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ORIGINAL PAPER

# Overexpression of a *Miscanthus lutarioriparius* NAC gene *MlNAC5* confers enhanced drought and cold tolerance in Arabidopsis

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**Abstract**

***Key message*** ***MLNAC5* functions as a stress-responsive NAC transcription factor gene and enhances drought and cold stress tolerance in transgenic Arabidopsis via the ABA-dependent signaling pathway.**

*Abstract* NAC transcription factors (TFs) play crucial roles in plant responses to abiotic stress. *Miscanthus lutarioriparius* is one of *Miscanthus* species native to East Asia. It has attracted much attention as a bioenergy crop because of its superior biomass productivity as well as wide adaptability to different environments. However, the functions of stress-related NAC TFs remain to be elucidated in *M. lutarioriparius*. In this study, a detailed functional characterization of *MlNAC5* was carried out. *MlNAC5* was a member of ATAF subfamily and it showed the highest sequence identity to *ATAF1*. Subcellular localization of MlNAC5-YFP fusion protein in tobacco leaves indicated that MlNAC5 is a nuclear protein. Transactivation assay in yeast cells demonstrated that *MlNAC5* functions as a transcription activator and its activation domain is located in the C-terminus. Overexpression of *MlNAC5* in Arabidopsis had impacts on plant development including dwarfism, leaf senescence, leaf morphology, and late flowering under normal growth conditions. Furthermore, *MlNAC5* overexpression lines in Arabidopsis exhibited hypersensitivity to abscisic acid (ABA) and NaCl. Moreover, overexpression of *MlNAC5* in Arabidopsis significantly enhanced drought and cold tolerance by transcriptionally regulating some stress-responsive marker genes. Collectively, our results indicated that *MlNAC5* functions as an important regulator during the process of plant development and responses to salinity, drought and cold stresses.

**Keywords** *Miscanthus lutarioriparius* · Abiotic stress · Abscisic acid · Bioenergy crop · NAC transcription factor

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## Introduction

Plants are regularly threatened by various abiotic stresses, such as drought, salinity and cold, which adversely affect plant growth and development and are responsible for major yield loss in agriculture (Bray et al. 2000). As a consequence, plants have developed multifaceted strategies at the physiological, biochemical, and molecular levels to

cope with these adverse stress factors, thus enabling them to survive under various environmental conditions (Bohnert et al. 2006; Broun 2004).

Plant stress responses are modulated by complex gene regulatory systems, in which transcription factors play essential roles in stress responses by regulating their target genes through specific binding to cis-acting elements in their promoters (Liu et al. 1998; Tran et al. 2004; Uno et al. 2000). Several types of transcription factors (TFs), such as NAC (NAM, ATAF1/2 and CUC2), WRKY, zinc finger, AP2/EREBP, and MYB family members, are known to transcriptionally regulate downstream gene expression under abiotic stresses in plants (Chen et al. 2003; Eulgem et al. 1999; Fujita et al. 2004; Liu et al. 1998; Mukhopadhyay et al. 2004; Tran et al. 2004; Uno et al. 2000).

NAC proteins comprise one of the largest families of plant-specific transcription factors. NAC family genes have been identified by genome-wide analysis from various plant species, such as Arabidopsis (Ooka et al. 2003), rice (Fang et al. 2008; Ooka et al. 2003), poplar (Hu et al. 2010), soybean (Le et al. 2011), etc. NAC proteins share a common structure consisting of a conserved DNA-binding NAC domain in the N-terminal region and a highly diversified C-terminal domain that confers transcriptional activator or repressor activity (Olsen et al. 2005). Based on the motif distribution, the NAC domain can be further divided into five subdomains (A–E) (Ooka et al. 2003).

NAC proteins play essential roles in diverse aspects of plant development, such as pattern formation in embryos (Souer et al. 1996), lateral root development (He et al. 2005; Xie et al. 2000), leaf senescence (Guo and Gan 2006; Uauy et al. 2006), flowering (Sablowski and Meyerowitz 1998) and secondary wall formation (Mitsuda et al. 2005). There is increasing evidence demonstrating that NAC family transcription factors are involved in responses to various biotic and abiotic stresses, including drought, salinity, cold, bacterial and fungal pathogens, low-oxygen stress, etc. (for reviews, see (Nuruzzaman et al. 2013; Puranik et al. 2012). For example, the expression of three Arabidopsis NAC genes, *ANAC019*, *ANAC055*, and *ANAC072* (*RD26*), are induced by drought, high salinity and abscisic acid (ABA), respectively. Overexpression of these three genes remarkably enhances tolerance to drought stress (Tran et al. 2004). Another Arabidopsis stress-inducible NAC gene, *AtNAC2* (*ANAC092*/*ORE1*), functions at the downstream of ethylene and auxin-signaling pathways and is required for salt stress response and lateral root development (He et al. 2005).

In Arabidopsis, *ATAF1* (*ANAC002*) and *ATAF2* (*ANAC081*), together with *ANAC102* and *ANAC032* are phylogenetically classified into a small subfamily (ATAF) (Christianson et al. 2009; Ooka et al. 2003). *ATAF1* was initially reported to play a negative role in response to drought stress by functional analysis of *ataf1* null mutants (Lu et al. 2007). However, later on another group reported that it is the overexpression of *ATAF1*, not the null mutation conferred the enhanced drought tolerance, revealing a positive role of *ATAF1* in plant drought response (Wu et al. 2009). In addition, *ATAF1* also functions as a negative regulator in defense responses against pathogen attack, indicating that *ATAF1* plays important roles in biotic stresses (Jensen et al. 2008; Wu et al. 2009). *ANAC102* was found to play an important role in regulating seed germination under low-oxygen stress (Christianson et al. 2009).

In rice (*Oryza sativa*), a subset of NAC transcription factors have been well characterized to participate in stress responses. Overexpression of an ATAF-like NAC gene, *SNAC2*/*OsNAC6*, in rice resulted in enhanced tolerance to dehydration, high salinity, cold stresses and blast disease (Hu et al. 2008; Nakashima et al. 2007). Another stress-responsive rice NAC gene, *SNAC1*/*OsNAC1*, confers enhanced drought and salt tolerance under field conditions by promoting stomatal closure (Hu et al. 2006). Overexpression of *OsNAC5* was shown to confer enhanced stress tolerance to rice via an ABA-dependent pathway (Song et al. 2011; Takasaki et al. 2010). Overexpression of *OsNAC10* driven by a root-specific promoter improves drought tolerance and grain yield in rice under field conditions (Jeong et al. 2010). In addition, improved tolerances to multiple abiotic stresses such as drought and salinity in transgenic rice plants overexpressing *OsNAC045*, *OsNAC052* or *OsNAC063* have been reported (Gao et al. 2010; Yokotani et al. 2009; Zheng et al. 2009).

Although tremendous research on NAC proteins has focused primarily on model plant species such as Arabidopsis and rice, more and more stress-related NAC genes were functionally characterized in agronomy crops such as wheat, barley, maize and soybean (Chen et al. 2013; Hao et al. 2011; Jensen et al. 2007; Lu et al. 2012; Mao et al. 2014, 2012). As for *Miscanthus lutarioriparius*, a potential lignocellulosic plant for bioenergy production, little information of NAC genes is available. In a previous study, we reported stress-inducible expression patterns of 13 stress-related NAC genes from *M. lutarioriparius* (Ji et al. 2014). In the present study, we further fulfilled a detailed functional analysis of *MlNAC5*. *MlNAC5* was demonstrated to function as a nucleus located transcriptional activator. Overexpression of the *MlNAC5* in transgenic Arabidopsis led to enhanced tolerance to drought and cold stress. These results suggest that *MlNAC5* functions as a stress-responsive transcription factor in response to abiotic stresses. Furthermore, *MlNAC5* overexpression in Arabidopsis affected diverse aspects of plant development under normal growth conditions, suggesting that *MlNAC5* not only played important roles in abiotic stress but also was involved in diverse plant development process.

## Materials and methods

### Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used for gene transformation in this study. Seeds were surface sterilized with 10 % sodium hypochlorite, rinsed several times with sterile distilled water and sown onto 1/2 MS plates for germination. At 8 days (d) after germination, seedlings were transferred to soil and grown in growth chamber at 21 ± 1 °C with relative humidity of 55 % under long-day (LD) conditions (16 h light/8 h dark).

### Multiple sequence alignment and phylogenetic analysis

Alignment of MlNAC5 and other NAC-domain protein sequences were performed with ClustalX program (ver 2.0) (Thompson et al. 2002) and adjusted by GeneDoc software (ver 2.5). The phylogenetic tree was constructed with MEGA program (ver 5.0) by Neighbor-Joining (NJ) algorithm (Tamura et al. 2011). The parameters pairwise deletion and p-distance model were used. Bootstrap test of phylogeny was performed with 1,000 replicates.

### Subcellular localization analysis

The full-length coding sequence of *MlNAC5* without a terminator codon was amplified by PCR and the fragment was cloned into pGWC-T (Chen et al. 2006) to generate an entry clone (pGWC-*MlNAC5*) and sequenced. LR reaction (Gateway, Invitrogen) was carried out with pGWC-*MlNAC5* and the binary vector pEarleyGate101 (Earley et al. 2006), which resulted in a plasmid (pEarleyGate101-*MlNAC5*) consisting of MlNAC5-YFP under the control of CaMV 35S promoter. The resulting construct was then sequenced to confirm an intact in-frame fusion. The construct and negative control (pEarleyGate101) were transformed into *Agrobacterium* strain *EHA105* and infiltrated into tobacco (*Nicotiana benthamiana*) leaves via *Agrobacterium*-mediated transformation. The YFP fluorescence signal was visualized and photographed with a Laser Scanning Confocal Microscope (Olympus, FluoView FV1000). Cells were labeled with the DNA dye 4,6-diamidino-2-phenylindole (DAPI) to visualize the nucleus.

