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RESEARCH PAPER

# *TaNAC2*, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in *Arabidopsis*

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## Abstract

**Environmental stresses such as drought, salinity, and cold are major factors that significantly limit agricultural productivity. NAC transcription factors play essential roles in response to various abiotic stresses. However, the paucity of wheat NAC members functionally characterized to date does not match the importance of this plant as a world staple crop. Here, the function of *TaNAC2* was characterized in *Arabidopsis thaliana.* A fragment of *TaNAC2* was obtained from suppression subtractive cDNA libraries of wheat treated with polyethylene glycol, and its full-length cDNA was obtained by searching a full-length wheat cDNA library. Gene expression profiles indicated that *TaNAC2* was involved in response to drought, salt, cold, and abscisic acid treatment. To test its function, transgenic *Arabidopsis* lines overexpressing *TaNAC2–GFP* controlled by the cauliflower mosaic virus 35S promoter were generated. Overexpression of *TaNAC2* resulted in enhanced tolerances to drought, salt, and freezing stresses in *Arabidopsis*, which were simultaneously demonstrated by enhanced expression of abiotic stress-response genes and several physiological indices. Therefore, *TaNAC2* has potential for utilization in transgenic breeding to improve abiotic stress tolerances in crops.**

**Key words:** Phenotype, physiological trait, stress response, transcription factor.

## Introduction

Environmental stresses such as drought, salinity, and extreme temperature impose osmotic stress on plants and significantly affect both biomass and grain yields of crops worldwide. To protect cellular activities and maintain whole plant integrity, plants have developed various mechanisms to cope with abiotic stresses. Many stress-induced genes have been identified, including those encoding key enzymes for abscisic acid (ABA) biosynthesis (Nambara and Marion-Poll, 2005), proteins involved in osmotic adaptation and tolerance to cellular dehydration (Yao *et al.*, 2011), cellular protective enzymes (Puckette *et al.*, 2007), numerous signalling proteins such as protein kinases/protein phosphatases (Zhu, 2002), and transcription factors (Mochida *et al.*, 2009).

Transcription factors have been grouped into diverse families on the basis of conserved structural domains involved in DNA binding to *cis*-elements in the promoters of target genes, or other functional modular structures. Increasing evidence is demonstrating that numerous transcription factors, such as DREB, CBF, bZIP, zinc-finger, MYB, and NAC, are directly or indirectly involved in the regulation of plant defence and stress responses (Thomashow, 1999; Zhu, 2002; Seki *et al.*, 2003; Shinozaki *et al.*, 2003; Fujita *et al.*, 2004; Mukhopadhyay *et al.*, 2004; Chen *et al.*, 2006).

The NAC (NAM, ATAF, and CUC) superfamily is one of the largest transcription factor families found only in plants. Proteins of this family are characterized by a highly conserved DNA-binding domain, known as the NAC domain, in the N-terminal region. The C-terminal region of NAC proteins, which usually contains a transcriptional activation domain, is highly diversified both in length and sequence (Ooka *et al.*, 2003). More than 100 members of this family have been identified in both *Arabidopsis* and rice

(Qu and Zhu, 2006; Fang *et al.*, 2008). NACs play important roles in diverse aspects of plant development, including pattern formation in embryos and flowers, formation of secondary walls, leaf senescence (Guo and Gan, 2006; Zhong *et al.*, 2006, 2007; Mitsuda and Ohme-Takagi, 2008), and lateral root development (Xie *et al.*, 2000; He *et al.*, 2005). Overwhelming data demonstrate that NACs are involved in responses to various biotic and abiotic stresses, including responses to a range of pathogens, drought, salt, cold, and low-oxygen stress. In *Arabidopsis*, three NAC members, ANAC019, ANAC055, and ANAC072, bind to the ERD1 promoter region to produce enhanced tolerance to drought stress (Tran *et al.*, 2004). ANAC2 is involved in response to plant hormones, such as ABA, 1-aminocyclopropane-1-carboxylic acid, and α-naphthaleneacetic acid, salt stress, and lateral root development (He *et al.*, 2005). ATAF1 and ATAF2, along with a barley counterpart known as HvNAC6, play negative roles in response to drought stress (Delessert *et al.*, 2005; Lu *et al.*, 2007) and enhance pathogen resistance (Jensen *et al.*, 2007). In addition, AtNAC102 is involved in regulating seed germination under low-oxygen conditions (Christianson *et al.*, 2009). Many NACs have been characterized in rice. *SNAC1* is involved in response to drought stress in guard cells and its overexpression results in significant enhanced drought tolerance at anthesis under field conditions (Hu *et al.*, 2006). Overexpression of the root-specific NAC transcription factor *OsNAC10* improves drought tolerance and grain yield in rice under field conditions (Jeong *et al.*, 2010). Overexpression of *SNAC2*/*OsNAC6*, *OsNAC045*, and *OsNAC063* enhances tolerance to multiple abiotic stresses (Nakashima *et al.*, 2007; Hu *et al.*, 2008; Yokotani *et al.*, 2009; Zheng *et al.*, 2009). *SINAC1* and *SINAM1* are involved in salt response in tomato (Yang *et al.*, 2010). Diverse expression patterns of nine NACs under various biotic and abiotic stresses were characterized in *Brassica napus* (Hegedus *et al.*, 2003). For wheat, it is well documented that GRAB1 and GRAB2 are involved in inhibition of DNA replication of a wheat dwarf geminivirus in cultured cells (Xie *et al.*, 1999); whereas *TaNAM-B1* participates in the promotion of leaf senescence and can improve grain protein, zinc, and iron contents (Uauy *et al.*, 2006). Recent research showed that *TaNAC4* and *TaNAC8* are involved in both biotic and abiotic stress responses in wheat (Xia *et al.*, 2010a, b). Collectively, many reports indicate that numerous characterized NAC members are involved in response to environmental stimuli and that different NAC members play various roles in response to abiotic stress. However, the paucity of wheat NAC members functionally characterized to date does not match the importance of this plant as a world staple crop.

In this study, *TaNAC2*, a NAC transcription factor member from common wheat, was cloned and its expression patterns in response to water deficiency, high salinity, low temperature, and ABA were identified. Transgenic experiments indicated that *TaNAC2* increases tolerance to drought, salt, and freezing stresses in *Arabidopsis*. Morphological assays revealed no obvious negative effects caused by *TaNAC2* overexpression, suggesting a potential for utilizing the gene in improving tolerance to abiotic stresses in crop plants.

## Materials and methods

### *Plant materials and abiotic stress experiments*

Wheat (*Triticum aestivum* L.) genotype 'Hanxuan 10' with a prominent drought-tolerant phenotype was utilized in this study. The growing conditions and stress treatment assays for wheat seedlings have been described previously (Mao *et al.*, 2010). To investigate the genomic origin of the target gene, 16 accessions of various wheat species – four A genome accessions (two *Triticum urartu* and two *Triticum monococcum*), three S genome accessions (*Aegilops speltoides*, the putative B genome donor), three diploid D genome accessions (*Aegilops tauschii*), three AB genome accessions (*T. dicoccoides*), and three hexaploid wheat accessions – were selected for PCR. To further identify the genomic location of the target gene, 38 nulli-tetrasomic (NT) lines of Chinese Spring were used for chromosome location.

*Arabidopsis thaliana* (ecotype Columbia), chosen for transgenic studies, was grown in a controlled environment chamber at 22 °C, with a 12/12 photoperiod, light intensity of 120 mmol $m^{-2}$ $s^{-1}$, and 70% relative humidity. Four T3 homozygous transgenic lines were randomly selected for functional analysis and all seeds used to perform phenotypic assays, including wild-type (WT) and green fluorescent protein (GFP) controls, were same-batch-harvested. To identify the expression pattern of drought stress response genes in *Arabidopsis*, transgenic lines and WT planted on MS medium were treated with –0.5 MPa polyethylene glycol (PEG) solution for 3 h.

