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ORIGINAL PAPER

A maize stress-responsive NAC transcription factor, ZmSNAC1, confers enhanced tolerance to dehydration in transgenic *Arabidopsis*

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Abstract NAC proteins are plant-specific transcription factors that play essential roles in stress responses. However, only little information regarding stress-related NAC genes is available in maize. In this study, a maize NAC gene, *ZmSNAC1*, was cloned and functionally characterized. Expression analysis revealed that *ZmSNAC1* was strongly induced by low temperature, high-salinity, drought stress, and abscisic acid (ABA) treatment, but downregulated by salicylic acid treatment. Subcellular localization experiments in *Arabidopsis* protoplast cells indicated that ZmSNAC1 was localized in the nucleus. Transactivation assays demonstrated that ZmSNAC1 functioned as a transcriptional activator. Overexpression of *ZmSNAC1* in *Arabidopsis* led to hypersensitivity to ABA and osmotic stress at the germination stage, but enhanced tolerance to dehydration compared to wild-type seedlings. These results suggest that ZmSNAC1 functions as a stress-responsive transcription factor in positive modulation of abiotic stress tolerance, and may have applications in the engineering of drought-tolerant crops.

Key message ZmSNAC1 functioned as a stress-responsive transcription factor in response to abiotic stresses, and might be useful for crop tolerance improvement.

Keywords Abiotic stress · Maize · NAC · Stress tolerance · Transgenic plant

Abbreviations

ABA	Abscisic acid
SA	Salicylic acid
NAC	NAM, ATAF1/2 and CUC2
ORF	Open reading frame
GFP	Green fluorescent protein
MeJA	Methyl jasmonic acid
SNAC	Stress responsive NAC
PCR	Polymerase chain reaction
2, 4-D	2, 4 Dichlorophenoxyacetic acid
CaMV	Cauliflower mosaic virus
UTR	Untranslated regions
SSR	Simple sequence repeats

Introduction

Abiotic stresses, such as drought, high-salinity, and cold, adversely affect the growth and productivity of plants, including crops (Nakashima et al. 2011). As a consequence, plants have evolved a series of mechanisms to cope with such environmental adversities (Qin et al. 2011). In recent years, great achievements have been made in increasing the tolerance of plants to abiotic stresses by identifying potential stress-related genes (Nakashima et al. 2011). For example, it has been revealed that transcription factors function as important regulators and play significant

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roles in the response to abiotic stresses (Shinozaki and Yamaguchi-Shinozaki 2007; Lata and Prasad 2011; Qin et al. 2011). Overexpression of some transcription factor genes may significantly enhance stress tolerance by regulating the expression of downstream genes (Yamaguchi-Shinozaki and Shinozaki 2006; Nakashima et al. 2009) such as *OsNAC10* (Jeong et al. 2010), *ZmDREB2A* (Qin et al. 2007), and *GmNAC11* (Hao et al. 2011). Thus, characterizing the functions of stress-related transcription factors is necessary not only to better understand the molecular mechanisms of plant responses to various abiotic stresses but also to improve crops.

NAC (*Petunia NAM*, *Arabidopsis ATAF1/2* and *CUC2*) proteins belong to a plant-specific transcription factor superfamily, whose members contain a conserved sequence known as the DNA-binding NAC-domain in the N-terminal region and a variable transcriptional regulatory C-terminal region (Souer et al. 1996; Aida et al. 1997). Based on its motif distribution, the NAC-domain can be divided into five sub-domains (A–E) (Ooka et al. 2003). Because of their highly diverse sequences, subdomains B and E were suggested to play different roles compared to subdomains C and D, which are reported to function in DNA binding (Kikuchi et al. 1999; Duval et al. 2002). At least 117 and 151 predicted NAC family members have been identified in *Arabidopsis* and rice, respectively (Nuruzzaman et al. 2010), and are classified into two large groups, including 18 subgroups, on the basis of predicted NAC-domain amino acid sequences (Riechmann et al. 2000; Ooka et al. 2003).

The NAC transcription factors regulate a number of biochemical processes that protect plants under different stress conditions (Tran et al. 2010). Overexpression of *NAC* genes significantly increases the drought and salt tolerance of a number of plants (Zheng et al. 2009; Gao et al. 2010; Xia et al. 2010; Le et al. 2011). For example, transgenic *Arabidopsis* plants overexpressing either *ANAC019*, *ANAC055*, or *ANAC072* show significantly improved drought tolerance (Tran et al. 2004). A stress-inducible rice *NAC* gene, *SNAC1*, strongly enhances the plant's drought resistance and salt tolerance, with no effect on yield (Hu et al. 2006). Recently, *OsNAC5*, a new ABA-dependent transcription factor gene, was reported to regulate stress-inducible genes and to confer enhanced stress tolerance to rice (Takasaki et al. 2010; Song et al. 2011).

As described above, there have been great achievements in research on the functions of NAC transcription factors in model plants, especially in rice and *Arabidopsis*. However, only a few NAC members have been characterized in maize (Zimmermann and Werr 2005; Liu et al. 2009; Verza et al. 2010). In this study, we cloned an *NAC* gene, *ZmSNAC1*, in the maize inbred line "CN165", which showed a high sequence similarity to *SNAC1* of rice. *ZmSNAC1* expression was regulated by various abiotic stresses and phytohormone treatments. Furthermore, ZmSNAC1 was demonstrated to function as a nucleus-located transcriptional activator. *ZmSNAC1*-overexpressing transgenic *Arabidopsis* showed enhanced sensitivity to ABA and osmotic stress in the germination stage, and exhibited increased tolerance to dehydration in the seedling stage, without any pleiotropic effects. These data suggest that ZmSNAC1 functions as a stress-responsive transcription factor in response to abiotic stresses, and might be useful in molecular breeding to improve crop stress tolerance.

Materials and methods

ZmSNAC1 isolation and sequence analysis

In order to identify maize NAC proteins, the PlantTFDB database (http://planttfdb.cbi.pku.edu.cn/) (Zhang et al. 2011) was searched, and a total of 190 maize NAC proteins were obtained. One of these, designated *ZmSNAC1*, was selected for further functional analysis. Specific primers were designed to amplify the full-length cDNA sequence of *ZmSNAC1* from the maize inbred line "CN165" by RT-PCR. Extracted and purified PCR products were cloned into pMD18-T, and then confirmed by sequencing. The PCRs were carried out as follows: 5 min at 94 °C; 35 cycles of 40 s at 94 °C, 40 s at 58 °C, and 1.5 min at 72 °C; and then 30 min at 72 °C.