### Transactivation assay

The coding sequences of *MlNAC5,* the fragments encoding the N-terminus (1–477 bp) and the C-terminus (478–873 bp) were amplified by PCR. The primer sequences are listed in Supplemental Table 1. The PCR products were inserted into the NdeI and EcoRI sites of the pGBKT7 vector to obtain pGBKT7-*MlNAC5*-FL (1–306aa), pGBKT7-*MlNAC5*-N (1–135aa) and pGBKT7-*MlNAC5*-C (136–306aa). These three constructs and the pGBKT7 empty vector (negative control) were transformed into the yeast strain *AH109*. The transformed strains were confirmed by PCR and then were streaked on SD/Trp and SD/-Trp/-His plates. The transactivation activity of each protein was evaluated according to its growth status and β-galactosidase filter lift assay.

### β-Glucuronidase assay

A 726 bp DNA fragment upstream from the translational initiation codon of the *MlNAC5* was amplified by hiTAIL-PCR (Wang et al. 2013). The fragment was inserted upstream GUS reporter gene in pCXGUS-P vector. The construct was introduced to *Agrobacterium* strain *EHA105* and transformed into Arabidopsis Col-0 by the floral dip method (Clough and Bent 1998). In situ GUS activity assays were performed for T1 plants as described previously (Beeckman and Engler 1994). Plant materials were stained in X-Gluc solution at 37 °C for 3 h. The stained materials were cleared with 75 % (v/v) ethanol and photographed using a dissecting microscope (Olympus BX51).

### In silico promoter sequence analysis

For detection of putative cis-acting regulatory elements in the promoter sequence, the online search tool of PlantCARE (plant cis-acting regulatory elements, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was utilized.

### Generation of transgenic Arabidopsis plants

The coding sequences of *MlNAC5* was amplified by PCR and cloned into the pGWC-T vector (pGWC-*MlNAC5*) to create an entry clone. The overexpression vector (pEarlayGate100-*MlNAC5*) under the control of the CaMV 35S promoter was constructed by LR recombination action (Gateway, Invitrogen). The construct was transferred into *Agrobacterium* strain *EHA105* and then transformed into Arabidopsis Col-0 plants using the floral dip method (Clough and Bent 1998). Transgenic plant seeds were screened in soil by spraying BASTA (25 mg/L). T3 homozygous lines were used for further analysis.

### Germination assay

Approximately 100 seeds of homozygous *MlNAC5* overexpressing lines and the wild type (WT) were surface sterilized and sown on 1/2MS agar medium (0.8 %) containing different concentrations of NaCl (50, 100 and 150 mM) or ABA (0.5 and 1.0 μM). Plates were stratified at 4 °C in the dark for 3 days, and then transferred to a

growth chamber to germinate. The germination rate was calculated based on radicle protrusion or the greening of expanded cotyledons at 10 days thereafter. Each experiment was performed in triplicates.

### Root length measurement

Seedlings grown on 1/2 MS plates for 3 or 6 days were transferred vertically to 1/2 MS plates supplemented with 10 μM ABA or 100 mM NaCl. The seedlings grown on 1/2 MS plates without stress were employed as the control. Root length was measured 5 days after transplantation for each plant. Each experiment was performed in triplicates.

### Abiotic stress tolerance assays

For dehydration treatment, eight-day-old seedlings of *MlNAC5* overexpression lines and the WT were transferred from 1/2 MS plates to water-saturated soil, water was withheld until the plants showed evident drought-stressed phenotypes, then the plants were re-watered. To characterize the function of *MlNAC5* in cold tolerance, 2-week-old potted plants were treated at −8 °C for 1.5 h. After that, the plants were incubated in a cold growth chamber (4 °C) for 3 h, then transferred to normal growth conditions (21 ± 1 °C) for recovery. For each stress assay, the survival rates of transgenic lines and the WT were calculated. Experiments were repeated independently three times. For each experiment, approximately 20 plants were used.

### RNA isolation and quantitative real-time RT-PCR (RT-qPCR) analysis

Total RNA was extracted using the RNAiso Plus kit (TaKaRa) and treated with RNAase-free DNase I (TaKaRa). Reverse transcription was performed with 2 μg RNA using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RT-qPCR was performed on the LightCycler 480 system (Roche) using SYBR Premix ExTaq (TaKaRa) as described previously (Ji et al. 2014). Each reaction was performed in triplicates, and products were verified by melting curve analysis. *Actin2* was used as an internal control for normalization. Relative gene expression levels are represented by relative quantification values calculated using the $2^{-ddCt}$ method. The primers are listed in Supplemental Table 1.

### Statistical analysis

Statistical analysis was calculated in SPSS 11.5 (SPSS, Inc., IL, USA) by Student's *t* test to determine significant differences.

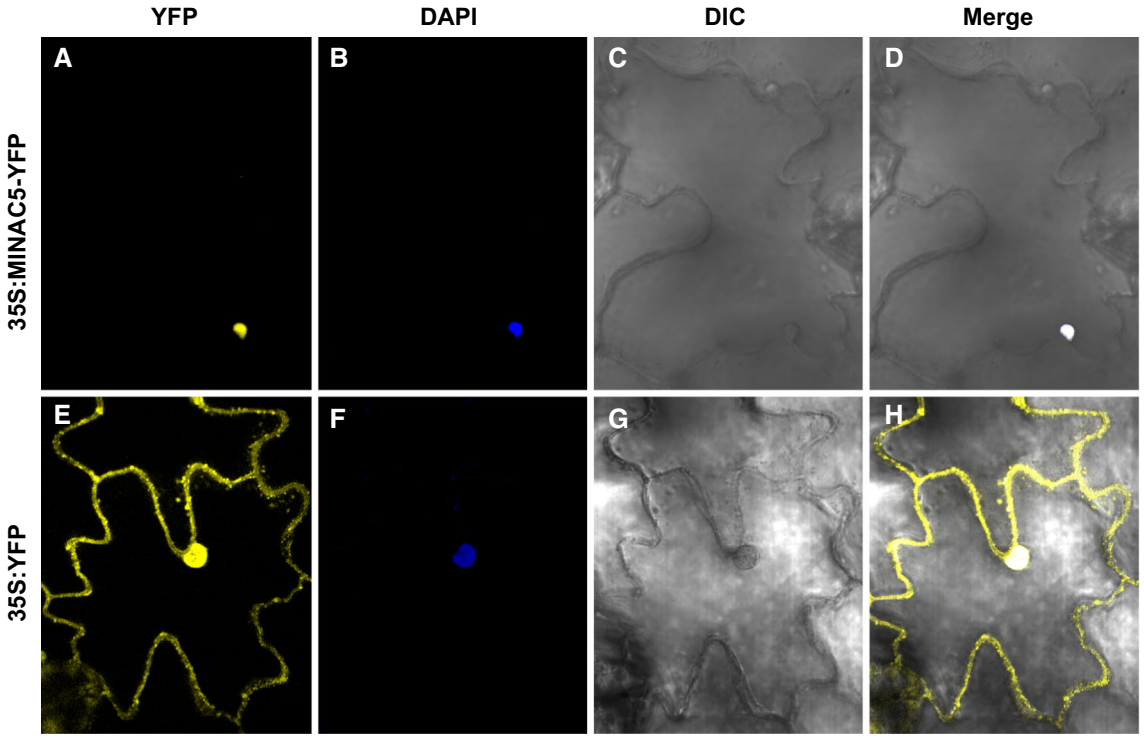


**Fig. 1** Nuclear localization of MlNAC5. YFP and MlNAC5-YFP fusion proteins were transiently expressed under control of the CaMV 35S promoter in tobacco leaves and observed under a laser scanning confocal microscope. **a**, **e**: fluorescent images of YFP. **b**, **f**: DAPI stained. **c, g**: Differential interference contrast (DIC) images in bright field. **d**, **h**: the merged images

## Results

### MlNAC5 belongs to ATAF subfamily of NAC proteins

In a previous study, we reported the expression analysis of 13 NAC stress-related NAC transcription factor genes in *M. lutarioriparius* (Ji et al. 2014). Phylogenetic analysis revealed that MlNAC5 was clustered together with Arabidopsis ATAF1 and rice orthologue OsNAC6/SNAC2. MlNAC5 had an overall sequence identity of 83.3 and 71.9 % with ZmSNAC1 and OsNAC6/SNAC2, respectively, at the amino acid level. Sequence alignments showed that MlNAC5 contained a conserved NAC domain in the N-terminal region, which was further divided into five subdomains (A–E) (Supplemental Fig. 1). To further reveal the divergence of MlNAC5 proteins during evolution, we analyzed the phylogenetic relationship of MlNAC5 with their ATAF1 orthologues as well as other NAC genes, which are functionally characterized in different plant species including Arabidopsis, rice, wheat and soybean (Supplemental Fig. 2). Phylogenetic analysis showed that all NACs surveyed were divided into four subgroups in the tree. MlNAC5 was located in the first subgroup of the tree, which included all the ATAF orthologues.

### MlNAC5 is localized in the nucleus

To determine the subcellular localization of MlNAC5, the MlNAC5-YFP fusion construct and the YFP control in pEarleyGate101 driven by CaMV 35S promoter were transiently expressed in tobacco epidermal cells and visualized under a confocal microscope. The YFP signal was consistently observed within both the cytoplasm and nucleus, whereas the MlNAC5-YFP fusion protein was exclusively localized in the nucleus that was confirmed by DAPI staining (Fig. 1). This result indicated that the *MlNAC5* encodes a nuclear protein.

