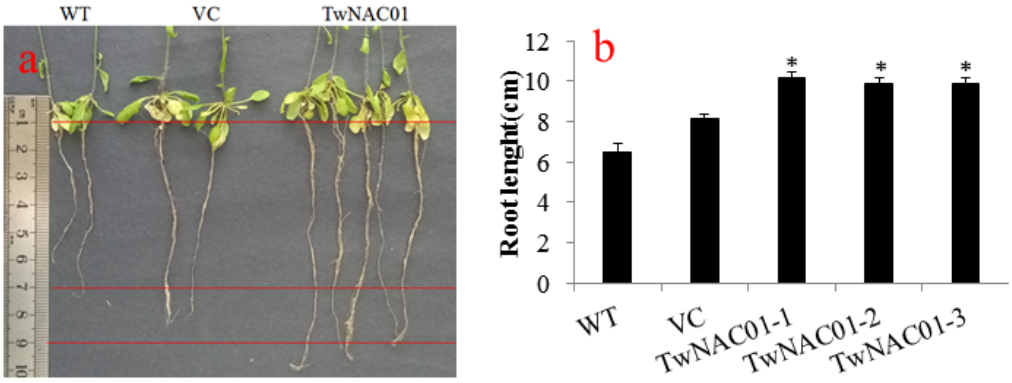

Fig 5

Figure 5

Root lengths in the WT, VC (vector control), and transgenic lines overexpressing TwNAC01. A. primary root length of WT, VC, and transgenic plants grown for 55 d. b. Statistical analysis of the primary root lengths of WT, VC, and transgenic plants grown for 55 d. Values shown are the means ± SE of three replicates of three independent samples. Asterisks indicate statistically significant differences from the WT (*P< 0.05).