We performed a BLAST search of the *ZmSNAC1* cDNA sequence in the maize sequence database (http://www.maizesequence.org), and found a corresponding genome sequence in B73. Specific PCR primers were designed to amplify the genomic DNA sequence of CN165. All primer sequences are listed in Supplementary Table S1. The Contig Express software (version 6.0, Invitrogen) was used to assemble sequences. DNAMAN (version 5.0) and ClustalW (version 2.0; Thompson et al. 1997) were used for multiple sequence alignments. A phylogenetic tree was built with MEGA4 (version 4; Saitou and Nei 1987; Tamura et al. 2007) using the neighbor-joining method (bootstrap: 1,000).

Expression analysis of *ZmSNAC1* under different treatments

Seeds of CN165 were surface-sterilized with 75 % ethanol for 5 min. After several rinses with tap water, the seeds were sown into pots containing vermiculite in a greenhouse under normal conditions. Plants in the three-leaf stage were subjected to a series of stress treatments. For NaCl, ABA, SA, MeJA, and 2, 4-D treatment, seedlings were

transferred to Hoagland solution supplemented with 200 mM NaCl, 100 μM (±) *cis*, *trans*-ABA, 100 μM SA, 100 μM MeJA, or 100 μM 2, 4-D, respectively. For the control sample, the maize seedlings without treatment were collected during the same time course. For cold and heat treatments, seedlings grown in pots at 22 °C were transferred to growth chambers at 4 and 42 °C, respectively. For dehydration treatment, seedlings were immediately removed from pots and dehydrated on Whatman 3MM paper at room temperature, with approximately 50 % humidity. Shoots and roots were harvested separately at the time points indicated, then immediately frozen in liquid nitrogen and stored at −76 °C.

In order to investigate the spatial expression pattern of *ZmSNAC1* in maize, we used the root, stalk, and leaf of seedlings in the three-leaf stage, and the aerial root, ear leaf (leaf in ear position), tassel and immature ear of seedlings in the pre-flowering stage. The silk in the flowering stage was also included in this study. Total RNA was extracted using TRIzol reagent (Invitrogen) and treated with DNase I (TaKaRa) to eliminate genomic DNA contamination. Then first-strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen).

Quantitative real-time PCR was used to quantify *ZmSNAC1* expression. A mixture of PCR reaction components was prepared according to the manufacturer's protocol of the SYBR *Premix Ex Taq*™ (TaKaRa). The maize *GAPDH* gene (NM_001111943) and *Arabidopsis Actin* gene (NM_112764) were used as internal controls for normalization. Thermocycling and fluorescence detection were performed with an ABI7300 instrument (Applied Biosystems). PCR was performed using a two-step method: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Relative expressions of *ZmSNAC1* under each condition were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Data are mean ± standard error of four biological replicates. Statistical significance was indicated for those upregulated at least twofold or downregulated less than 0.5-fold.

Subcellular localization of ZmSNAC1

The full-length coding sequence of *ZmSNAC1* without a terminator codon (TGA) was amplified from CN165, and the fragment was cloned into the pGreen-GFP-N vector to generate a ZmSNAC1-GFP fusion protein. After sequencing confirmation, the construct and negative control (empty vector) were transformed into *Arabidopsis* protoplasts and cultured at 24 °C for 16 h. The GFP fluorescence signal was visualized and photographed with a laser scanning confocal microscope (Zeiss LSM 710). The excitation lines were configured at 488 and 633 nm.

Transactivation assay

As shown in Fig. 4, the sequencing-confirmed full-length coding *ZmSNAC1* sequence was cloned into the pGBKT7 DNA-BD vector (Clontech). Empty pGBKT7 vector was used as a negative control. Then the constructs were transformed into yeast strain AH109, according to the manufacturer's instructions (Clontech). Positive transformants were selected on SD/-Trp and SD/-Trp/-His/30 mM 3-AT, respectively. A colony-filter assay was performed according to the yeast protocol handbook (Clontech).

Generation of transgenic *Arabidopsis* plants

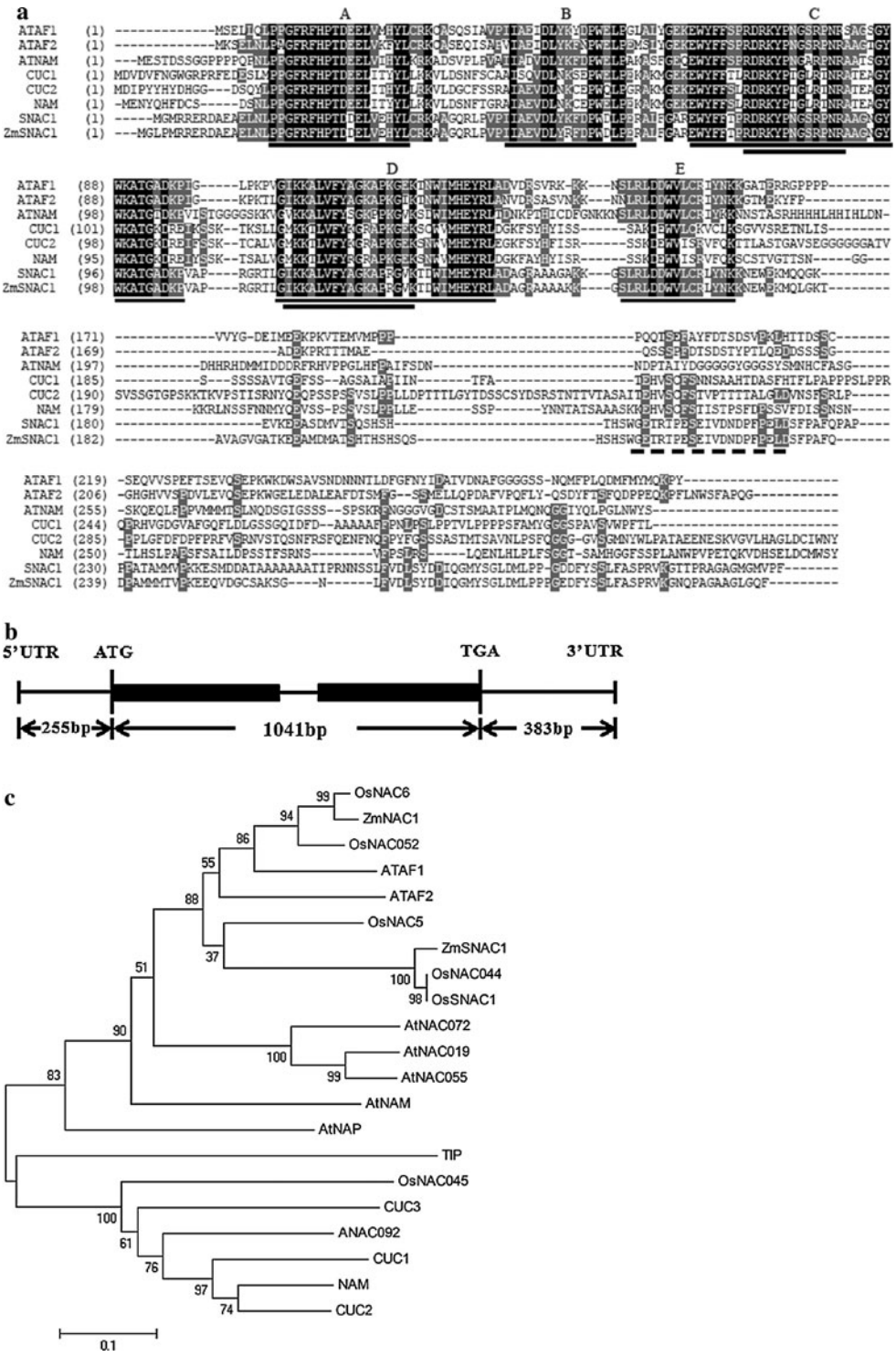
The sequencing-confirmed open reading frame (ORF) of *ZmSNAC1* was cloned into the pCAMBIA3301 plasmid (CAMBIA, Canberra, Australia), which was controlled by a Cauliflower mosaic virus (CaMV) 35S promoter. The constructs were transferred into *Agrobacterium tumefaciens* GV3101 and then transformed into *Arabidopsis* using the floral dip method (Clough and Bent 1998). Seeds of the T_0 and T_1 generation were screened on MS agar medium (Murashige and Skoog 1962) containing 7.5 mg/L phosphinothricin (PPT). Positive transgenic plants were selected according to a segregation ratio (resistant:sensitive = 3:1), and confirmed by genomic PCR. The selected T_3 generation transgenic lines that displayed 100 % resistance to PPT were considered homozygous, and thus were harvested individually for further analysis.