### MlNAC5 is a transcriptional activator

To investigate whether the deduced MlNAC5 protein had transcriptional activity, the entire coding region, N-terminal domain and C-terminal domain were cloned into pGBKT7, which contains the GAL4 DNA-binding domain, respectively, The constructs and empty vector pGBKT7 (negative control) were transformed into yeast strain *AH109*. All the transformed yeast cells grew well on SD medium lacking tryptophan (-Trp) (Fig. 2). The transformants containing the full-length MlNAC5 (pGBKT7-MlNAC5-FL) and the C-terminus of MlNAC5 (pGBKT7-MlNAC5-C) could grow on selection medium SD/-Trp/-His/5 mM 3-AT, whereas the cells with the N-terminus of

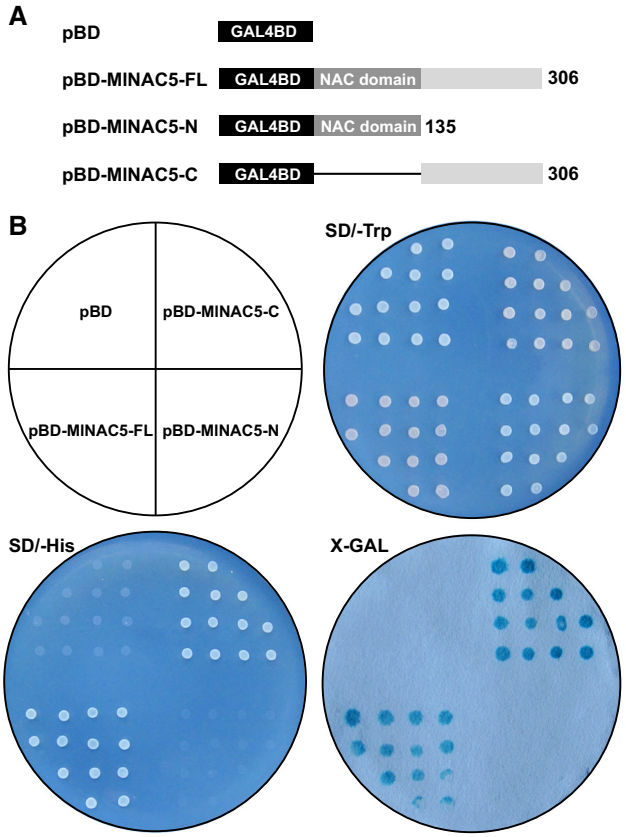


**Fig. 2** Transactivation assay of MlNAC5 in yeast cells. The full-length proteins (MlNAC5-FL), N-terminal fragment (MlNAC5-N) and C-terminal fragment (MlNAC5-C) were fused with GAL4 DNA-binding domains and expressed in yeast strain AH109. The pGBKT7 vector was used as a negative control. The transformed yeasts were streaked on the SD/-Trp and SD/-His medium. LacZ activity was assessed by β-galactosidase filter lift assay

MlNAC5 (pGBKT7-MlNAC5-N) and pGBDT7 control failed to grow. Furthermore, the yeast cells that grew well on the SD/-His medium turned blue in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), indicating that the reporter gene LacZ was activated. These results indicated that MlNAC5 functions as a transcriptional activator and its transactivation domain is located in the C-terminus.

### Morphological characteristics of *MlNAC5*-overexpressing Arabidopsis plants

To analyze the functions of *MlNAC5*, we generated transgenic Arabidopsis plants overexpressing *MlNAC5* under the control of CaMV 35S promoter. More than 30 transgenic lines were obtained and the expression of *MlNAC5* was examined by RT-qPCR. Two homozygous lines (*MlNAC5*-OX-4 and *MlNAC5*-OX-18) at T3 generation with relatively high expression levels were selected for further phenotypic analysis.

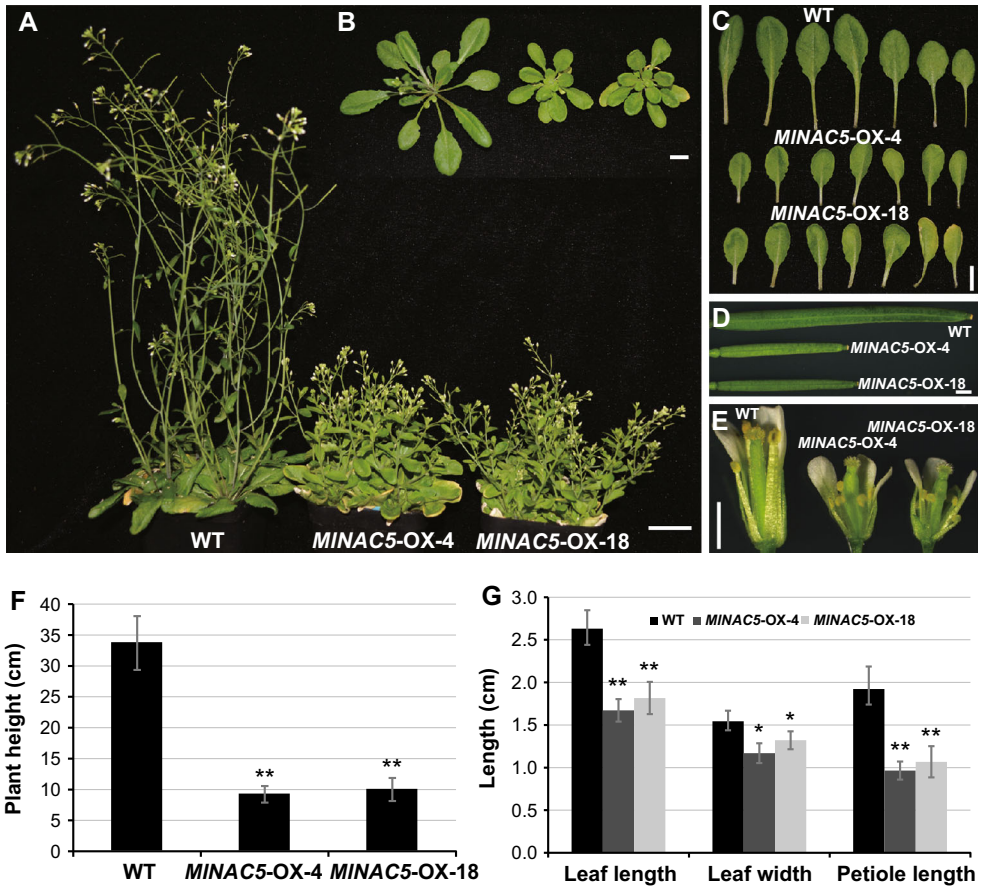


**Fig. 3** Phenotypic analysis of *MlNAC5*-overexpression lines. **a** Seven-week-old WT and *MlNAC5*-overexpression plants grown under long-day (LD) conditions. Two independent *MlNAC5*-overexpression lines (OX-4 and OX-18) display growth retardation and the plant height was significantly reduced compared to WT. **b** Five-week-old WT and *MlNAC5*-overexpression plants grown under LD conditions. The growth of *MlNAC5*-overexpression lines was retarded and the plants displayed precocious leaf senescence. **c** Detached leaves of WT and *MlNAC5*-overexpression plants grown for 5 weeks under LD conditions. **d** Siliques of WT and *MlNAC5*-overexpression plants, the siliques of *MlNAC5*-overexpression plants were shorter compared to WT. **e** Flower morphology of WT and *MlNAC5*-overexpression lines. Some petals and sepals were removed. The length of stamens of *MlNAC5*-overexpression lines was significantly decreased compared to that of WT. **f** Plant height of 8-week-old *MlNAC5*-overexpression and WT plants. Plant height was measured using 20 plants for each genotype. *Asterisks* indicate statistically significant differences compared with WT as determined by Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation. **g** Leaf length, width and petiole length of rosette leaves from 4-week-old *MlNAC5* overexpression and WT plants. Measurement was performed using 30 leaves (leaf 5 and leaf 6) for each genotype. *Asterisks* indicate statistically significant differences compared with WT values based on Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation

The *MlNAC5* overexpressing lines displayed multiple phenotypic changes at various developmental stages (Fig. 3). The transgenic plants exhibited retarded growth compared to WT, and the size of seedling was much smaller than WT. The rosette leaf morphology was also significantly altered in *MlNAC5*-overexpressing plants. The transgenic lines had much smaller rosette leaf size compared to WT under long-day conditions, and length to width ratio dramatically reduced.

The inflorescence stem growth was also retarded in *MlNAC5* overexpression lines, which resulted in a moderately dwarf phenotype at maturity. In addition, the flowering time was delayed in *MlNAC5*-overexpressing plants compared to WT. Moreover, the fertility of *MlNAC5*-overexpressing plants was significantly affected and few seeds were generated at maturity. Anatomy analysis revealed that pistils and stamens developed normally in *MlNAC5*-overexpressing plants. However, the length of stamens in *MlNAC5* transgenic plants was dramatically reduced compared to that of WT, which may hinder the successful pollination thus leading to the higher sterility in transgenic line.