### *Construction and screening of a full-length cDNA library database*

Tissues from wheat seedlings at various growth stages and from mature plants were collected to extract total RNA with TRIZOL reagent (Invitrogen); mRNA was isolated with oligo(dT) cellulose (Qiagen). Several full-length cDNA libraries of wheat in Lambda Zap II (Stratagene) were constructed by the optimized Cap-trapper method (Mao *et al.*, 2005). A full-length wheat cDNA library was generated with the 3′ end and 5′ sequencing data of full-length cDNA clones. A 349-bp cDNA fragment encoding a NAC-like C-terminal domain was obtained by sequencing from suppression subtractive cDNA libraries of wheat treated with PEG (Pang *et al.*, 2007) and used as a query probe to screen the full-length wheat cDNA library. Four candidate clones were obtained by blastn and the full-length cDNA of the target gene was also identified by blastn.

Database searches of the nucleotide and deduced amino acid sequences were performed through NCBI/GenBank/Blast. Alignment and similarity analysis of sequences from different species were performed by the MegAlign program in DNAStar. Signal sequence and transmembrane regions were predicted with SignalP (http://genome.cbs.dtu.dk/services/SignalP) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The secondary structure was predicted with PREDATOR (http://bioweb.pasteur.fr/seqanal/protein/intro-uk.html), and the functional region was identified using PROSITE (http://web.expasy.org/docs/swiss-prot_guideline.html).

### *Construction of a phylogenetic tree of* TaNAC2

Phylogenetic analysis was performed to understand the relationship between TaNAC2 and NAC members from wheat and other plant species. A maximum likelihood tree based on putative amino acid sequences was constructed with the proml program in the PHYLIP software package (version 3.69). The bootstrap parameter was set at 100.

### *Genetic characterization of* TaNAC2

To analyse the structure of *TaNAC2*, three pairs of primers flanking the open reading frame were designed and one pair of primers specifically amplifying the A genome allele of *TaNAC2* was obtained (forward, 5′-TGCAGAGTTCCACGATAGGCCG-3′; reverse, 5′-CCTACCGACCCAACGAACGAG-3′). The primers were further used to amplify the *TaNAC2* genomic sequence for gene structure analysis and chromosome identification.

### *Subcellular localization of* TaNAC2 *protein*

The full-length open reading frame of *TaNAC2* was fused upstream of the GFP gene and put under the control of the constitutive cauliflower mosaic virus 35S promoter in the pJIT163–GFP expression vector to construct a 35S::TaNAC2–GFP fusion protein. Restriction sites were added to the 5′ and 3′ ends of the coding region by PCR; the oligonucleotides for fusion GFP subcloning were: forward, 5′-CTCT**AAGCTT**TCATCGGCAGCGGAGCGATT-3′ (*Hin*dIII site in bold); reverse, 5′-CTCT**GGATCC**GCGGACACGGGGGGA-3′ (*Bam*HI site in bold). The PCR product obtained was digested with the relevant restriction endonucleases, ligated with the pJIT163–GFP plasmid, and cut with the corresponding enzyme to create a recombinant plasmid for expressing the fusion protein. Positive plasmids were confirmed by restriction analysis and sequenced. Recombinant constructs were transformed into living onion epidermal cells by biolistic bombardment with a GeneGun (Biorad Helios) according to the instruction manual (helium pressure, 150–300 psi). The subcellular location of *TaNAC2* was detected by monitoring the transient expression of GFP in onion epidermal cells as described.

### *Quantitative real-time PCR*

After treatment with deoxyribonuclease I, RNA samples were used as templates for cDNA synthesis by the Superscript First-Strand Synthesis System kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed in triplicate with an ABI Prism 7900 system using the SYBR Green PCR Master Mix kit (Applied Biosystems), according to the manufacturer's instructions. *Tubulin* transcript was used to quantify relative transcript levels. The qRT-PCR primers were: forward, 5′- ATCGGCAGCGGAGCGATT-3′; reverse, 5′- AGGGGTCGAAGCGGTAGAGG-3′. Relative gene expression levels were detected using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen; 2001), using the following formula:

$$\Delta\Delta C_T = \left(C_{T,\text{Target}} - C_{T,\text{Tubulin}}\right)_{\text{Time}\times} - \left(C_{T,\text{Target}} - C_{T,\text{Tublin}}\right)_{\text{Time 0.}}$$

The $C_T$ (cycle threshold) values for both target and internal control genes were means of triplicate independent PCRs. Time X is any treated time point (1, 3, 6, 12, 24, 48, or 72 h) and Time 0 represents the untreated time (0 h).

*Actin* transcript was used to quantify the expression levels of *TaNAC2* and abiotic stress-response genes in transgenic *Arabidopsis* plants. Two transgenic lines, with the lowest and highest *TaNAC2* expression levels, were selected to measure expression levels of the abiotic stress response genes. The oligonucleotides for abiotic stress-response genes have been described elsewhere (Ding *et al.*, 2009).

### *Generation of transgenic plants*

The coding region of *TaNAC2* cDNA was amplified by reverse-transcription PCR using primers 5′-CTCT**AAGCTT**TCATCGGCAGCGGAGCG-3′ (*Hin*dIII site in bold) and 5′-CTCT**GGATCC**GCGGACACGGGGGGAGGCG-3′ (*Bam*HI site in bold) and cloned into the pPZP211 vector as a GFP-fused fragment driven by the CaMV 35S promoter (Hajdukiewicz *et al.*, 1994). The transformation vectors harbouring a 35S::*GFP* or 35S::*TaNAC2*–GFP, were introduced into *Agrobacterium* and transferred into WT *Arabidopsis* plants by the floral dip transformation method (Bent, 2006). Positive transgenic lines were screened on kanamycin plates and then identified by reverse-transcription PCR and fluorescence detection.

### *Morphological characterization of transgenic plants*

Transgenic plants were characterized for morphological changes under short days (12/12 light/dark cycle) in a growth chamber with a constant temperature of 22 °C. Root morphologies were examined on plants grown on MS medium solidified with 1.0% agar. Briefly, T3 homozygous transgenic and WT seeds were germinated on MS medium and grown vertically for 8 d before measurement of primary root lengths. For biomass measurement, transgenic plants and the two controls were planted in identical pots filled with vermiculite/humus (1:1) and cultured under well-watered conditions.

### *Water loss rate determination*

Water loss rates were measured using 10 plants each of WT and transgenic plants (including GFP transgenic plants). Four-week-old plants were detached from roots and weighed immediately (fresh weight, FW), the plants were then left on the laboratory bench (humidity, 45–50%, 20–22 °C) and weighed at designated time intervals (desiccated weights). The proportions of fresh weight loss were calculated relative to the initial weights. Plants were finally oven-dried for 24 h at 80 °C to a constant dry weight (DW). Water contents (WC) were measured according to the formula: WC (%) = (desiccated weight – DW)/(FW – DW) × 100.

### *Cell membrane stability measurement*

Plant cell membrane stabilities (CMS) were determined with a conductivity meter (DDS-1, YSI), CMS (%) = (1 – initial electrical conductivity/electrical conductivity after boiling) × 100. Seven-day-old seedlings (grown on 1 × MS medium, 0.8% agar) were transferred to a horizontal screen; seedling roots were completely submerged in PEG-6000 (25.4%, –1.4 MPa) or NaCl (250 mM). When signs of stress began to appear on WT plants, seedlings were removed and immediately thoroughly rinsed with double distilled water ($ddH_2O$) prior to immersion in 20 ml $ddH_2O$ at room temperature. After 2 h the initial conductivities of the solutions were recorded. Samples were then boiled for 30 min, cooled to room temperature and the final conductivities were measured.