Germination assay

Harvested seeds were surface-sterilized with 75 % ethanol (v/v) for 1 min, 5 % sodium hypochlorite (v/v) for 10 min, and several rinses with sterile distilled water. Fifty seeds of each homozygous transgenic line and the wild-type (WT) were placed on MS agar medium (0.9 %) containing 350 mM D-Mannitol, 150 mM NaCl and 1.5 μM ABA, respectively. Seeds were vernalized at 4 °C in the dark for 2 days, and then transferred to a greenhouse (22 °C, humidity 40–50 %, 120–150 μmol/m^{-2} s^{-1} under 16 h light/8 h dark, and 8 h dark at 18 °C). The germination ratio was calculated daily for 7 days thereafter.

Dehydration assay

For dehydration treatment, seeds were plated on MS media and grown for approximately 3 weeks on vertically



oriented plates. Transgenic and WT plants were extracted from MS agar medium simultaneously, dehydrated in air for 40 min, and photographed at the time points indicated. Dehydration-stressed seedlings were submerged in deionized water for 2 h to rehydrate. The relative humidity was ~40 % and the temperature was 25 °C during the stress period. For each repeated experiment, approximately 60 plants of each line were used for test. Experiments were repeated independently three times and the results were consistent.

◀ **Fig. 1** Sequence analysis of *ZmSNAC1*. **a** Comparison of the amino acid sequences of NAC proteins, i.e. ATAF1 (GenBank ID: NP_171677), ATAF2 (GenBank ID: CAA52772), CUC1 (GenBank ID: BAB20598), CUC2 (GenBank ID: BAA19529) and AtNAM (GenBank ID: AAD17314) from *Arabidopsis*; NAM (GenBank ID: CAA63102) from petunia, SNAC1 (GenBank ID: ABD52007) from rice, and ZmSNAC1 (GenBank ID: AEY78612) from maize. *Black boxes* indicate identical residues, and *gray boxes* indicate residues conserved among these proteins. The conserved sub-domain of NAC is *single-underlined*, and the nucleus localization signal (NLS) is *double-underlined*. The predicted motif in the C-terminal is *dot-underlined*. **b** *ZmSNAC1* gene structure. *Filled boxes* indicate exons and *lines between boxes* indicate introns. **c** Phylogenetic relationship between ZmSNAC1 and typical and stress-responsive NAC proteins. The multiple sequence alignment was performed using ClustalX 1.83, and the phylogenetic tree analysis was constructed by the neighbor-joining method using the amino acid sequence of each NAC protein. The GenBank IDs of each selected NAC proteins are listed as follows: the *Arabidopsis* ATAF1 (NP_171677); ATAF2 (CAA52772); CUC1 (BAB20598); CUC2 (BAA19529); CUC3 (AAP82630); NAP (CAA10955); TIP (AAF87300); AtNAM (AAD17314); ANAC019 (NP_175697); ANAC055 (NP_188169); ANAC072 (NP_567773); ANAC092 (NP_198777); the rice SNAC1 (ABD52007); OsNAC5 (BAA89799); OsNAC6 (BAA89800); OsNAC052 (BAA89800); OsNAC045 (NP_001065629); OsNAC044 (BAG90542); the petunia NAM (CAA63102); the maize ZmNAC1 (ABY67929)

Water loss measurement

Wild-type and *ZmSNAC1*-overexpressed transgenic plants were grown in soil under identical controlled conditions. To measure water loss, leaves of a similar size, age and position were detached from the roots and placed on Whatman filter papers. Their fresh weight was determined at the time points indicated. The relative humidity was ~45 % at 23 °C. Experiments were repeated independently three times.

Results

Isolation and sequencing of *ZmSNAC1*

In total, 190 putative maize NAC proteins containing an NAC conserved domain were found in the PlantTFDB database. One of these, whose deduced complete protein sequence has the highest identity (81.1 %) to the rice SNAC1 protein (GenBank ID: ABD52007), was designated *ZmSNAC1* (GenBank ID: JQ217429) for further study (Supplementary Fig. S1).

Genomic DNA sequence analysis revealed that *ZmSNAC1* had 1,679 base pairs (bp), containing two exons and one intron (Fig. 1a); with a 939 bp ORF that encodes a putative protein of 312 amino acids. The protein contained a typical NAC-domain in the N-terminal region, a 255 bp 5′-untranslated region (UTR), and a 383 bp 3′-UTR (Fig. 1b). By searching for its genome DNA in MaizeGDB, *ZmSNAC1* was mapped in bin 1.11 (GenBank ID: AC195355.3) of the genetic linkage map IBM2 between simple sequence repeats (SSR) markers umc2242 and umc1118.