## *MlNAC5* promoter harbors stress-responsive related cis-acting elements

We analyzed the promoter fragment of the *MlNAC5* for the presence of putative cis-acting regulatory elements. A

**Table 1** Stress-related cis-acting regulatory elements identified in the promoter region of *MlNAC5*

| Site Name | Species | Position | Strand | Matrix score | Sequence | Function |
|---|---|---|---|---|---|---|
| ABRE | *Triticum aestivum* | 410 | − | 9 | GACACGTGGC | cis-acting element involved in the abscisic acid responsiveness |
| ABRE | *Hordeum vulgare* | 480 | + | 9 | CGTACGTGCA | cis-acting element involved in the abscisic acid responsiveness |
| ABRE | *Arabidopsis thaliana* | 412 | + | 6 | CACGTG | cis-acting element involved in the abscisic acid responsiveness |
| TC-rich repeats | *Nicotiana tabacum* | 156 | + | 9 | GTTTTCTTAC | cis-acting element involved in defense and stress responsiveness |
| LTR | *H. vulgare* | 600 | − | 6 | CCGAAA | cis-acting element involved in low-temperature responsiveness |
| MBS | *A. thaliana* | 21 | − | 6 | CAACTG | MYB binding site involved in drought inducibility |
| MBS | *A. thaliana* | 256 | + | 6 | TAACTG | MYB binding site involved in drought inducibility |
| MBS | *A. thaliana* | 223 | + | 6 | CAACTG | MYB binding site involved in drought inducibility |
| TGACG-motif | *H. vulgare* | 372 | − | 5 | TGACG | cis-acting regulatory element involved in the MeJA responsiveness |
| TGACG-motif | *H. vulgare* | 425 | + | 5 | TGACG | cis-acting regulatory element involved in the MeJA responsiveness |
| CGTCA-motif | *H. vulgare* | 372 | + | 5 | CGTCA | cis-acting regulatory element involved in the MeJA responsiveness |
| CGTCA-motif | *H. vulgare* | 425 | − | 5 | CGTCA | cis-acting regulatory element involved in the MeJA responsiveness |
| GC-motif | *Zea mays* | 352 | + | 6 | CCCCCG | enhancer-like element involved in anoxic-specific inducibility |
| GC-motif | *Z. mays* | 669 | − | 6 | CCCCCG | enhancer-like element involved in anoxic-specific inducibility |

number of stress response-related cis-acting elements were present in the *MlNAC5* promoter. These include three ABA response elements (ABREs), two TGACG-motifs and two CGTCA-motifs involved in the MeJA responsiveness, one LTR motif involved in low-temperature stress, and two GC-motifs involved in anoxic-specific inducibility (Table 1). These stress-related cis-acting elements may be responsive for stress-regulated expression of *MlNAC5*.

## Histochemical assay of *MlNAC5*::GUS expression in transgenic Arabidopsis

The expression pattern of *MlNAC5* was determined by histochemical β-glucuronidase (GUS) staining of transgenic Arabidopsis plants that harbored an *MlNAC5* promoter::GUS reporter construct. The results indicated that *MlNAC5* was mainly expressed in the hypocotyl of 5-day-old seedlings. High level of GUS expression was also detected in the main veins of rosette leaves and at the junction of the stem and the petiole of the 2-week-old seedlings. When plants grew to maturation, strong GUS activity was observed in anthers and the abscission zone of mature siliques (Fig. 4).

## *MlNAC5* overexpression confers to ABA hypersensitivity in transgenic Arabidopsis

To elucidate the role of *MlNAC5* in ABA signaling, two *MlNAC5*-overexpressing lines and WT seeds were germinated on 1/2 MS agar plates supplemented with 0.5 and 1.0 μM ABA for 10 days. Under normal growth conditions, the germination rate of WT reached to nearly 97 %, and the two *MlNAC5*-overexpressing lines exhibited comparable germination rates to WT. In the presence of 0.5 μM ABA, the generation rates of *MlNAC5*-overexpressing seeds ranged from 10 to 20 %. By contrast, the germination rate of WT was much higher, reaching to 90 %. In the presence of 1.0 μM ABA, the germination rate of WT seeds dropped to about 45 %, while only 4 % of *MlNAC5* overexpression lines were germinated (Fig. 5).

To further reveal the functional roles of *MlNAC5* in ABA signaling pathway during the post-germination stage, root elongation inhibition was analyzed for these plants. Six-day-old seedlings were transferred to 1/2 MS agar medium with or without 10 μM ABA and root length was measured at 5 days. The results showed that exogenous

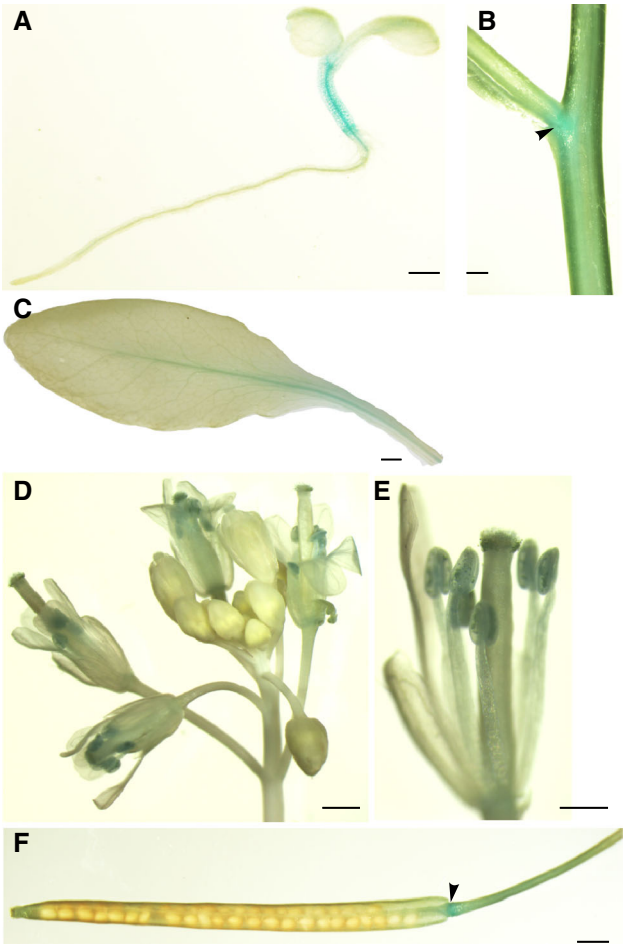


**Fig. 4** Expression of GUS gene driven by *MlNAC5* promoter in transgenic Arabidopsis. **a** 5-day-old seedling. **b** Stem. **c** Leaf. **d** Floral buds. **e** Close-up view of an opened flower. **f** Mature silique. *Scale bars* **a**, **b**, **e**: 500 μm; **c**, **d**, **f**: 1 mm

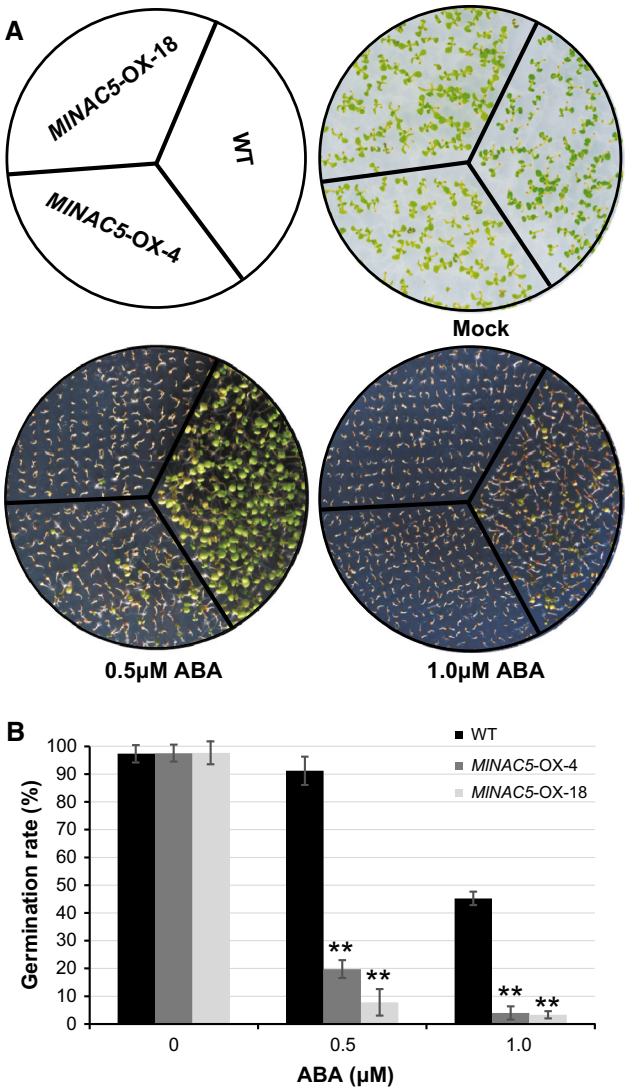


**Fig. 5** Effects of ABA treatment on seed germination of *MlNAC5*-overexpression lines and WT plants. **a** Germination assay on 1/2 MS plate supplemented with different concentrations of ABA. Seeds were sowed on 1/2 MS plates supplemented with or without different concentrations of ABA as indicated and cultured under LD conditions for 10 days. **b** Quantification of germination rates under ABA treatment. The germination rates were scored 10 days after planting on the plates supplemented with the indicated ABA concentrations. Three independent experiments were performed with 100 seeds for each genotype per experiment. *Asterisks* indicate statistically significant differences compared with WT values based on Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation

ABA substantially inhibited the root elongation for all the plants, however, the root growth inhibition was more pronounced for *MlNAC5*-overexpressing lines than that of WT plants (Fig. 6). Taken together, these results indicate that *MlNAC5* overexpression leads to ABA hypersensitivity in transgenic Arabidopsis during germination and the root elongation stage.