### *Determination of osmotic potential*

Osmotic potential (OP) was measured with a Micro-Osmometer (model 210, Fiske Associates). Measurements were taken in the freezing point mode at room temperature. Five plants of each line were pooled as a sample, which was finely ground using a mortar and pestle before being transferred to a microcentrifuge tube. The supernatant tissue sap was obtained after centrifuging at 12 000 rpm for 5 min at room temperature. Three replications were made for each line and the osmotic potential for each sample was measured three times. Free proline was extracted and quantified from fresh tissues of well-watered seedlings (0.5 g) as described by Hu *et al.* (1992).

### *Chlorophyll fluorescence assays*

Chlorophyll fluorescence was measured with a portable chlorophyll fluorescence meter (OS 30P, Opti-sciences) after applying stress treatment of 350 mM NaCl for 24 h. Fully expanded leaves of stressed plants were selected to determine the chlorophyll fluorescence parameters; three measurements were made for each plant and 20 plants were used for WT and transgenic samples. The maximum efficiency of photosystem II photochemistry, $F_v/F_m$

$= (F_m - F_0)/F_m$, was determined to assess changes in the primary photochemical reactions determining photosynthetic potential at an early stage of salinity stress.

### Abiotic stress tolerance assays

Drought tolerance assays were performed on seedlings. Both WT and transgenic seeds were grown on MS medium. Seven-day-old seedlings were planted in sieve-like rectangular plates (3 cm deep) filled with soil mixture and well watered. Seedlings were cultured in a greenhouse (22 °C, 70% relative humidity, 120 μmol $m^{-2}$ $s^{-1}$, 12/12 light/dark cycle) without watering.

For salt tolerance assays, seedlings were planted in sieve-like plates and well watered as described for the drought tolerance treatment. Water was withheld for 2 weeks before irrigation with NaCl solution (250 mM) from the bottoms of the plates. When the soil was completely saturated with salt water, the NaCl solution was removed and the plants were cultured normally.

For cold tolerance assays, four seedlings were planted in identical pots and cultured as described above. Seedlings at 4 weeks were stressed in a –10 °C freezer for 1.5 h and then cultured at 15 °C for 24 h to facilitate recovery before culturing under normal growing conditions.

## Results

### Molecular characterization of TaNAC2

A full-length cDNA of the target gene was obtained by screening full-length wheat cDNA libraries. Blast results showed it was highly homologous to *SNAC1* and almost identical to *TaNAC2* (AAU08786) in the 5′ and 3′ untranslated regions and the open reading frame (Xue, 2005). It was therefore considered to be *TaNAC2* and named accordingly. The *TaNAC2* cDNA is 1559 bp in length and consists of 203 bp of 5′ untranslated region, 990 bp of open reading frame, and 366 bp of the 3′ untranslated region. The open reading frame encodes a polypeptide of 329 amino acid residues with a predicted molecular mass of 36.75 kD and pI value of 6.55. The deduced amino acid sequence shows relatively high homologies with counterpart monocot NAC family members, viz. those of *Oryza sativa* and *Zea mays*, and lower homologies with other NAC family members from dicot species, such as *Glycine max* and *A. thaliana*. *TaNAC2* has 74.8% identity to OsNAC1 (ABD52007), 74.7% to ZmNAC1 (NP_001123932), 60.1% to OsNAC3 (NP_001059213), 57.1% to TaGRAB1 (CAA09371), and very low identity with NACs from dicot species, including ANAC102 and ATAF2.

Scansite analysis indicated that *TaNAC2* has characteristic N-terminal and C-terminal regions. The N-terminal region contains a NAC domain (19–172 amino acid residues), which is highly conserved across both monocots and dicots, and functions as a DNA-binding domain (Fig. 1A). The C-terminal transcription activation region is extraordinarily divergent and is thought to be involved in regulation of downstream gene expression under different environmental stimuli. Secondary structure prediction revealed that the *TaNAC2* sequence forms seven α-helixes and four β-pleated sheets.

### Phylogenetic analysis

A phylogenetic tree was constructed with the putative amino acid sequences of TaNAC2 and some NAC family members from other species. TaNAC2 clustered in the same clade as OsNAC1/SNAC1 and ZmNAC1 (Fig. 1B); SNAC1 had earlier been shown to enhance tolerance to drought and salt stress in transgenic rice (Hu *et al.*, 2006).

### Genetic characterization of TaNAC2

The genomic sequence of *TaNAC2* was amplified with genome-specific primer pairs as described in materials and methods. The genomic sequence of *TaNAC2* was about 1.4 kb, consisting of two exons and one intron, with all splicing sites complying with the GT-AG rule. To investigate the genomic origin of *TaNAC2*, 16 accessions of various wheat species were subjected to PCR. As shown in Fig. 2A, the target fragment was amplified by all accessions carrying the A genome, indicating that *TaNAC2* originated from the A genome. This result was confirmed by PCR results for 38 nulli-tetrasomic Chinese Spring wheat lines (Fig. 2B). *TaNAC2* was amplified in all lines except NT5A5B and NT5A5D, indicating that *TaNAC2* was located on chromosome 5A.

### Early response of TaNAC2 to hyperosmotic stresses

Diverse expression levels of *TaNAC2* were characterized by qRT-PCR of seedling leaves. Different expression patterns were observed under water deficiency, salt, low temperature (LT), and ABA treatment (Fig. 3). *TaNAC2* was significantly activated by salt, LT, and water-deficit stresses, but relatively weakly by ABA. Among the four stresses, *TaNAC2* was extremely sensitive to NaCl and LT stresses at the early stage of treatment. The expression patterns and maximum expression levels differed for each stress. The expression levels peaked at 3 h for NaCl, 12 h for ABA, 24 h for PEG, and 72 h for cold, with the corresponding maxima being 116, 9, 29, and 160 greater, respectively, than the control.

### Subcelluar localization of TaNAC2

Transcription factors are typically localized in cell nuclei where they perform DNA binding and transcriptional activation roles. However, the *TaNAC2* polypeptide sequence had no signal peptide and transmembrane region, suggesting that it might not interact with the cell membrane. To identify the cellular localization of *TaNAC2*, the expression and distribution of *TaNAC2* were examined in both transgenic *Arabidopsis* roots and onion epidermal cells by expression of the fusion protein with GFP using fluorescence microscopy. As Fig. 4A shows, *TaNAC2*–GFP was highly expressed in transgenic *Arabidopsis* roots, especially in young root tips, and the well-organized distribution pattern suggested that *TaNAC2* might localize in the nucleus. To further identify the subcelluar localization, *TaNAC2*–GFP was transiently expressed in onion epidermis, and microscopy showed that *TaNAC2* was localized in nuclei (Fig. 4B).