As shown in Fig. 1a, the consensus N-terminal region (NAC-domain) of ZmSNAC1 could be further divided into five conserved sub-domains. The C-terminal region, which contained a WGETRTPESxxVD [N/S] D [P/A] FPE [L/M] D motif, was highly divergent. Compared to previously reported NAC proteins (Ooka et al. 2003; Tran et al. 2004; Jeong et al. 2010), phylogenetic analysis indicated that ZmSNAC1 belonged to a subgroup that also contains several stress-responsive NAC proteins, such as OsNAC5 and OsNAC6 (Fig. 1c).

Expression pattern of *ZmSNAC1*

In order to investigate the function of the *ZmSNAC1* gene, we performed expression analysis under various abiotic stresses. As shown in Fig. 2a, the *ZmSNAC1* transcript level was significantly upregulated by low temperature, because its expression increased gradually and peaked (more than eightfold) in both roots and shoots 24 h after treatment. However, under dehydration and salt treatment, the relative level of *ZmSNAC1* expression increased by more than 15- and 28-fold, respectively, at 12 h and then decreased at 48 h in roots (Fig. 2b, c). *ZmSNAC1* expression was not induced by heat stress (Fig. 2d). The water control experiment was performed to show no influence on the expression of *ZmSNAC1* (Supplementary Fig. S2). Furthermore, we examined the expression patterns of *ZmSNAC1* in response to various phytohormones. *ZmSNAC1* transcript levels were significantly induced by ABA and were repressed by SA, while no obvious changes were detected in response to methyl jasmonic acid (MeJA) or dichlorophenoxyacetic acid (2, 4-D) (Fig. 2e).

In addition, we determined tissue-specific *ZmSNAC1* expression in maize at different developmental stages. *ZmSNAC1* expression was detected in all tissues tested. Expression levels in the roots of seedlings in the three-leaf stage and in the ear leaf (leaf in ear position) of seedlings in the pre-flowering stage were markedly higher than those in other tissues (Fig. 2f).

Subcellular localization of ZmSNAC1

According to the results of sequence analysis, two putative nuclear localization signals, located at Arg79–Arg91 and IIe116–Lys132, were found in the conserved NAC-domain of ZmSNAC1 (Fig. 1a). To examine the subcellular localization of ZmSNAC1, a *ZmSNAC1-GFP* fusion construct, driven by a 35S-Omega promoter, was introduced into *Arabidopsis* protoplasts. The ZmSNAC1-GFP protein

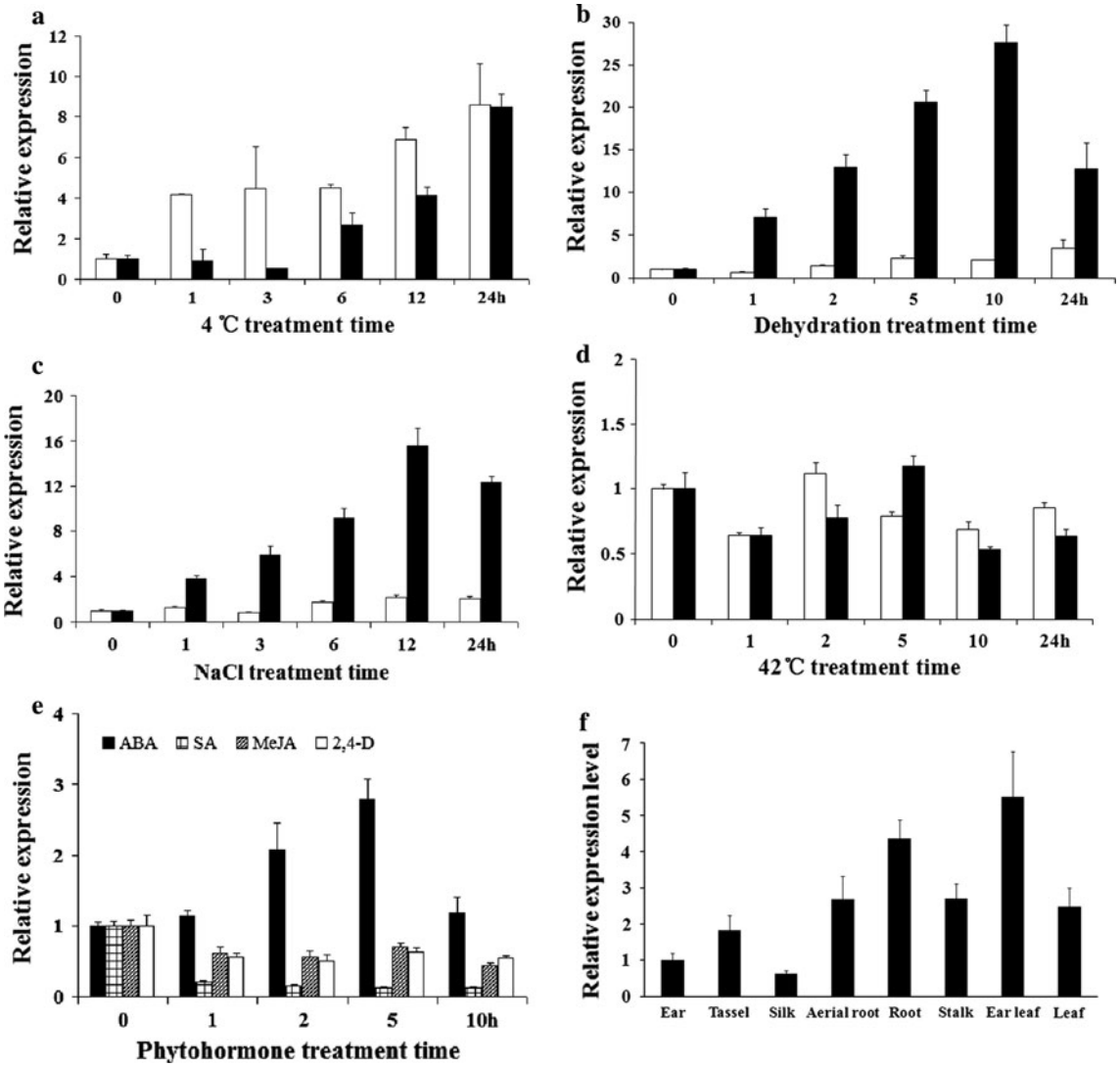


Fig. 2 Expression pattern of *ZmSNAC1* gene in maize. **a–d** Expression of *ZmSNAC1* in shoots (*white bars*) and roots (*black bars*), respectively, under low temperature, dehydration, high-salinity and heat stress conditions. **e** Expression of *ZmSNAC1* in shoots under ABA, MeJA, SA, and 2, 4-D treatments. *ZmSNAC1* transcript level at 0 h (untreated control) was used as the calibrator. **f** Tissue-specific expression of *ZmSNAC1* at different developmental stages. Transcript level in the ear was used as the calibrator. The maize *GAPDH* gene (GenBank ID: NM_001111943) was used as an internal control for normalization. Each experiment was performed at least three times, and *error bars* represent standard deviation