## Overexpression of *MlNAC5* enhances drought tolerance in transgenic Arabidopsis

We further tested the effect of *MlNAC5* overexpression on dehydration stress tolerance via a whole-plant drought assay in soil (Fig. 7). Ten-day-old *MlNAC5*-overexpressing and WT plants were transferred from 1/2 MS plates to water-saturated soil. Hereafter water was withheld to gradually reduced water availability in soil. After 14 days, the relative soil water content was decreased to about 10 %, and most of the WT plants had wilted because of the extreme water deficit. In contrast, the *MlNAC5*-overexpressing plants did not show serious wilting except for a slight leaf senescence phenotype. After re-watering, approximately 90 % of *MlNAC5*-overexpressing plants remained vigorous, whereas almost 65 % of WT plants did not recover from the drought stress. In addition, *MlNAC5*-overexpression plants showed significantly lower water loss rates than WT during a 2-h dehydration stress. These results indicated that overexpression of *MlNAC5* could enhance plant drought tolerance.

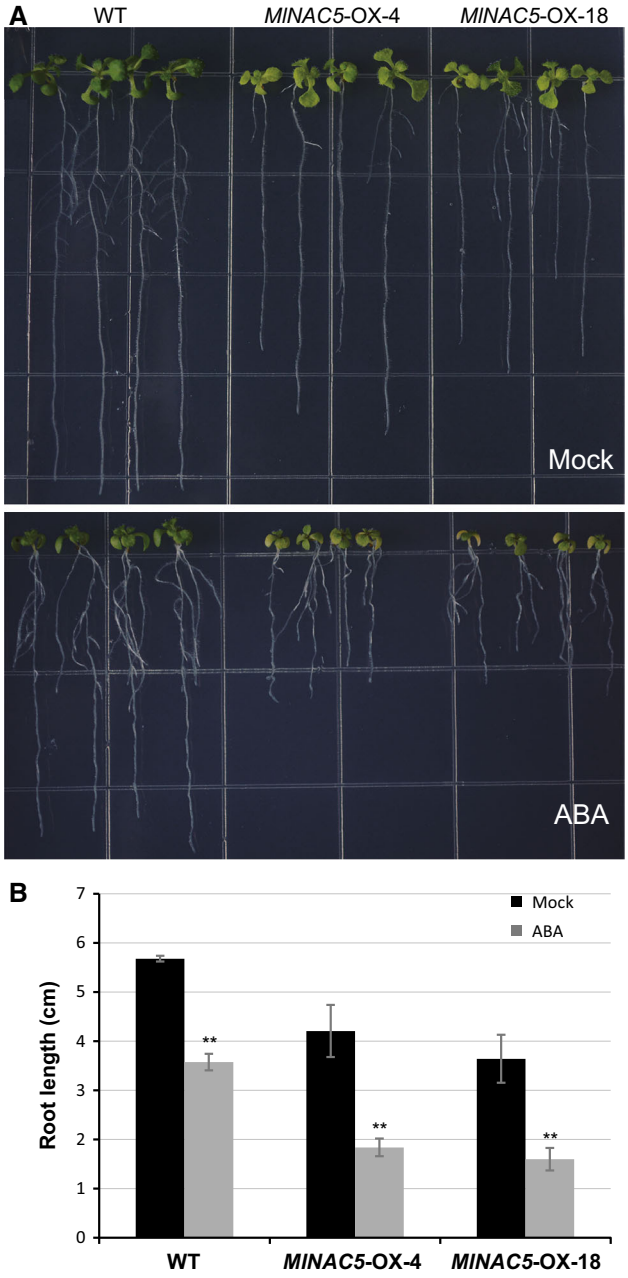


**Fig. 6** *MlNAC5* overexpression led to enhanced ABA sensitivity in root growth. **a** Comparison of primary root length of transgenic and WT Arabidopsis seedlings with ABA treatment. Seedlings grown on 1/2 MS plates for 3 days were transferred vertically to 1/2 MS plates supplemented with or without 10 μM ABA. **b** Quantification of primary root length. Root length was measured in a triplicates experiment using 20 plants for each genotype per experiment. *Asterisks* indicate statistically significant differences compared with WT as determined by Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation

## Overexpression of *MlNAC5* confers hypersensitivity to salinity in transgenic Arabidopsis

The effect of *MlNAC5* overexpression on salt tolerance was also investigated by measuring the germination rates under 50, 100 and 150 mM NaCl treatments. As shown in Fig. 8, there were no significant differences in terms of germination rates between WT and *MlNAC5* overexpression seeds under normal growth conditions. Under 50 mM NaCl treatment, the germination rates of *MlNAC5* overexpression lines and WT exhibited no significant differences. When exposed to 100 mM NaCl treatment, approximately 60–70 % of *MlNAC5* overexpression seeds germinated, whereas the germination rate of WT seeds was much higher, reaching to 95 % at day 10. Under 150 mM NaCl treatment, only 10–15 % seeds of *MlNAC5*-overexpression lines germinated, while the germination rate of WT seed reached to approximately 90 %.

To further reveal the functional roles of *MlNAC5* in salt response during the post-germination stage, root elongation inhibition was analyzed for *MlNAC5* overexpression and WT plants. Three-day-old seedlings were transferred to 1/2 MS agar medium with or without 100 mM NaCl and root length was measured at 5 days. NaCl treatment substantially inhibited the root elongation for all the plants. Although the root length of two *MlNAC5*-overexpressing lines was shorter than that of the WT plants under normal conditions, root growth retardation was more pronounced in *MlNAC5*-overexpressing lines compared to that of WT plants on 1/2 MS medium with 100 mM NaCl (Fig. 9).

## Overexpression of *MlNAC5* improves cold tolerance of transgenic Arabidopsis

To characterize the function of *MlNAC5* in cold tolerance, 2-week-old potted plants were subject to freezing at −8 °C for 1.5 h. The plants were incubated in a cold growth chamber (4 °C) for 3 h, then transferred to normal growth conditions (21 ± 1 °C) for recovery. The results showed that the survival ratios of the two *MlNAC5* transgenic lines (approximately 80 %) were much higher than those of the WT (50 %) (Fig. 10). These results indicated that overexpression of *MlNAC5* improves plant tolerance to low-temperature stress.

## Overexpression of *MlNAC5* induces expression of stress-responsive genes

To elucidate the possible molecular mechanisms underlying drought and cold tolerance conferred by overexpression of *MlNAC5*, we investigated the expression of several abiotic stress-responsive genes in transgenic plants under drought, cold, and ABA treatments (Figs. 11, 12). The RT-qPCR analysis showed that transcripts of all genes examined were significantly up-regulated in WT and *MlNAC5*-overexpressing plants following ABA treatment. However, the fold change of up-regulation for these genes after ABA treatment for 0.5 and 1 h was much higher in *MlNAC5*-

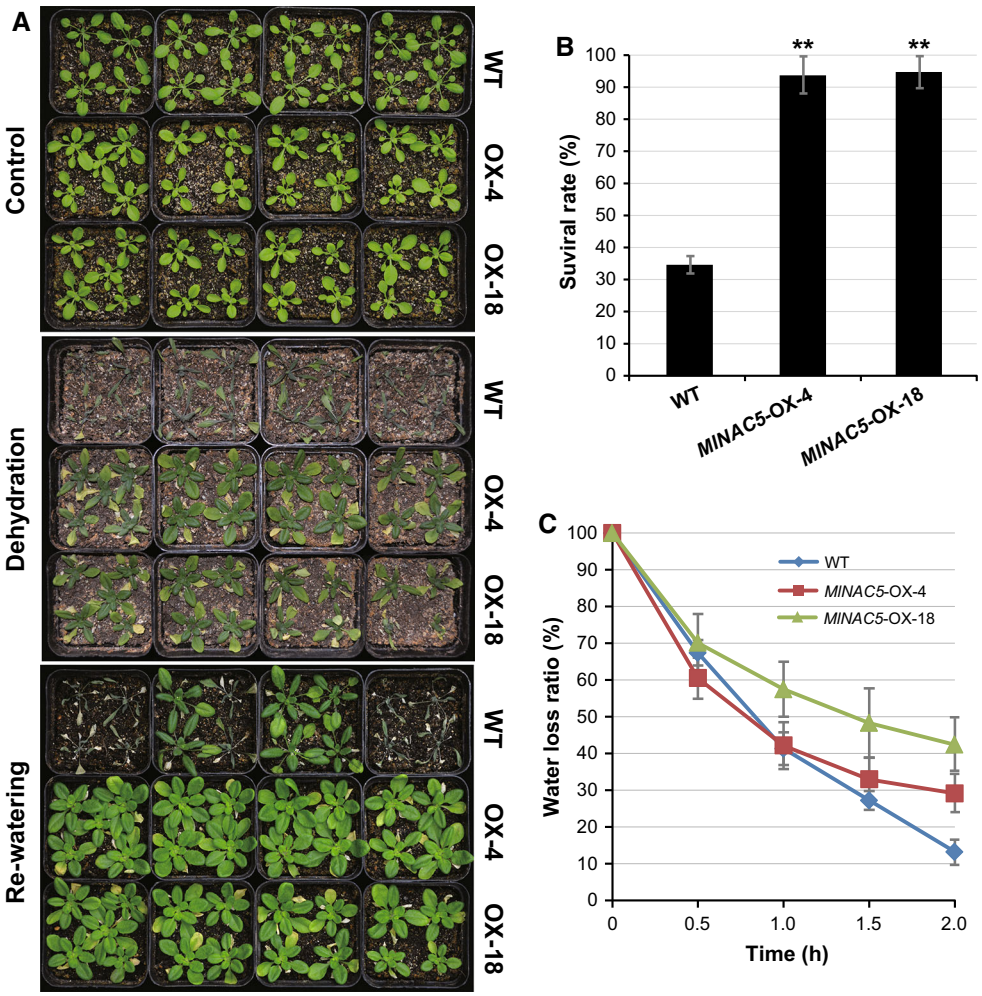


**Fig. 7** Overexpression of *MlNAC5* enhanced drought tolerance in Arabidopsis. **a** Phenotypes of *MlNAC5*-overexpression lines and WT during drought stress. *MlNAC5*-transgenic lines and WT plants were grown for 2 weeks in soil under LD conditions, subjected to 14 days drought stress followed by 2 days of re-watering. **b** Percentage of survival plants in drought-tolerance assays. Experiments were performed in triplicates with 20 plants for each genotype per experiment. *Asterisks* indicate significantly higher survival rates compared to WT control as determined by Student's *t* test. *Bars* indicate standard deviation. **c** Water loss assay from 2-week-old seedlings of *MlNAC5* transgenic and WT plants grown on 1/2 MS plates. The seedlings were excised from 1/2 MS plate, and exposed to dehydration stress for 2 h on the dry filter paper at room temperature. Each data point represents the means of triplicate measurements