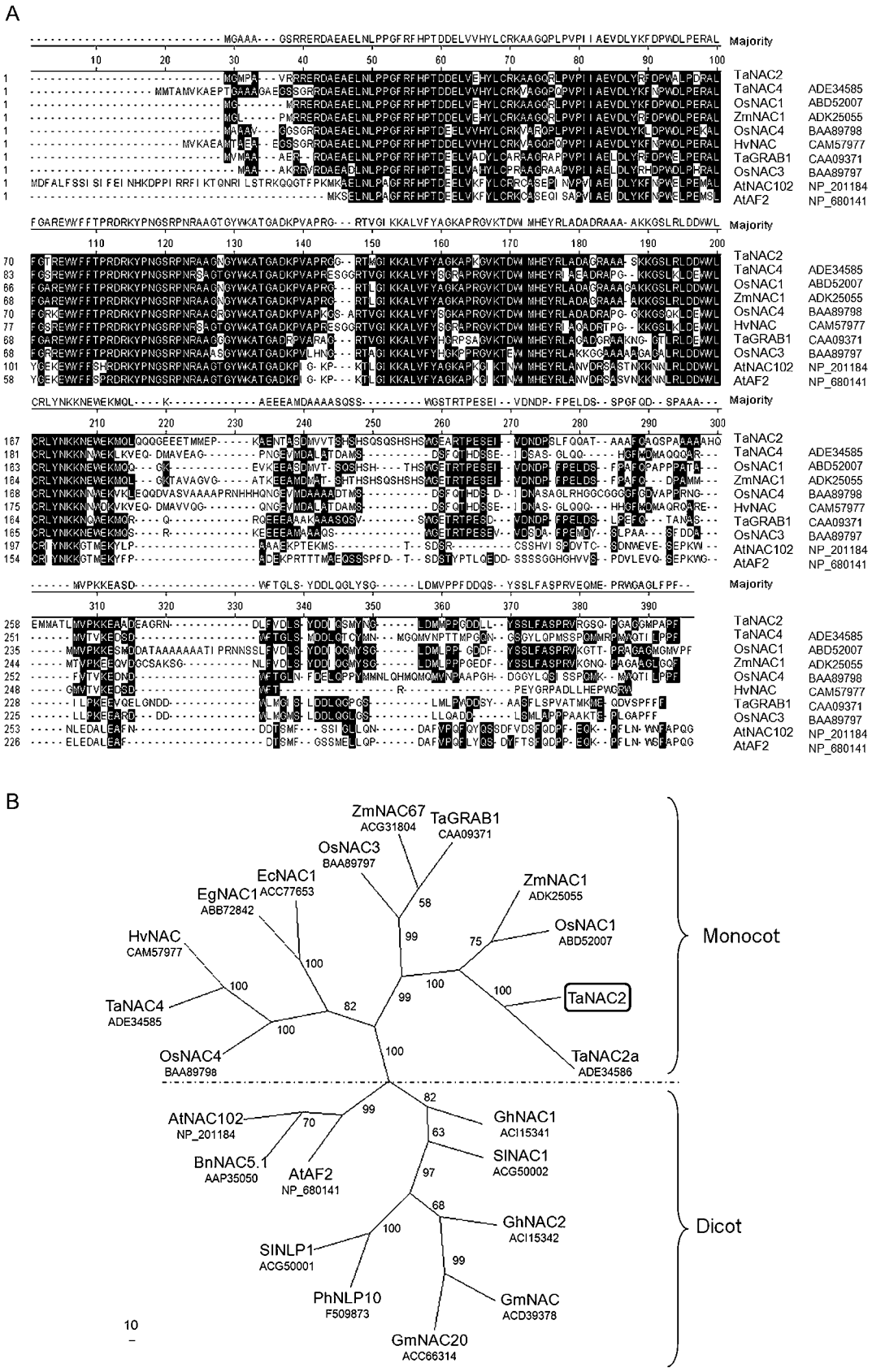


**Fig. 1.** Sequence alignment of *TaNAC2* and NACs in various plant species. (A) Amino acid alignment of *TaNAC2* and other NAC family members from selected plant species. Gaps (dashed lines) were introduced for optimal alignment. The numbers on the left indicate amino acid positions. Identical amino acid residues are shaded black. The region underlined indicates the conserved NAC-domain. Alignments were performed using MegAlign of DNAStar. (B) Phylogenetic tree of *TaNAC2* and NAC members from other plant species.

### *Morphological characteristics of* TaNAC2-*overexpressing* Arabidopsis *plants*

The phenotypes of transgenic *Arabidopsis* plants with *TaNAC2* were assessed at different developmental stages. The expression levels of *TaNAC2* in four T3 homozygous lines were measured prior to phenotypic assays (Supplementary Fig. S1, available at *JXB* online). Primary roots of *TaNAC2* transgenic lines were longer than the WT and GFP controls, and in three of the four lines the differences were significant (F-test, $*P < 0.05$; Fig. 5). There was no difference between the transgenic lines and WT control for germination rate, lateral root number, and seedling size. For seedlings (4 weeks old) grown in soil, there was no obvious difference between transgenic and WT plants under well-watered conditions, except that the flowering date of *TaNAC2* transgenic lines was 3–5 days earlier than WT (data not shown). The final biomass per plant of *TaNAC2* transgenic lines was higher than the WT control, but the differences were not significant (Supplementary Fig. S2).

### TaNAC2 *transgenic lines have improved physiological traits for abiotic stress*

To assess water retention ability of transgenic *Arabidopsis* plants, the four transgenic lines, along with WT and GFP controls were subjected to a detached-rosette rate of water loss assay. Fresh weights were recorded seven times over a 5.5 h period. The four transgenic lines showed lower rates of water loss at each time point (Fig. 6A). The final relative water contents of *TaNAC2* rosettes were significantly higher than those of the WT and GFP controls (F-test, $**P < 0.01$; Fig. 6B).

The four transgenic lines grown under well-watered conditions were tested for osmotic potential (OP). The OP of three of the transgenic lines were significantly lower than those of WT and GFP (F-test, $*P < 0.05$, $**P < 0.01$); the difference between WT and GFP transgenic lines was not significant. Thus overexpression of *TaNAC2*, but not GFP alone, appeared to reduce OP (Fig. 7A). To probe the reason for OP reduction in transgenic plants, free proline contents were determined. In this series of experiments there was no difference between *TaNAC2* transgenic lines and controls in terms of free proline content (data not shown).

Chlorophyll fluorescence is an effective parameter for revealing early signs of stress and is a suitable way to screen for stress tolerance in plants (Chaerle *et al.*, 2007). To further evaluate photosynthetic potential, the four transgenic lines were subjected to a chlorophyll fluorescence assay. Under normal conditions, $F_v/F_m$ ratios for the transgenic lines were similar to the controls (data not shown). Under severe salt stress conditions, $F_v/F_m$ ratios for all *TaNAC2* transgenic lines were significantly higher than the controls (F-test, $**P < 0.01$; Fig. 7B).

To examine the response of *TaNAC2* transgenic lines against hyperosmotic stress, the lines were subjected to physiological assays of drought and salt stresses. After germination on MS medium, 7-day-old seedlings were treated separately with 25.4% (–1.4 MPa) PEG-6000 and NaCl (250 mM). Twenty hours later, when signs of PEG stress began to appear on WT and GFP plants, samples were collected for CMS measurements. The CMS of transgenic lines were significantly higher than the controls (F-test, $*P < 0.05$; Fig. 7C), indicating that PEG stress damage on WT plants was more severe than on *TaNAC2* plants. For salt stress, symptoms appeared on control plants 4 h after NaCl treatment, but no symptoms were identified on *TaNAC2* transgenic lines. CMS determinations revealed that the CMS for most *TaNAC2* plants was higher than that of WT and GFP, and the CMS of three transgenic lines reached significant levels (F-test, $*P < 0.05$, $**P < 0.01$; Fig. 7C).

### TaNAC2 *transgenic lines have pronounced tolerance to multi-abiotic stresses*

To characterize the performance of *TaNAC2* transgenic lines under drought stress in soil, the four lines were tested at the seedling stage. After a 30-day water-withholding period, the rosette leaves of WT and GFP plants became dark, whereas the *TaNAC2* transgenic lines remained green. On the 35th day, all WT and GFP plants displayed severe wilting (all rosette leaves were severely curled and some were dead), whereas only some of the *TaNAC2* transgenic lines showed signs of severe water stress and the rosette leaves of some transgenic plants were still green and fully expanded. Three days after rewatering, all WT and GFP plants were dead, whereas 30–60% of *TaNAC2* transgenic lines survived the stress (Fig.8A, D).

To determine whether *TaNAC2* overexpression enhances tolerance to salt stress, *Arabidopsis* seedlings grown in soil were exposed to 250 mM NaCl solution. About 20 h later, the leaf tips of control plants began to crimple, but no signs of salt stress were observed on the transgenic lines. Three days later, the rosette leaves of WT began to bleach, and again there no signs of salt stress on the transgenic lines. Seven days later, it was evident that most transgenic plants were much greener than the control plants. Fifteen days after stress (withholding water), more than 90% of WT and GFP plants were dead, whereas 37–53% of transgenic plants were still alive (Fig. 8B, D).