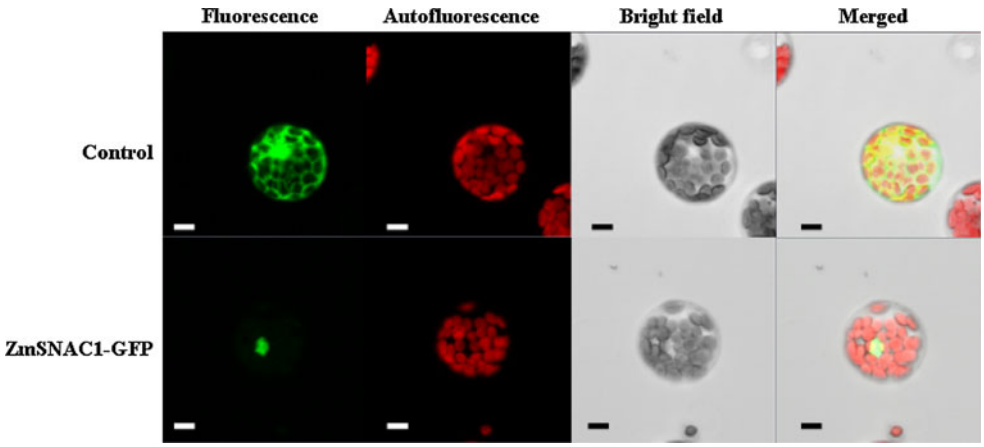


Fig. 3 Subcellular localization of ZmSNAC1 in *Arabidopsis* leaf protoplast cells. *Bar* indicates 10 μm

was clearly visible in the nucleus, while the control (transformed with a GFP construct) was observed in whole cells (Fig. 3).

Transactivation activity analysis of ZmSNAC1

In order to investigate whether the deduced ZmSNAC1 protein had transcriptional activity, the entire coding region was fused to the GAL4 DNA-binding domain (DBD) in the pGBKT7 vector. The construct and empty vector pGBKT7 (negative control) were transformed into yeasts. Transformants containing pGBKT7-ZmSNAC1 grew well on the selection medium, and the colony-filter assay supported this result (Fig. 4), suggesting that ZmSNAC1 exhibited transactivation activity in yeast.

Germination sensitivity of *ZmSNAC1*-overexpressing transgenic plants to ABA and osmotic stress

In order to evaluate the function of *ZmSNAC1* in vivo, we generated six *ZmSNAC1*-overexpressing transgenic *Arabidopsis* lines. WT and transgenic plants displayed comparable phenotypes under normal growth conditions. The expression levels of *ZmSNAC1*-overexpressing lines were examined by quantitative RT-PCR (Fig. 5a). Two independent transgenic lines, OX-5 and OX-7, were randomly chosen for further phenotypic analysis.

The germination test indicated that overexpression of *ZmSNAC1* significantly elevated the sensitivity of transgenic plants to osmotic stress, whereas no difference was found in seed germination between the WT and transgenic plants under normal conditions (Fig. 5b). For example, in the presence of 350 mM D-mannitol, the germination percentages of transgenic seeds ranged from 12 to 16 %, while those of the WT reached 63 % at day 7. Under 150 mM NaCl treatment, approximately 50–76 % of *ZmSNAC1*-overexpressing seeds germinated, compared to 88 % of the WT.

A similar phenomenon was observed when *ZmSNAC1*-overexpressing seeds were germinated on MS agar medium supplemented with 1.5 μM exogenous ABA. Transgenic plants were more sensitive to ABA than was the WT (Fig. 5b). Thus, *ZmSNAC1*-overexpressing transgenic plants were hypersensitive to ABA and osmotic stresses during the germination stage.

Performance of *ZmSNAC1*-overexpressing transgenic *Arabidopsis* under dehydration treatment

Because significant variation in expression was detected under dehydration (Fig. 2b; Supplementary Fig. S3), the dehydration tolerance of *ZmSNAC1*-overexpressing transgenic *Arabidopsis* was further examined. As shown in Fig. 6a, after dehydration in air for 5 min, leaves of the WT began to wither and exhibited a dark green color, whereas the transgenic seedlings retained bright green leaves that were spread out. After 40 min dehydration, the WT exhibited curved leaves while transgenic leaves withered only a little. After rehydration for 2 h, approximately 68 % (41/60) of the transgenic plants recovered compared to 39 % (23/59) of the WT.

Besides phenotypic performance water loss rate is an important physiological parameter to investigate plants' dehydration tolerance. Significantly, lower water loss rates were detected in *ZmSNAC1*-overexpressed plants compared to the WT under dehydration stress treatment (Fig. 6b). These results indicate that *ZmSNAC1* enhances the dehydration tolerance of transgenic plants.

Discussion

NAC proteins constitute a plant-specific superfamily whose members participate in many regulatory and developmental processes. They have been well investigated in rice and *Arabidopsis* (Nuruzzaman et al. 2010). In maize, a total of 190 predicted NAC members have been identified. In the present study, sequence analysis showed that ZmSNAC1, one of these 190 proteins, contained a typical NAC conserved domain located in the N-terminal region. Five subdomains were identified in the NAC-domain (Fig. 1a). There is no difference between the ZmSNAC1 sequences from "CN165" and "B73" according to sequence