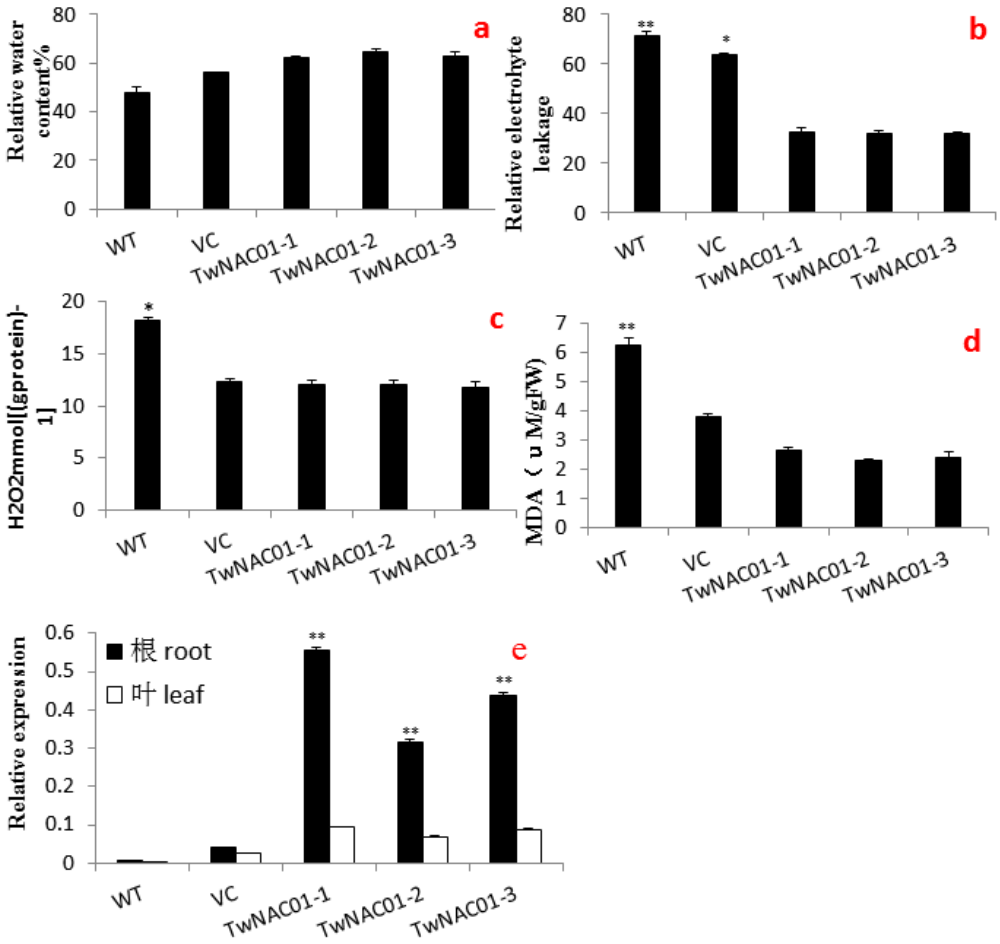

Fig 6

Figure 6

Analysis of physiological indices under drought stress conditions. Analyses of leaf relative water content (a), leaf electrolyte leakage, (b), H2O2 content (c), and MDA content (d) in WT and transgenic lines overexpressing TwNAC01 lines under normal and drought-stress conditions. Relative expression of TwNAC01 in the roots and leaves of transgenic Arabidopsis thaliana lines (e). Values are means ± SE of three replicates. Asterisks indicate statistically significant differences from the WT (*P< 0.05)

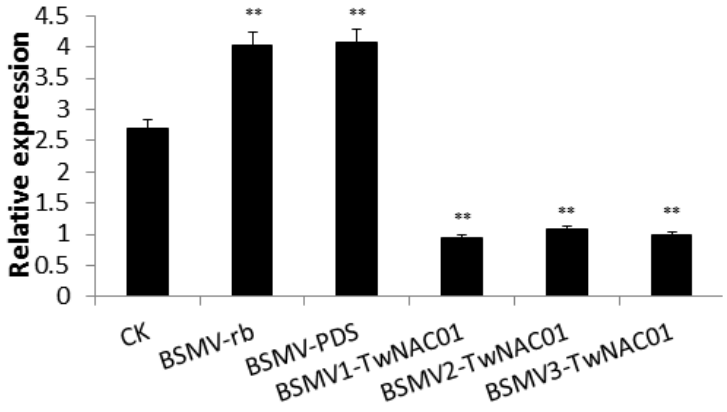

Fig 7

Figure 7

Relative TwNAC01 expression patterns in triticale after TwNAC01 gene silencing, as determined using qRT-PCR and the 2−ΔΔCT method. Transcript levels were normalized to TaActin. CK, vector control; BSMV-γb, empty vector; BSMV-PDS, BSMV plus indicator gene; BSMV-TwNAC01, vector carrying silenced TwNAC01. Values shown are the means ± SE of three replicates of three independent samples. Asterisks indicate statistically significant differences from the CK (vector control) plants (* $P < 0.05$; ** $P < 0.01$).