overexpressing plants compared to that of WT. Generally, these genes had three to tenfold higher expression in the transgenic plants than that of the WT (Fig. 11). Similarly, all genes examined were significantly up-regulated in WT and *MlNAC5*-overexpression plants under drought treatments. The fold changes of up-regulation for all genes except *COR47* were much higher in *MlNAC5*-overexpression plants compared to WT control (Fig. 11).

The expressions of six cold-responsive marker genes were significantly up-regulated in *MlNAC5*-overexpression lines and WT plants during a 24-h cold treatment. Although the transcripts of these genes peaked at different time points after the cold treatment, the fold change of up-regulation of these genes was much higher at almost all the time points in two *MlNAC5* transgenic lines compared to WT (Fig. 12).

## Discussion

We previously reported stress-inducible expressions of 13 NAC transcription factor genes in *Miscanthus lutarioriparius* (Ji et al. 2014), a candidate bioenergy plant for lignocellulosic biomass production. In the present study, we overexpressed *MlNAC5* in transgenic Arabidopsis to further identify its functional roles in response to abiotic stresses. Our results showed that the transgenic plants have improved drought and cold tolerance, as assessed by a whole-plant abiotic stress assay.

The N-terminal domain of the NAC protein is crucial for recognition of and binding to specific cis-elements in their target genes, while the C-terminal region is considered to be essential for transcriptional activation. We showed that MlNAC5 is exclusively located in the nucleus in a transient assay using tobacco leaves, which is in agreement with the characteristic of a transcription factor with DNA-binding activity. Furthermore, a transactivation assay was conducted in yeast, and the results showed that MlNAC5 functions as a transcriptional activator, with transcriptional activation domain located in the C-terminal region. These results were in accordance with other NAC members reported previously (Fujita et al. 2004; Hao et al. 2011; He et al. 2005; Jeong et al. 2010; Mao et al. 2012; Nakashima et al. 2007; Olsen et al. 2005; Wu et al. 2009).

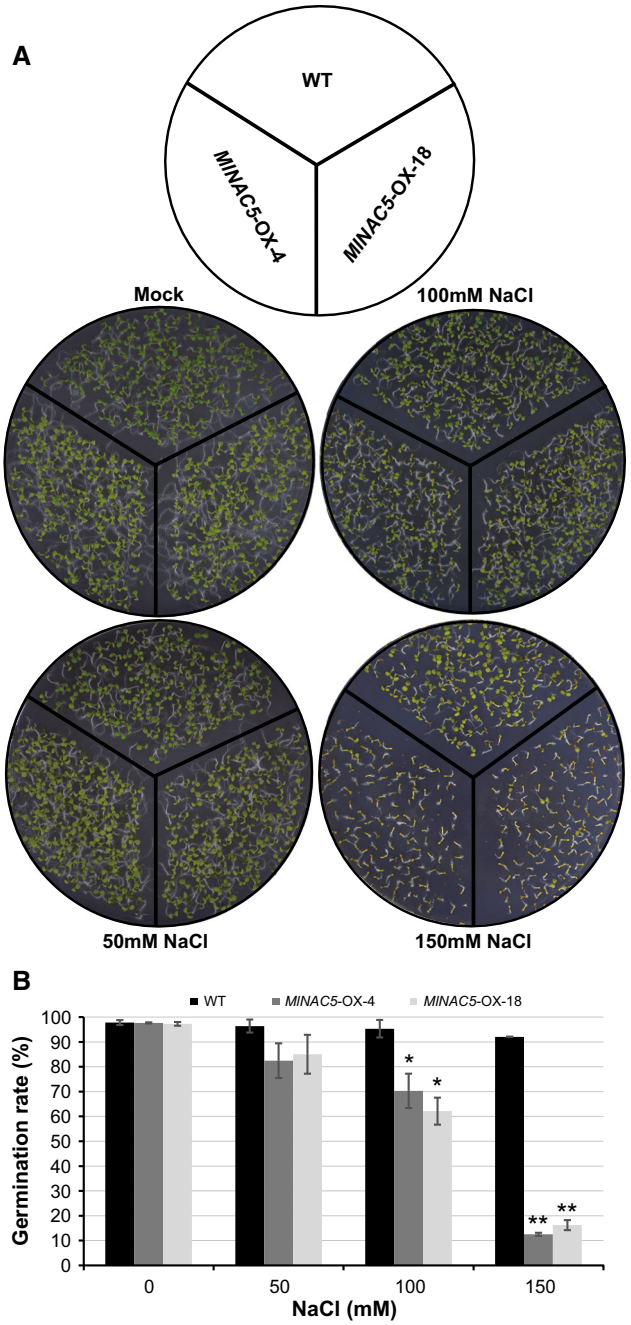


**Fig. 8** Effects of NaCl treatment on seed germination of *MlNAC5*-overexpression lines and WT plants. **a** Germination assay on 1/2 MS plate with different concentrations of NaCl. Seeds were sowed on 1/2 MS plates with or without different concentrations of NaCl as indicated and cultured under LD conditions for 10 days. **b** Quantification of germination rates under NaCl treatment. The germination rates were scored 10 days after planting on the plates with different NaCl concentrations. Three independent experiments were performed with 100 seeds for each genotype per experiment. *Asterisks* indicate statistically significant differences compared with WT values based on Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation

Phylogenetic analysis further revealed that MlNAC5 was clustered into the ATAF subgroup of NAC protein family and was one of the closest orthologues of ATAF1.

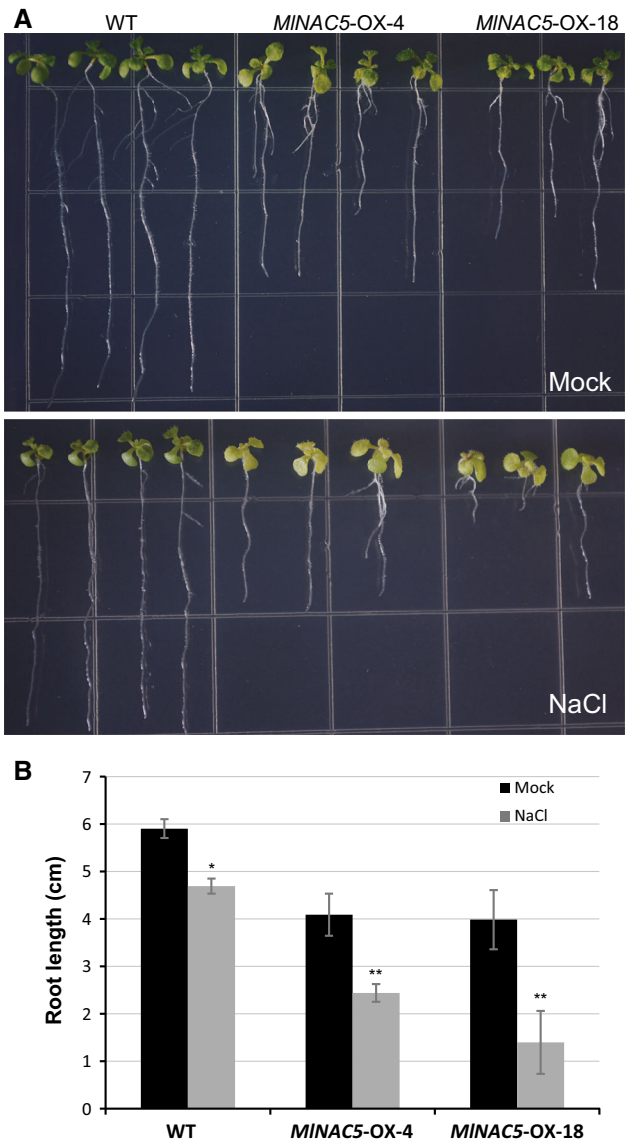


**Fig. 9** *MlNAC5* overexpression led to hypersensitivity to NaCl in root growth. **a** Comparison of primary root length of transgenic and WT Arabidopsis seedlings under NaCl treatment. Seedlings grown on 1/2 MS plates for 3 days were transferred vertically to 1/2 MS plates supplemented with or without different concentrations of NaCl as indicated. **b** Quantification of primary root length under NaCl treatment. The experiment was performed in triplicates using 20 plants for each genotype per experiment. *Asterisks* indicate statistically significant differences compared with WT based on Student's *t* test ($P < 0.01$). *Bars* indicate standard deviation