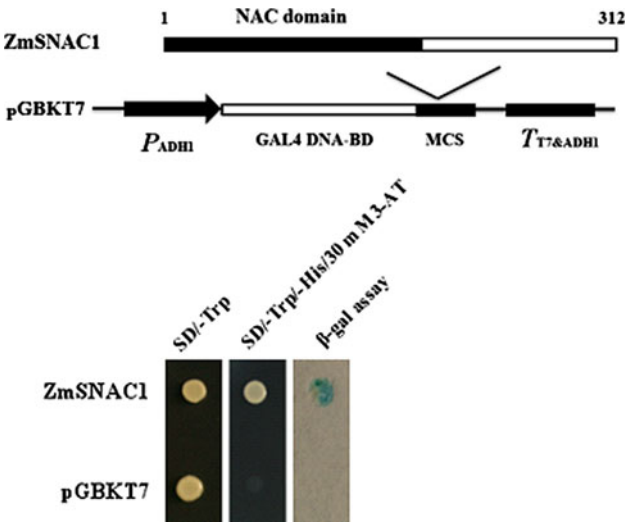


Fig. 4 Transactivation activity analysis of ZmSNAC1 in yeast. A fusion protein of the GAL4 DNA-binding domain and full-length *ZmSNAC1* was checked for its transactivation activity in yeast strain AH109. The pGBKT7 vector was used as a negative control. Experiments were repeated three times independently, and the results were consistent

alignment (Supplementary Fig. S4). Phylogenetic analysis showed that the deduced ZmSNAC1 protein exhibited the highest (81.1 %) sequence identity to rice SNAC1, both of which belong to an identical subgroup OsNAC3 (Fig. 1c, Supplementary Fig. S1). In this subgroup, *OsNAC5*, *OsNAC6* and *SNAC1* are strongly induced by various abiotic stresses. Overexpression of these stress-related genes could significantly enhance stress tolerance (Hu et al. 2006; Jeong et al. 2010; Nakashima et al. 2011; Song et al. 2011; Takasaki et al. 2010). Thus, we speculated that *ZmSNAC1*, as with its homology, might play an important role in the response to various environmental stresses.

Expression analyses showed that the *ZmSNAC1* gene was strongly induced by low temperature, NaCl, dehydration and exogenous ABA treatment (Fig. 2). Moreover, when maize plants underwent long-term soil-drying stress, the level of *ZmSNAC1* expression increased with the degree of stress, and then decreased to the same level as that in untreated plants after re-watering (see Supplementary Fig. S3). These data are similar to some previous reports of functional *NAC* genes, suggesting that *ZmSNAC1* might be involved in response to abiotic stresses. On the other hand, our data suggest that *ZmSNAC1* was responsive to SA, which is believed to play an essential role in the

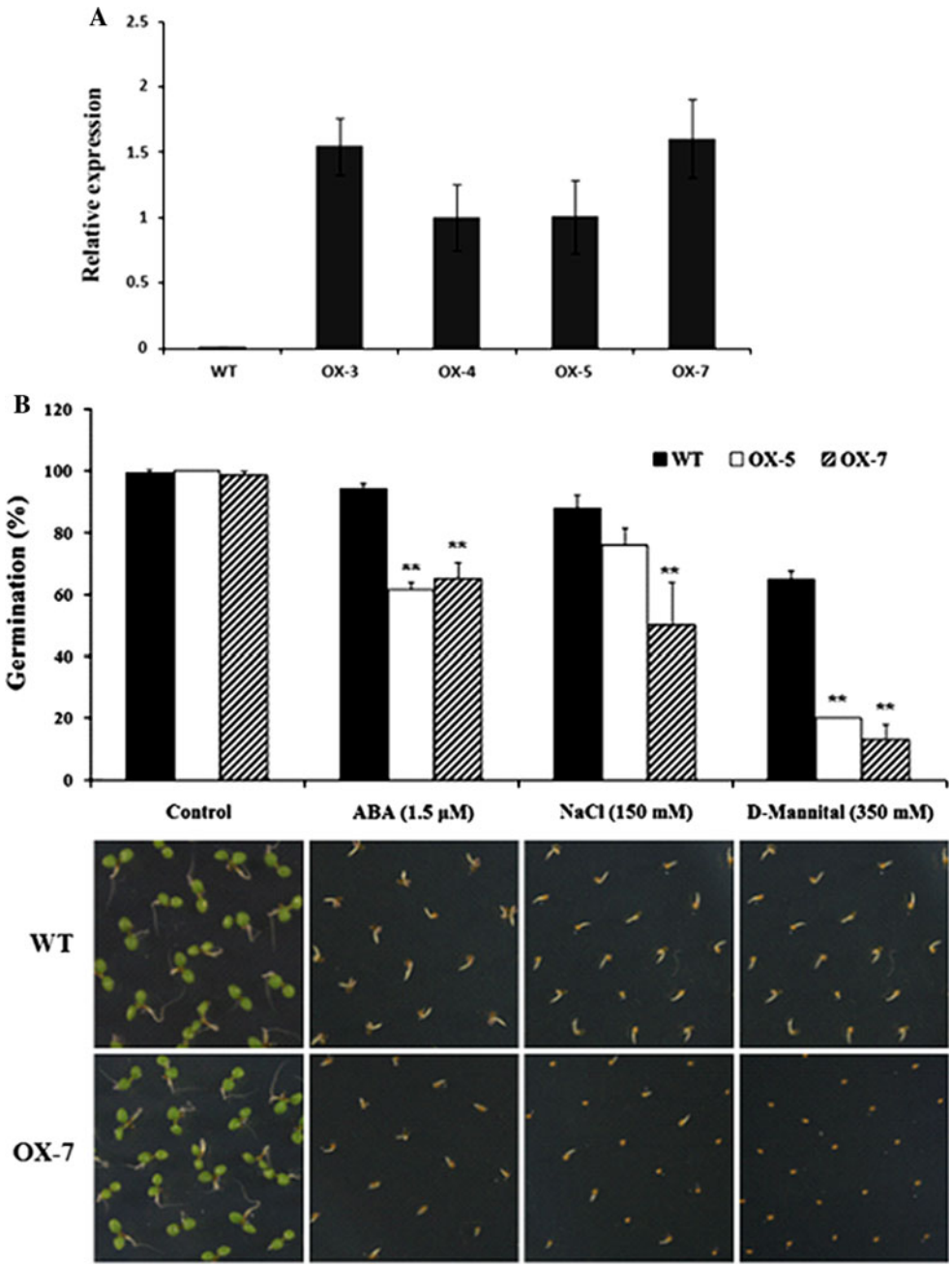


Fig. 5 Germination assays of ZmSNAC1-overexpressed plants. **a** Quantitative RT-PCR analysis of ZmSNAC1 expression in WT *Arabidopsis* and the homozygous transgenic lines OX-3, OX-4, OX-5, and OX-7. The Arabidopsis Actin gene (NM_112764) was used as an internal control. **b** Germination assay in the presence of 1.5 μM ABA, 150 mM NaCl, or 350 mM D-mannitol, respectively, on day 7 after incubation at 22 °C. Each measurement consisted of 50 seeds. *Error bars* indicate standard deviation ($n = 4$). * and ** represent significant differences from the WT at values of $P < 0.05$ and <0.01, respectively, as determined by Student's *t* test

signaling pathways involved in the response to biotic stresses (Fig. 2). Some NAC members are involved in the responses to pathogens and wounding (Delessert et al. 2005; Selth et al. 2005). Thus, ZmSNAC1 may also function in the response to pathogens. However, further studies are needed to clarify the role of ZmSNAC1 in SA-dependent pathways.