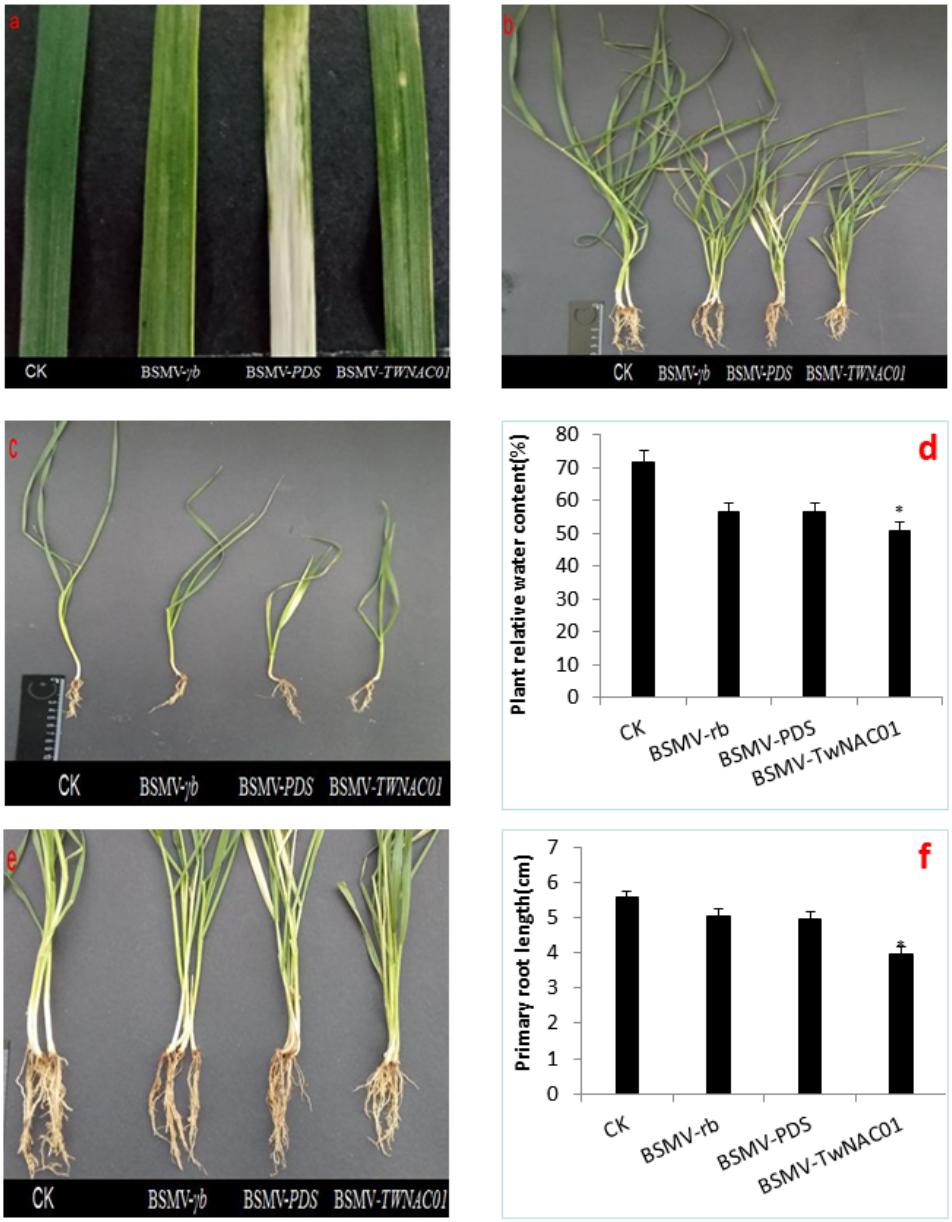

Fig 8

Figure 8

Phenotypic differences among triticale plants inoculated with different BSMV vectors for the VIGS of TwNAC01. a. Leaves, showing viral infection. b, c. Biomass comparison. d. Relative water contents. e. Root lengths, visual comparison. f. Root length quantification. Values shown are means ± SE of three replicates. Asterisks indicate statistically significant differences from control (uninfected) plants (* $P < 0.05$; ** $P < 0.01$). CK, control; BSMV-γb, empty vector; BSMV-PDS, BSMV plus indicator gene; BSMV-TwNAC01, vector carrying silenced TwNAC01.