To examine response to cold stress, plants of the same lines were put into a freezer and subjected to freezing tress. Only 18–22% WT and GFP rosette leaves survived the severe cold stress, whereas the survival rates of rosette leaves on *TaNAC2* transgenic lines reached 32–71%, and the survival rates of transgenic plants (79–85%) were also much higher than WT (56%) and GFP (67%; Fig. 8C, D).

At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Ec, *Eleusine coracana*; Eg, *Elaeis guineensis*; Gh, *Gossypium hirsutum*; Gm, *Glycine max*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; Ph, *Petunia hybrida*; Sl, *Solanum lycopersicum*; Ta, *Triticum aestivum*; Zm, *Zea mays*. The tree was constructed with the PHYLIP 3.69 package. Bootstrap values are in percentages.

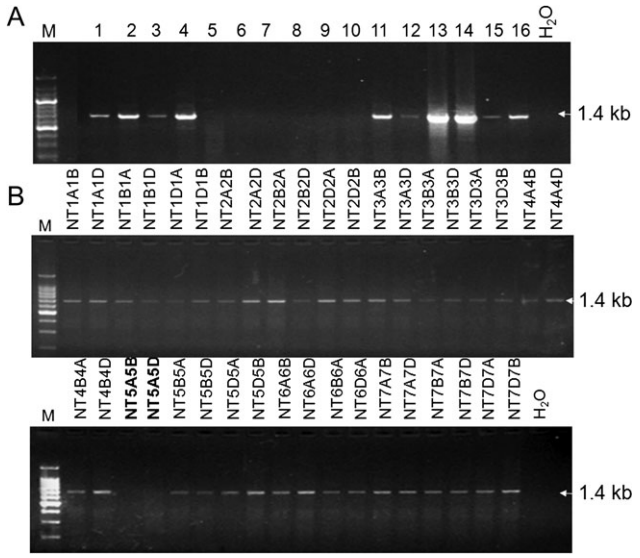


**Fig. 2.** Chromosome location of *TaNAC2*. (A) Genomic origin of *TaNAC2* among 16 accessions of wheat and related species. Lanes: 1, 2, *Triticum urartu*; 3, 4, *Triticum monococcum*; 5–7, *Aegilops speltoides*; 8–10, *Aegilops tauchii*; 11–13, *Triticum durum*; 14–16, *Triticum aestivum*; M, 200-bp DNA ladder. (B) Chromosome location of *TaNAC2* using 38 nulli-tetrasomic (NT) lines of Chinese Spring. The gene was not amplified by NT5A5B or NT5A5D. M, 200-bp DNA ladder.

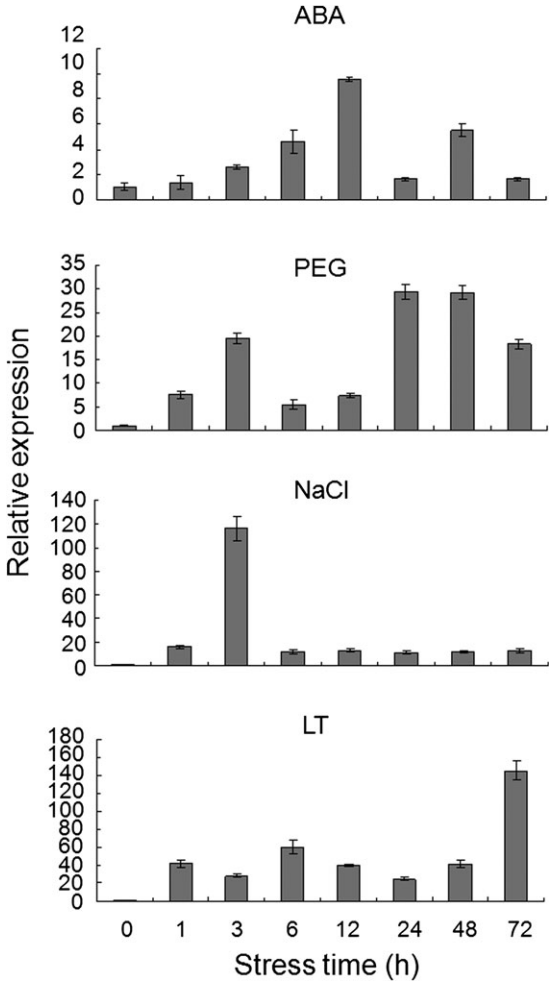


**Fig. 3.** Expression patterns of *TaNAC2* in response to stress treatments: abscisic acid (ABA), polyethylene glycol (PEG, –0.5 MPa), salt (NaCl), and low temperature (LT) Two-leaf seedlings of common wheat cv. Hanxuan 10 were exposed to abiotic stresses as described in Materials and methods. The $2^{-\Delta\Delta CT}$ method was used to measure the relative expression levels of the target gene in stressed and non-stressed leaves. Means were generated from three independent measurements; bars indicate standard errors.

### *Enhanced expression of abiotic stress-response genes in* TaNAC2 *plants*

Morphological assays indicated that *TaNAC2* transgenic lines had enhanced tolerance to drought, salt, and cold stresses. To reveal the underlying molecular mechanisms, transgenic lines L2 and L3, with the lowest and highest *TaNAC2* expression, were selected for expression pattern assays under normal and water-deficit stress conditions with 10 abiotic stress-response genes – *DREB1A*, *DREB2A*, *CBF1*, *CBF2*, *RD29A*, *RD29B*, *RD22*, *COR15*, *COR47*, and *Rab18* – and four ABA synthesis or response genes – *ABA1*, *ABI1*, *ABI2*, and *ABI5*. Transcripts of four genes (*DREB2A*, *RD22*, *ABI2*, and *ABI5*) were consistently and significantly higher under both stressed and non-stressed conditions, whereas expression levels of four genes (*RD29B*, *RD29A*, *Rab18*, and *ABI1*) were significantly higher in PEG stressed plants (Fig. 9). Transcript levels of the other six genes (*DREB1A*, *CBF1*, *CBF2*, *COR15*, *COR47*, and *ABA1*) were not significantly changed (data not shown).

## Discussion

### TaNAC2 *overexpression has no adverse effects in* Arabidopsis

To investigate the *in vivo* role of *TaNAC2* in plant abiotic stress resistance, a fused *TaNAC2*–GFP was transformed into *Arabidopsis*. Before undertaking functional analyses, overexpression of *TaNAC2* was confirmed by reverse-transcription PCR and microscopy (data not shown). Growth retardation is a common phenomenon in transgenic plants, restricting the utilization of target genes for plant breeding. To evaluate the applicability of *TaNAC2* for transgenic breeding, morphological features of transgenic *TaNAC2* plants were closely monitored, but no adverse effects were identified. Root length determinations indicated that most *TaNAC2* transformed plants had longer primary roots than WT and GFP controls (Fig. 5A). A longer root system should facilitate water absorption from deeper soils, and thus strengthen drought tolerance and increase biomass under water-deficit conditions. The biomass of transgenic lines was higher than that of WT control (Supplementary Fig. S2). Overexpression of *TaNAC2* resulted in earlier flowering (data not shown), perhaps suggesting that NAC transcription factors are involved in floral development (Souer *et al.*, 1996). Future research should address this issue and a better understanding of