The N-terminal region of the NAC protein is critical for recognition of and binding to a specific DNA sequence (Duval et al. 2002; Tran et al. 2004; Olsen et al. 2005; Hu et al. 2006; Takasaki et al. 2010), and the C-terminal region of NAC transcription factors is considered to be essential for transcriptional activation (Takasaki et al. 2010). We found that ZmSNAC1 showed transactivation activity in yeast (Fig. 4), whereas no activation was detected in the predicted N-terminal domain (Supplementary Fig. S5). Our results suggest that ZmSNAC1 might function as a transcription factor in regulatory pathways. Further research is required to identify the sequences in the C-terminal region of ZmSNAC1 essential for transactivation activity.

Because *ZmSNAC1* expression was significantly induced by various abiotic stresses, we speculated that it might also be involved in response to abiotic stresses in maize. As a result, we further analyzed its function in vivo. Overexpression of *ZmSNAC1* may result in hypersensitivity to ABA and osmotic stresses in the seed germination stage (Fig. 5b). The lower germination rate of *ZmSNAC1*-overexpressed transgenic seeds may be due to their higher ABA sensitivity under osmotic stress conditions. Therefore, we speculate that ZmSNAC1 might act as a transcription factor in ABA-dependent signaling pathways in response to abiotic stresses.

Further, we verified that the *ZmSNAC1*-overexpressing transgenic plants exhibited enhanced tolerance to dehydration in the seedling stage. The detached leaves of transgenic plants showed a slower rate of water loss than those of WT plants (Fig. 6). Tissue water potential and water content are maintained by limiting water loss, the rate of which is determined largely by stomatal conductance (Verslues et al. 2006).

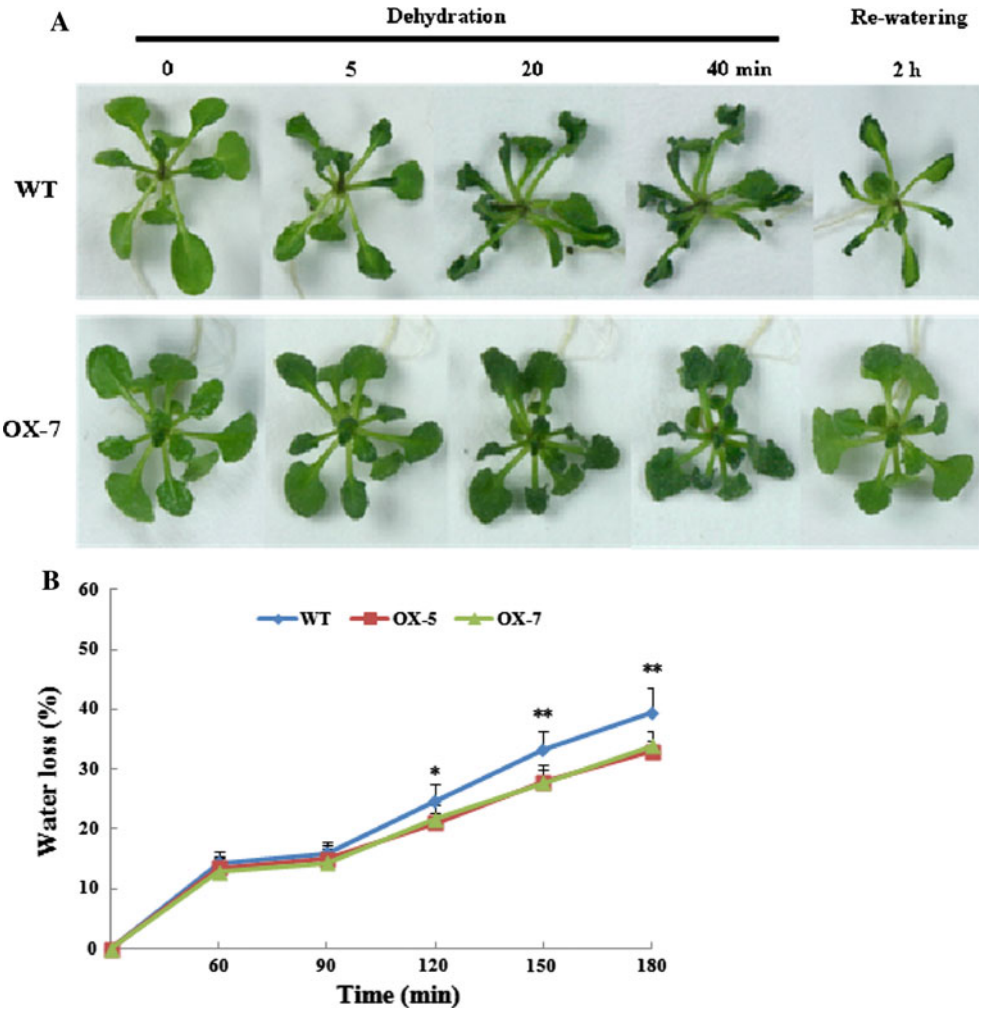


Fig. 6 Dehydration assays of *ZmSNAC1*-overexpressing plants. **a** Dehydration experiments were performed as described in the "Materials and methods". Photographs were obtained at the times indicated. The same results were observed in both OX-5 and OX-7 transgenic lines. OX-7 transgenic plants were presented here. **b** Water loss from detached leaves of WT and ZmSNAC1 transgenic plants. Each data point represents the mean of duplicate measurements ($n = 3$ each). * and ** represent significant differences from the WT at values of $P < 0.05$ and <0.01, respectively

Similarly, it has recently been demonstrated that overexpression of stress-responsive *NAC* genes in the same subgroup can improve the stress tolerance of transgenic plants (Hu et al. 2006, 2008; Yokotani et al. 2009; Zheng et al. 2009; Takasaki et al. 2010; Hao et al. 2011; Song et al. 2011). Based on microarray data, OsNAC5 was demonstrated to activate the expression of several stress-responsive genes in transgenic plants via binding to the specific DNA sequence in their promoter region, resulting in enhanced stress tolerance (Takasaki et al. 2010). Compared to the WT, *OsNAC052*-overexpressing transgenic plants were hypersensitive to ABA and had a higher water content, indicating better water-retaining ability due to activation of drought-induced genes under dehydration conditions (Gao et al. 2010). Extensive analysis will be necessary to investigate the particular regulatory mechanism of the dehydration tolerance. Our current results suggested that *ZmSNAC1* might play important roles in ABA-dependent signaling pathways in response to abiotic stresses by regulating the expression of functional protein genes. Moreover, despite the fact that overexpression of some NAC genes has detrimental effects on the growth and development of plant (Fujita et al. 2004; Yoon et al. 2008), *ZmSNAC1* transgenic lines exhibited no obvious morphological changes compared to WT, indicating it might be useful for improving the drought tolerance of crops.