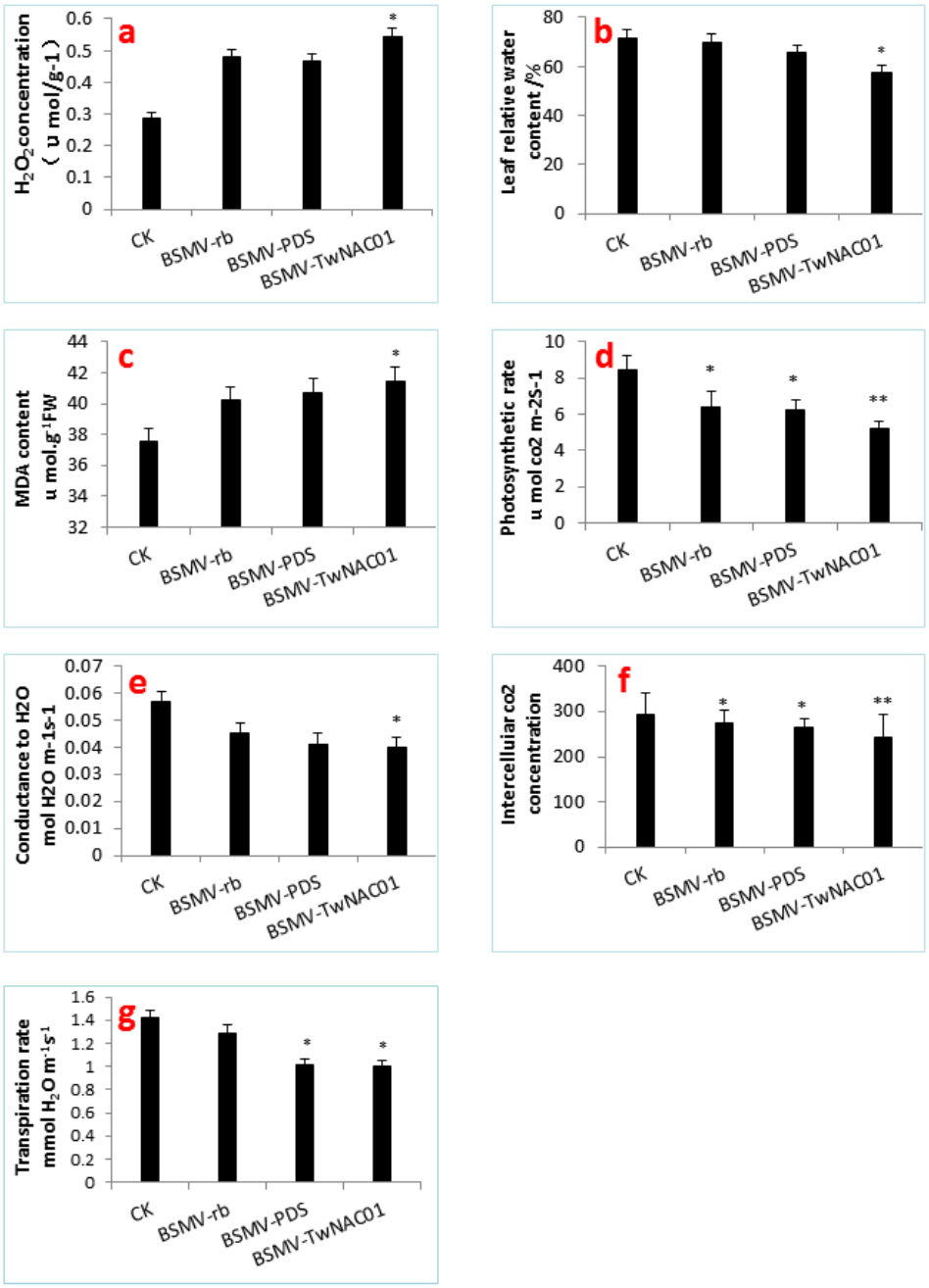

Fig 9

Figure 9

Physiological indexes of drought stress and photosynthesis in triticale leaves after virus-induced gene silencing using BSMV. a. H2O2 concentration. b. Relative water content (RWC). c. Malondialdehyde (MDA) content. d. Photosynthetic rate. e. Stomatal conductance to H2O. f. Intercellular CO2. g. Transpiration rate. Values shown are means ± SE of three replicates. Asterisks indicate statistically significant differences from control (uninfected) plants (* P < 0.05; ** P < 0.01). CK, control; BSMV-γb, empty vector; BSMV-PDS, BSMV plus indicator gene; BSMV-TwNAC01, vector carrying silenced TwNAC01.

Supplementary Files

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