The expression of *ATAF1* was induced by wounding, pathogen infection, drought, and ABA (Jensen et al. 2008; Lu et al. 2007; Wu et al. 2009). *ATAF1* was shown to mediate efficient penetration resistance in Arabidopsis upon pathogen attack (Jensen et al. 2008; Wu et al. 2009). On the other hand, *ATAF1* was also reported to function as a negative regulator in drought signal transduction pathways as *ataf1-1* mutant showed increased tolerance to

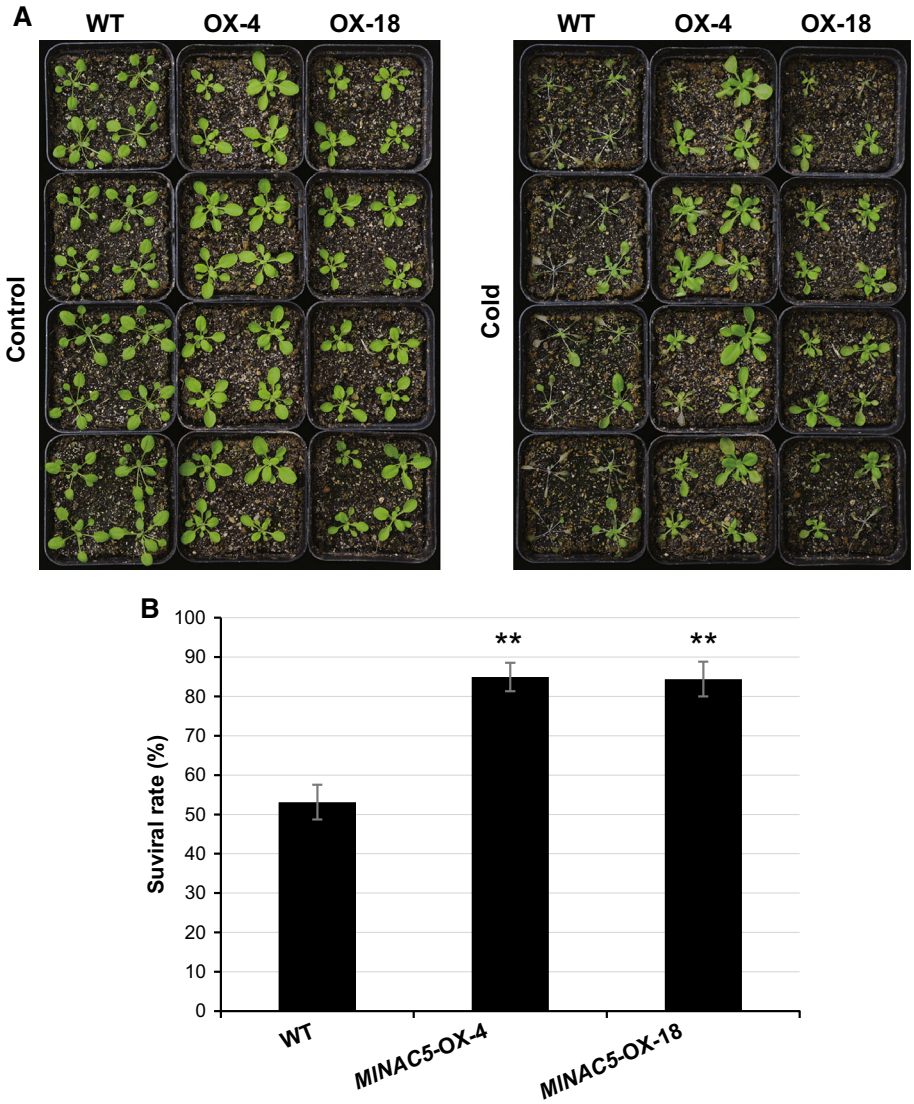


**Fig. 10** Overexpression of *MlNAC5* enhanced cold tolerance in Arabidopsis. **a** Phenotypes of *MlNAC5*-overexpression lines and WT controls during cold stress. Two-week-old potted plants were treated at −8 °C for 1.5 h, then incubated at 4 °C for 3 h, and transferred to normal growth conditions (21 ± 1 °C) for recovery. **b** Quantitative analysis of the plant survival rate in cold-tolerance assays. Experiments were performed in triplicates with 20 plants for each genotype per experiment. *Asterisks* indicate significant differences compared to WT as determined by Student's *t* test. *Bars* indicate standard deviation

drought stress (Lu et al. 2007). However, overexpression of *ATAF1* was later reported to enhance the tolerance to drought (Wu et al. 2009). *ATAF1* has been thought to respond to both abiotic and biotic stress despite of a controversy role of *ATAF1* in drought stress (Nuruzzaman et al. 2013).

The orthologues of *ATAF1* were also reported in other plant species, including rice (*OsNAC6*/*SNAC2* and *OsNAC52*) (Gao et al. 2010; Hu et al. 2008; Nakashima et al. 2007), barley (*HvNAC6*) (Chen et al. 2013; Jensen et al. 2007), soybean (*GmNAC20*) (Hao et al. 2011), maize (*ZmSNAC1*) (Lu et al. 2012) and chickpea (*CarNAC5*) (Peng et al. 2009). The expression of *OsNAC6*/*SNAC2* was significantly induced by drought, salt, cold wounding, and pathogen attack (Hu et al. 2008; Nakashima et al. 2007). *OsNAC6*/*SNAC2*-overexpression plants not only showed improved tolerance to drought and salinity stresses, but also exhibited increased tolerance to blast disease (Hu et al. 2008; Nakashima et al. 2007). Overexpression of *OsNAC52* conferred hypersensitivity to ABA and enhanced dehydration tolerance in transgenic Arabidopsis plants (Gao et al. 2010). Transient overexpression of *HvNAC6* in barley enhanced the tolerance to powdery mildew attack, whereas knockdown of *HvNAC6* by RNAi led to reduced basal resistance (Chen et al. 2013; Jensen et al. 2007). *GmNAC20* was shown to be regulated by various stresses, and its overexpression improved salinity and freezing tolerance in transgenic Arabidopsis plants through activation of the DREB/CBF-COR pathway (Hao et al. 2011). Meanwhile, *GmNAC20* was also shown to promote the lateral root formation probably via the auxin-signaling pathway (Hao et al. 2011). Overexpression of *ZmSNAC1* conferred hypersensitivity to ABA at germination as well as enhanced tolerance to dehydration in transgenic

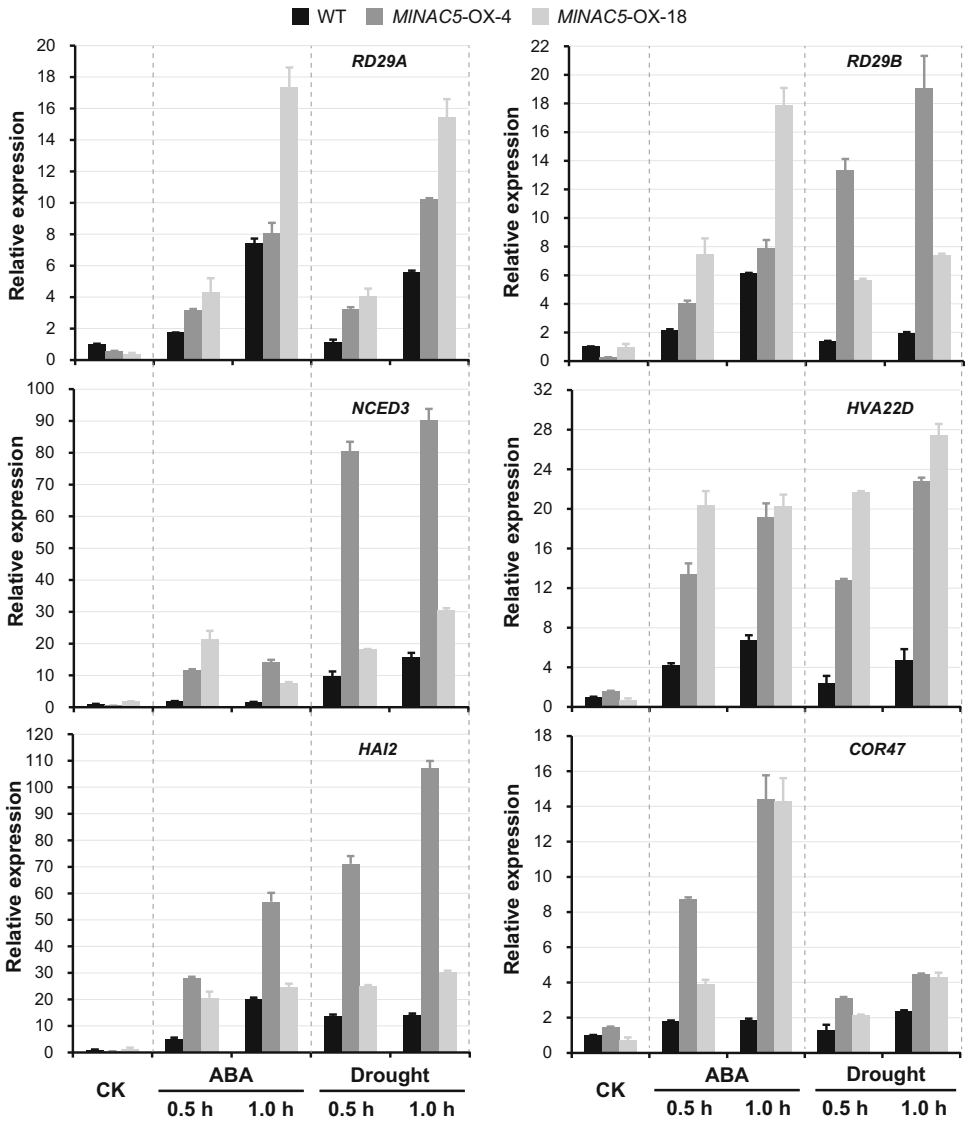


**Fig. 11** Expression analysis of stress-responsive genes in *MlNAC5*-overexpression and WT plants. Transcript levels of six stress-responsive genes were analyzed by RT-qPCR using *actin2* as a reference. Seedlings before treatment were used as controls. Data represent means of three independent assays. *Bars* indicate standard deviation

Arabidopsis (Lu et al. 2012). *CarNAC5* from chickpea was reported to be induced by various stress treatments including drought, heat, wounding, salicylic acid (SA), and indole-3-acetic acid (IAA) treatments (Peng et al. 2009). All these data suggest that *ATAF1* and its orthologues share a conserved role in both biotic and abiotic stresses. Our results showed that overexpression of *MlNAC5*, one of the closest orthologues of *ATAF1* in *Miscanthus*, significantly improved the drought and cold tolerance in transgenic Arabidopsis via an ABA-dependent signaling pathway, which further supports a positive role of *ATAF1* and its orthologues in abiotic stresses. However, the functional roles of *MlNAC5* in responses to biotic stresses remain to be elucidated.