In conclusion, we characterized ZmSNAC1 as a stress-responsive NAC-type transcription factor in maize. Overexpression of *ZmSNAC1* in *Arabidopsis* led to hypersensitivity to ABA and osmotic stress, and conferred tolerance to dehydration. Although the detailed mechanisms underlying the functions of ZmSNAC1 remain unclear, the data we present here indicate its potential for engineering stress-tolerant crops.

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References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in *Arabidopsis*: an analysis of the cup-shaped cotyledon mutant. Plant Cell 9:841–857

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Delessert C, Kazan K, Wilson IW, Van Der Straeten D, Manners J, Dennisand ES, Dolferus R (2005) The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. Plant J 43:745–757

Duval M, Hsieh TF, Kim SY, Thomas TL (2002) Molecular characterization of AtNAM: a member of the *Arabidopsis* NAC domain superfamily. Plant Mol Biol 50:237–248

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 39:863–876

Gao F, Xiong AS, Peng RH, Jin XF, Xu J, Zhu B, Chen JM, Yao QH (2010) OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. Plant Cell Tissue Organ Culture 100:255–262

Hao YJ, Wei W, Song QX, Chen HW, Zhang YQ, Wang F, Zou HF, Lei G, Tian AG, Zhang WK, Ma B, Zhang JS, Chen SY (2011) Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. Plant J 68:302–313

Hu HH, Dai MQ, Yao JL, Xiao BZ, Li XH, Zhang QF, Xiong LZ (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 35:12987–12992

Hu HH, You J, Fang YJ, Zhu XY, Qi ZY, Xiong LZ (2008) Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice. Plant Mol Biol 67:169–181

Jeong JS, Kim YS, Baek KH, Jung H, Ha Sun-Hwa, Choi YD, Kim M, Reuzeau C, Kim JK (2010) Root-specific expression of *OsNAC10* improves drought tolerance and grain yield in rice under field drought conditions. Plant Physiol 153:185–197

Kikuchi K, Ueguchi-Tanaka M, Yoshida TK, Nagato Y, Matsusoka M, Hirano HY (1999) Molecular analysis of the NAC gene family in rice. Mol Gen Genet 262:1047–1051

Lata C, Prasad M (2011) Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot 62:4731–4748

Le DT, Nishiyama R, Watanabe Y, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS (2011) Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. DNA Res 18:263–276

Liu ZJ, Shao FX, Tang GY, Shan L, Bi YP (2009) Cloning and characterization of a transcription factor ZmNAC1 in maize (*Zea mays*). Hereditas (Beijing) 31:199–205

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta DDCt}$ method. Methods 25:402–408

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. Plant Physiol 149:88–95

Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2011) NAC transcription factors in plant abiotic stress responses. Biochim Biophys Acta 1819:97–103

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S (2010) Genome-wide analysis of NAC transcription factor family in rice. Gene 465:30–44

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci 10:79–87

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K (2003) Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Res 10:239–247

Qin F, Kakimoto M, Sakuma Y, Maruyama K, Osakabe Y, Tran LS, Shinozaki K, Yamaguchi-Shinozaki K (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. Plant J 50:54–69

Qin F, Shinozaki K, Yamaguchi-Shinozaki K (2011) Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant Cell Physiol 52:1569–1582

Riechmann JL, Heard J, Martin G, Reuber L, Jiang CZ, Keddie J, Pineda O, Ratcliffe OJ, Samaha RR, Adam L, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu GL (2000) *Arabidopsis* transcription factors, genome-wide comparative analysis among eukaryotes. Science 290:2105–2110

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. Plant Cell 17:311–325

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227

Song SY, Chen Y, Chen J, Dai XY, Zhang WH (2011) Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234:331–345

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The no apical meristem gene of *Petunia* is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 85:159–170

Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K, Nakashima K (2010) The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. Mol Genet Gen 284:173–183

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (b) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress inducible NAC transcription factors that bind to a drought responsive *cis*-element in the early responsive to dehydration stress. Plant Cell 16:2481–2498

Tran LS, Nishiyama R, Yamaguchi-Shinozaki K, Shinozaki K (2010) Potential utilization of NAC transcription factors to enhance abiotic stress tolerance in plants by biotechnological approach. GM Crops 1:32–39

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu JH, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539

Verza NC, Figueira TRS, Sousa SM, Arruda P (2010) Transcription factor profiling identifies an aleurone-preferred NAC family member involved in maize seed development. Ann Appl Biol 158:115–129

Xia N, Zhang G, Sun YF, Zhu L, Xu LS, Chen XM, Liu B, Yu YT, Wang XJ, Huang LL, Kang ZS (2010) *TaNAC8*, a novel NAC transcription factor gene in wheat, responds to stripe rust pathogen infection and abiotic stresses. Physiol Mol Plant Pathol 74:394–402

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803

Yokotani N, Ichikawa T, Kondou Y, Matsui M, Hirochika H, Iwabuchi M, Oda K (2009) Tolerance to various environmental stresses conferred by the salt-responsive rice gene ONAC063 in transgenic *Arabidopsis*. Planta 229:1065–1075

Yoon HK, Kim SG, Kim SY, Park CM (2008) Regulation of leaf senescence by *NTL9*-mediated osmotic stress signaling in *Arabidopsis*. Mol Cells 25:438–445

Zhang H, Jin J, Tang L, Zhao Y, Gu X, Luo J (2011) PlantTFDB 2.0: update and improvement of the comprehensive plant transcription factor database. Nucleic Acids Res 39:D1114–D1117

Zheng XN, Chen B, Lu GJ, Han B (2009) Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. Biochem Biophys Res Commun 379:985–989

Zimmermann R, Werr W (2005) Pattern formation in the monocot embryo as revealed by NAM and CUC3 orthologues from *Zea mays* L. Plant Mol Biol 58:669–685