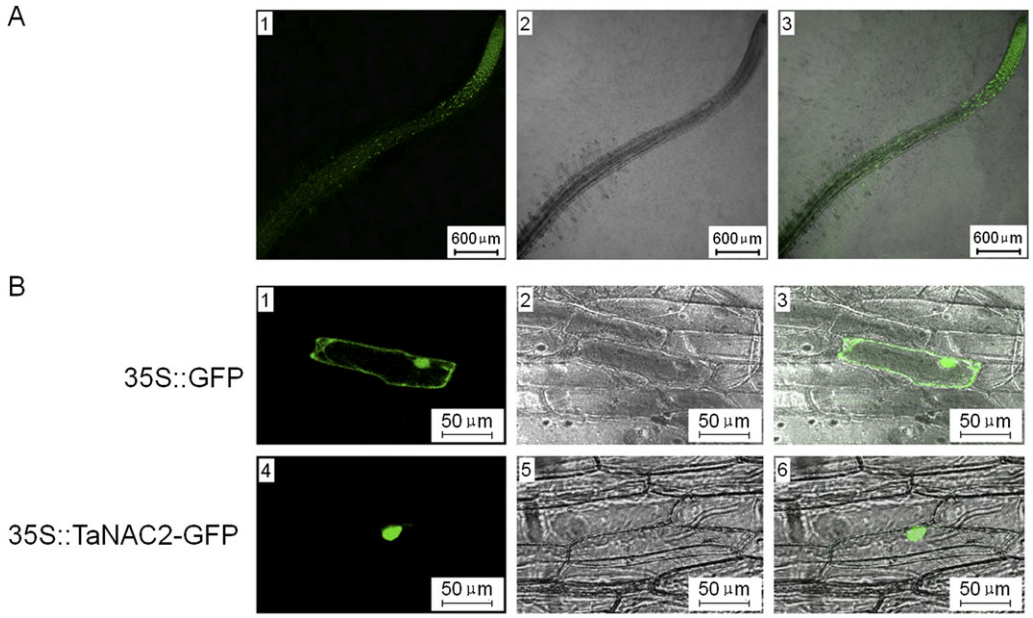


**Fig. 4.** Subcellular localizations of *TaNAC2* in transgenic *Arabidopsis* root and onion epidermal cells. (A) A construct harbouring *35S::TaNAC2–GFP* was introduced into *Agrobacterium* and transferred into *Arabidopsis* by floral infiltration. Positive transgenic lines were screened with kanamycin and then examined with a confocal microscope. Images are dark field for green fluorescence (1), bright field (2), and combined (3). (B) Cells were bombarded with constructs carrying *GFP* or *TaNAC2–GFP*. *GFP* and *TaNAC2–GFP* fusion proteins were transiently expressed under the control of the cauliflower mosaic virus 35S promoter in onion epidermal cells and observed with a laser scanning confocal microscope. Images are dark field (1, 4), bright field (2, 5), and combined (3, 6).

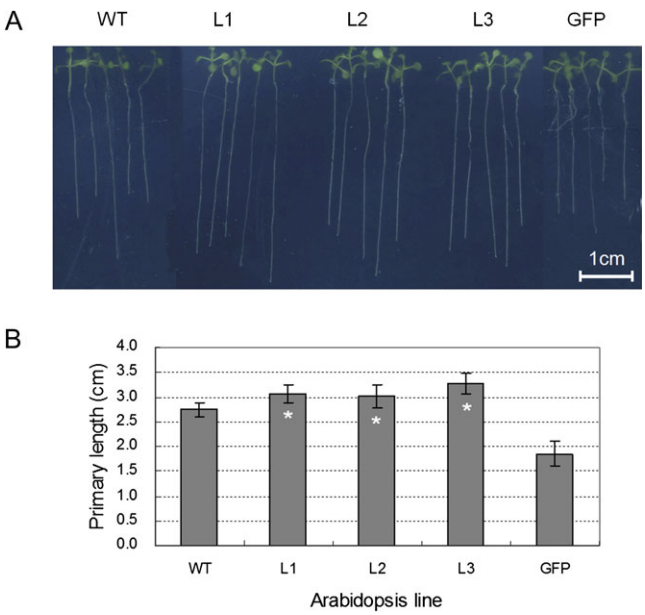


**Fig. 5.** Comparison of primary root lengths of *TaNAC2* plants. Seeds of four *TaNAC2* transgenic *Arabidopsis* lines and wild-type (WT) and green fluorescent protein (GFP) controls were planted on MS agar and cultured under short day conditions. Five seeds of each line were planted in triplicate and root lengths were measured after 8 days. (A) Three typical lines with significantly longer primary root lengths. (B) Comparison of measurements, calculated from three independent assays. *, Significantly different from wild type ($P = 0.05$).

its molecular mechanism might be beneficial in fine-tuning early maturity in crops.

### *Physiological changes in transgenic* TaNAC2 *plants under various conditions*

Environmental stresses often cause physiological changes in plants. Physiological indices, including CMS, OP, chlorophyll fluorescence, and rate of water loss, are typical physiological parameters for evaluating abiotic stress tolerance and resistance in crop plants. Plants with higher CMS and photosynthetic capacity and lower rates of water loss and OP often have enhanced tolerance or resistance to environmental stresses.

Detached-leaf water loss rate is an important parameter of plants under water-deficit conditions and has been proposed as an indicator of water status (Clarke *et al.*, 1989; Dhanda and Sethi, 1998). In the present work, the detached-leaf water loss rate of *TaNAC2* transgenic lines was lower than the WT and GFP controls, and the final water contents for transformed seedlings were significantly higher than those of the controls (Fig. 6B), strongly indicating that the transgenic lines had higher water retention capacity.

Plant survival depends on maintenance of positive turgor pressure, which is important for cell expansion and stomatal opening. Osmotic adjustment is a fundamental cell tolerance response to osmotic stress, and can be realized by the accumulation of osmoprotectants. Generally, a higher ability for osmotic adjustment means stronger adaptation and more

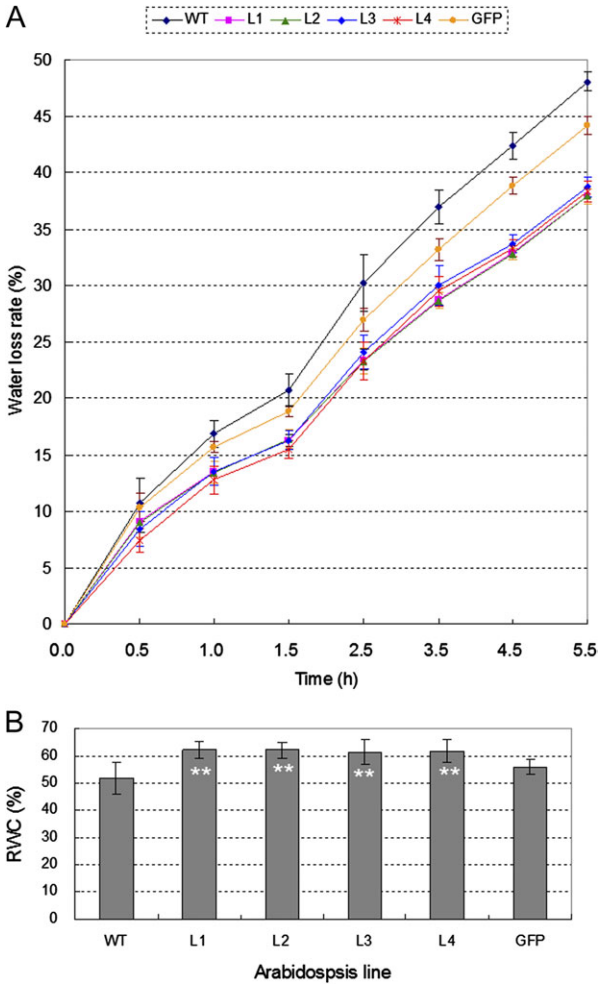


**Fig. 6.** *TaNAC2* plants have stronger water retention ability. (A) Comparison of water loss rates for detached rosettes between transgenic plants and wild-type (WT) and green fluorescent protein (GFP) controls. (B) Comparison of relative water contents (RWC) of detached rosettes of transformed plants and controls after a 5.5-hour treatment. Ten separate plants were used; values are mean ± SE.

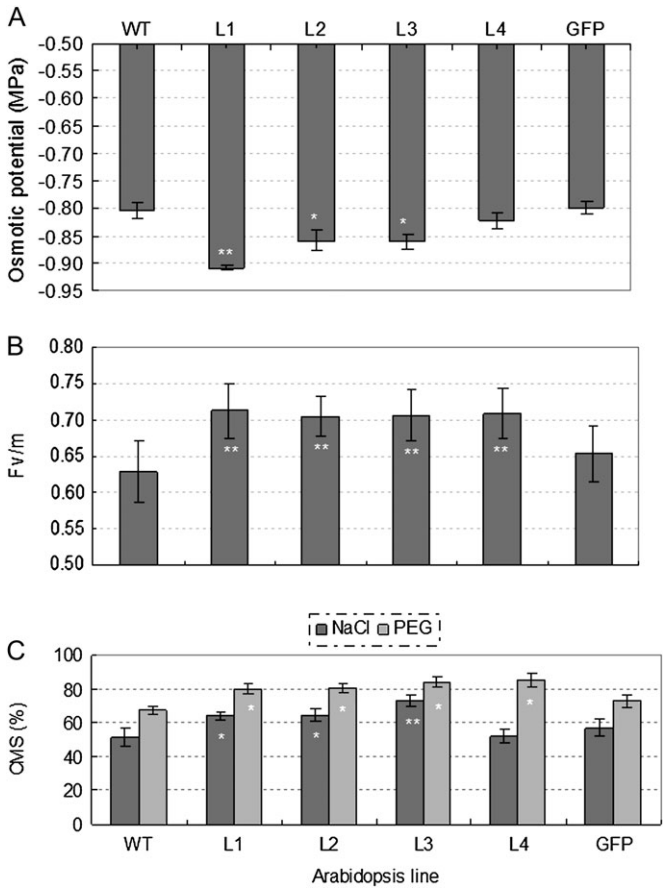


**Fig. 7.** Comparisons of physiological indices related to abiotic stress response of *TaNAC2* transgenic lines under stress conditions. (A) Transgenic *TaNAC2* plants had lower osmotic potentials than the wild-type (WT) and green fluorescent protein (GFP) controls. Four *TaNAC2* transgenic lines and controls were cultured under well-watered conditions and selected to perform osmotic potential assays. Five plants of each line were pooled as one sample and three samples were measured for each line. *, **, significantly different from wild type: *, $P = 0.05$; **, $P = 0.01$. (B) Comparison of photosynthetic potentials of *TaNAC2* transgenic lines and controls under high salinity stress. The $F_v/F_m$ ratios of four transgenic lines were significantly higher than the two controls. Twenty plants were measured; values are mean ± SE. **, significantly different from wild type, $P = 0.01$. (C) Comparison of cell membrane stability (CMS) for *TaNAC2* transgenic lines and controls following drought and salt stresses. Fifteen seedlings were pooled as one sample and three samples were measured for each line. *, ** significantly different from wild type: *, $P = 0.05$; **, 0.01. Values are mean ± SE.

tolerance to osmotic stress. Osmotic potential is a direct reflection of osmotic adjustment capability at the physiological level and has been used as an effective index to assess crop genotypes for osmotic stress tolerance. This work indicated that the OP of all transgenic lines were lower than the WT and GFP controls under well-watered conditions (Fig. 7A), indicating that the reduction of OP in transgenic plants was due to overexpression of *TaNAC2*. Decreased OP is primarily attributed to accumulation of osmoprotectants, including amino acids, quaternary amines, and various sugars. Numerous studies have shown that free proline is the most widely distributed multifunctional osmolyte in many organisms and plays important roles in enhancing osmotic stress tolerance (Bartels and Sunkar, 2005). However, increased free proline levels were not detected in transgenic plants (results not shown), suggesting that proline was not the cause of OP reduction and that *TaNAC2* was probably not involved in proline metabolism. Lower OP commonly predicts higher water retention capacity and a lower rate of water loss, as well as higher water use efficiency. The results of OP analyses were consistent with the above detached-leaf water loss rate and water content results (Fig. 6B) and partially explained the enhanced tolerances to drought, salt, and cold stresses.

Chlorophyll fluorescence from intact leaves, especially fluorescence induction patterns, is a reliable, non-invasive method to evaluate the physiological status of plants (Strasser *et al.*, 2002). The ratio of variable to maximal

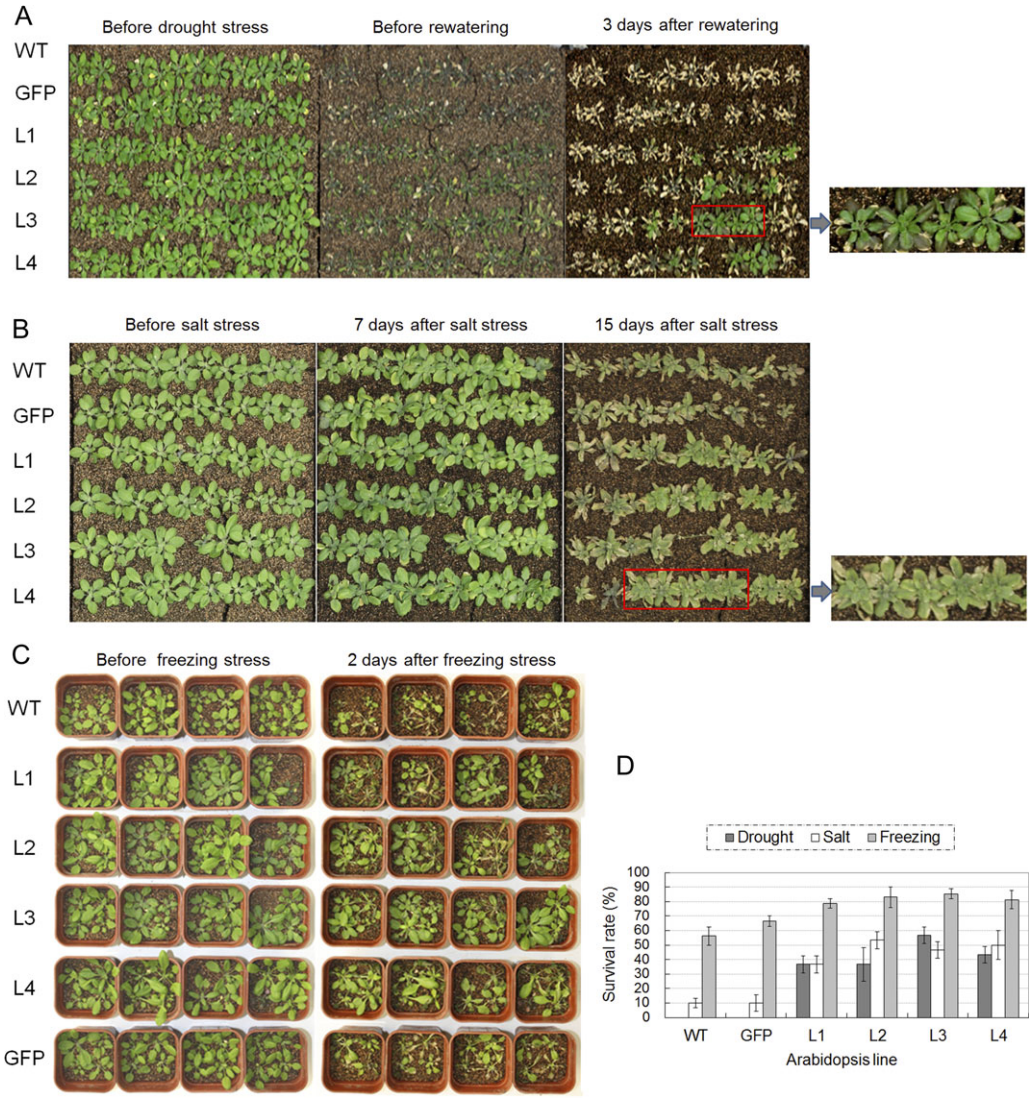


**Fig. 8.** Transgenic *TaNAC2 Arabidopsis* lines have enhanced tolerance to drought, salt, and freezing stress. (A) Phenotypes of four *TaNAC2* transgenic lines and wild-type (WT) and green fluorescent protein (GFP) controls following drought stress (A), salt stress (B), and freezing stress (C). For drought and salinity stress, ten plants of each line were grown for each treatment. Experiments were performed in triplicate. For freezing stress, pot-cultured transgenic seedlings at 4 weeks were divided into three replicates, and each replicate was stressed at −10 °C for 1.5 h. Twenty plants (five pots) of each line were used for each experiment.

fluorescence is an important parameter used to assess the physiological status of the photosynthetic apparatus. Environmental stresses that affect photosystem II efficiency are known to provoke decreases in $F_v/F_m$ ratio (Krause and Weis, 1991). In this research, lower $F_v/F_m$ ratios were evident in WT and GFP plants (Fig. 7B), suggesting that *TaNAC2* plants had more robust photosynthetic capabilities than the controls at the early stages of severe salt stress.

Cell membranes are among the first targets of adverse stresses and the maintenance of membrane integrity and stability under abiotic stress conditions is a major component of environmental stress tolerance in plants (Levitt, 1980). CMS was used for assessing tolerance to frost, heat, and desiccation (Farooq and Azam, 2006). In most of these studies, CMS exhibits a positive correlation with several physiological and biochemical parameters conditioning plant responses to environmental conditions such as water use efficiency (Franca *et al.*, 2000), OP and leaf rolling index, $K^+$ concentration, and osmotic adjustment (Munns, 2002). In this study, the CMS of *TaNAC2* transformants under both osmotic and salinity stress conditions were higher than the WT and GFP controls, demonstrating that CMS enhancement was caused by overexpression of *TaNAC2*. Because CMS has a positive relationship with several physiological and biochemical parameters, it was predicted that *TaNAC2* transformants might have strong capacities to tolerate environmental stresses, as verified by the current functional assays in *Arabidopsis* (Fig. 8).

### *Overexpression of* TaNAC2 *enhanced multi-environmental stress responses in* Arabidopsis

Numerous studies show that the plant NAC family plays critical roles in responses to hyperosmotic stress. ANAC019,

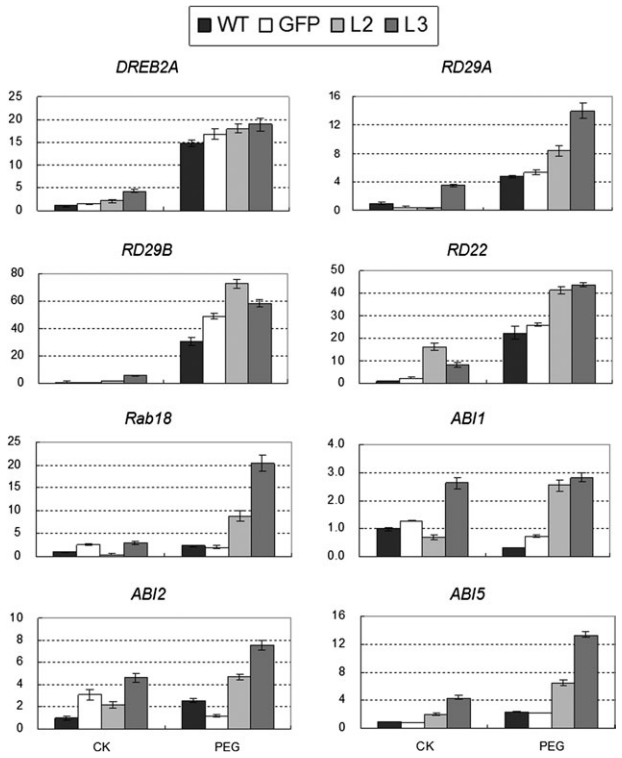


**Fig. 9.** Comparisons of relative transcript levels of *DREBA*, *RD29A*, *RD29B*, *RD22*, *Rab18*, *ABI1*, *ABI2*, and *ABI5* in control plants and *TaNAC2* transgenic lines treated with polyethylene glycol (PEG, –0.5 MPa). Vertical columns indicate relative transcript levels. Seedlings harvested before drought stress were used as controls (CK). Ten seedlings were pooled as a sample and three samples of each line were prepared for quantitative real-time PCR. Values are mean ± SE.

ANAC055, and ANAC072 confer enhanced tolerance to drought stress (Tran *et al.*, 2004). *SNAC1* and *OsNAC10* confer significant enhancement of drought tolerance (Hu *et al.*, 2006; Jeong *et al.*, 2010). Overexpression of *SNAC2*/*OsNAC6*, *OsNAC045*, and *OsNAC063* results in enhanced tolerance to multiple abiotic stresses (Nakashima *et al.*, 2007; Hu *et al.*, 2008; Yokotani *et al.*, 2009; Zheng *et al.*, 2009). In this study, the dynamic expressions of *TaNAC2* under different abiotic stresses were assessed; and overexpression led to enhanced tolerance to drought, salinity, and freezing. Both morphological and physiological evidence strongly demonstrated that the transgenic lines were more tolerant to drought, salinity, and freezing stresses than WT plants. Interestingly, there seemed to be a positive correlation between the expression levels of *TaNAC2* and improved abiotic stress tolerances in transgenic lines. Among the four selected transgenic lines, the transcripts of line 3 was significantly higher than those of the other three lines, and the tolerances of line 3 to drought, salt, and freezing stresses were better than the others (Supplementary Fig. S2, Fig. 8). It is speculated that the enhanced tolerances to abiotic stresses are mainly attributable to consistently and significantly increased expression of abiotic stress-response genes, including *DREB2A*, *ABI2*, *ABI5*, and *RD22*. *DREB2A* is a crucial regulatory element involved in drought response (Liu *et al.*, 1998). Its consistent upregulation undoubtedly increases the expression level of downstream drought stress-response genes and enhances tolerance to drought and/or other abiotic stresses due to 'cross talk' between various environmental stresses (Seki *et al.*, 2002; Xiong *et al.*, 1999). *ABI2* encodes a type-2C protein phosphatase, involved in ABA signalling (Finkelstein and Somerville, 1990); and *ABI5* encodes a basic leucine zipper transcription factor involved in altering expression of ABA-regulated genes (Finkelstein and Lynch, 2000). Their consistent high expression levels probably lead to upregulation of genes controlled by *ABI2* and *ABI5* in an ABA-dependent pathway and possibly enhance integrative tolerance to multiple abiotic stresses. Additionally, significant increases in expression of *RD29A*, *RD29B*, and *Rab18* were observed. These genes encode low-molecular-weight hydrophilic proteins (Lang and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 1993) and their significantly enhanced transcription undoubtedly leads to increases of solute in tissue sap, resulting in decreased OP of cells and reduced rates of water loss under stress conditions.

This study concerned the morphological and physiological features of *TaNAC2* overexpression in *Arabidopsis* under normal and adverse conditions, as well as potential molecular mechanisms for dynamic expression patterns of abiotic stress-response genes. The results were helpful to understanding the mechanisms of environmental stress on plants. Further ongoing research on transgenic wheat will enable the validation the functions of *TaNAC2* in enhancing tolerance to abiotic stress in crops.

## Supplementary material

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1**. Expression levels of *TaNAC2* in transgenic *Arabidopsis* lines.

**Supplementary Fig. S2**. Comparison of biomass for *TaNAC2* transgenic lines and controls.

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