Plant response to abiotic stresses via both ABA-dependent and ABA-independent signal transduction pathways. ABA acted as the signaling molecule in responses to abiotic stresses in plants. NAC TFs have been demonstrated to regulate the expression of both ABA-dependent and ABA-independent genes during abiotic stress responses (Tran et al. 2004; Hao et al. 2011; Jensen et al. 2008; Puranik et al. 2012). The expressions of *ATAF1* and its orthologues reported previously and herein could be induced by exogenously applied ABA, which suggested that *ATAF1* functions to abiotic stresses through an ABA-dependent pathway. *MlNAC5* overexpression plants exhibited hypersensitivity to exogenous ABA and enhanced tolerance to dehydration stress. A higher ABA sensitivity may stimulate

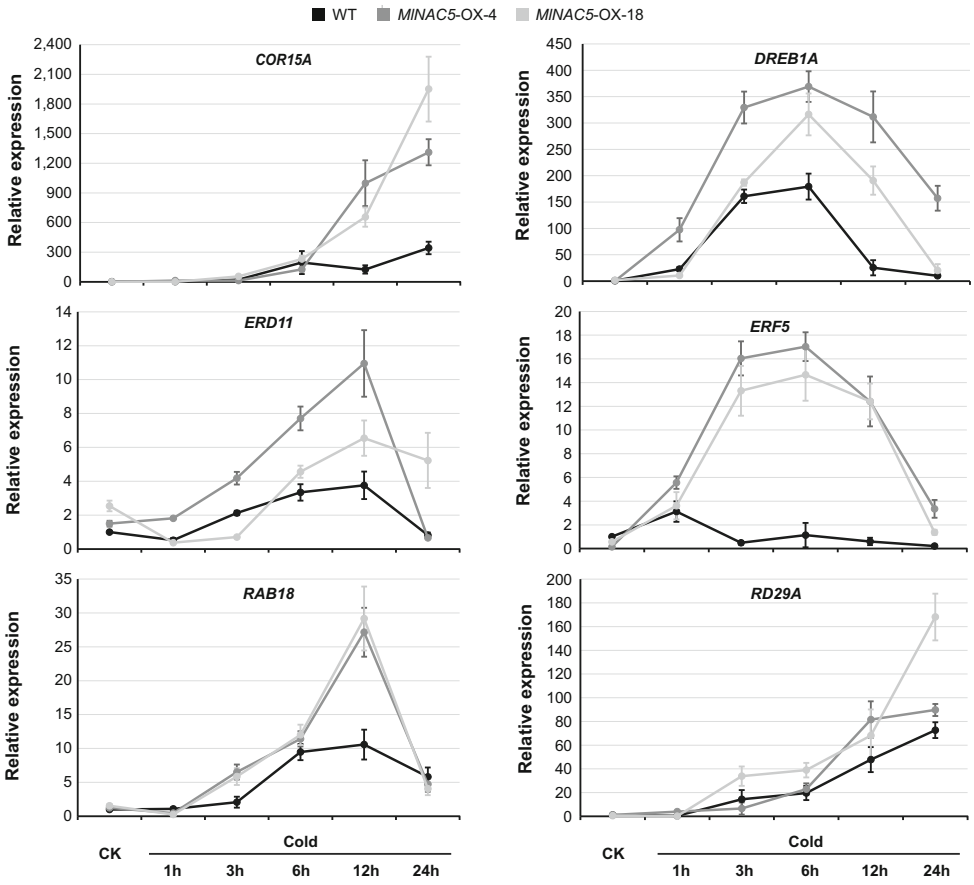


**Fig. 12** Time-course expression of cold-responsive genes in *MlNAC5*-overexpression and WT plants. Expressions of six cold-responsive genes were analyzed by RT-qPCR using *actin2* as a reference. The plants were grown at 21 ± 1 °C for 10 days, and treated at 4 °C for 1, 3, 6, 12 and 24 h, respectively. Plants before treatment were used as controls. Data represent means of three independent assays. *Bars* indicate standard deviation

stomatal closure to retain water and increase drought tolerance in plants. Similar results have been reported in Arabidopsis and maize (Lu et al. 2012; Wu et al. 2009).

ABA response elements (ABRE) and Dehydration responsive element (DRE) are typical cis-acting elements that mediate ABA-dependent signaling. Consistent with a role for *MlNAC5* in abiotic stress responses via ABA-dependent pathway, various cis-acting regulatory elements, including four ABRE motifs, are present in the *MlNAC5* promoter region.

To reveal the underlying molecular mechanisms in abiotic stress tolerance, we examined the expression levels of six abiotic stress responsive genes in *MlNAC5*-overexpression lines in Arabidopsis, and found that these genes were significantly up-regulated. In Arabidopsis, these genes are ultimately induced by a drought signaling pathway (Ingram and Bartels 1996). The products of these genes are low molecular weight hydrophilic proteins and may be helpful for water retention under stressed conditions. However, further extensive analysis will be necessary to elucidate the specific regulatory mechanism of *MlNAC5* in abiotic stress.

Abiotic stresses such as drought, salinity and cold, are the main adverse environmental factors and adversely affects all aspects of plant phenology and metabolism. To cope with these challenges, plants have evolved a range of complex regulatory mechanisms. Growth suppression is commonly deemed as such an adaptable strategy for plants to combat against abiotic stresses. In the present study, overexpression of *MlNAC5* in Arabidopsis affected many aspects of plant developmental processes, such as leaf morphology, dwarfism, late flowering and sterility. These phenotypes are similar to the previously reported transgenic plants of *ATAF1* and its orthologues under the control of the constitute CaMV 35S promoter. For instance, overexpression of the Arabidopsis *ATAF1* reduced the rosette size and plant height, and caused yellowing of the rosette leaves (Jensen et al. 2013). The *OsNAC6*-overexpression plants exhibited growth retardation and lower productivity (Hu et al. 2008; Nakashima et al. 2007). Overexpression of *TaNAC2* resulted in earlier flowering and longer primary roots (Mao et al. 2012). To circumvent this problem, stress-inducible promoters should be used to suppress the detrimental effects of NAC TFs on plant

growth under normal growth conditions, as well as improve stress tolerance. Supporting this claim are studies on *OsNAC6/SNAC2*, in which overexpression of *OsNAC6* driven by stress-inducible promoters, including the LIP9 promoter and especially its own promoter, simultaneously improved stress tolerance without growth retardation effects (Hu et al. 2008; Nakashima et al. 2007). However, whether stress-inducible promoters can be used in *MlNAC5* overexpression to improve stress tolerance while simultaneously suppress adverse growth effects awaits investigation.

In conclusion, we characterized *MlNAC5* as a stress-responsive NAC-type transcription activator in *Miscanthus*. Overexpression of *MlNAC5* in Arabidopsis led to hypersensitivity to ABA and NaCl, and conferred enhanced tolerance to drought and cold stresses. In addition, the data indicated that *MlNAC5* is also involved in various developmental processes.

**Author contribution statement** Conceived and designed the experiments: RH ZY GZ. Performed the experiments: XY XW. Analyzed the data: XY RH. Contributed reagents/materials/analysis tools: LJ JR CF. Wrote the paper: RH GZ.

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**Conflict of interest** The authors declare that they have no conflict of interest.

## References

Beeckman T, Engler G (1994) An easy technique for the clearing of histochemically stained plant tissue. Plant Mol Biol Rep 12:37–42

Bohnert HJ, Gong Q, Li P, Ma S (2006) Unraveling abiotic stress tolerance mechanisms—getting genomics going. Curr Opin Plant Biol 9:180–188

Bray E, Bailey-Serresand J, Weretilnyk E (2000) Responses to abiotic stresses. In: Buchanan B, Gruissem W, Rockville JR (eds) Biochemistry and molecular biology of plants. American Society of Plant Biologists, Rockville, pp 1158–1203

Broun P (2004) Transcription factors as tools for metabolic engineering in plants. Curr Opin Plant Biol 7:202–209

Chen JQ, Dong Y, Wang YJ, Liu Q, Zhang JS, Chen SY (2003) An AP2/EREBP-type transcription-factor gene from rice is cold-inducible and encodes a nuclear-localized protein. Theor Appl Genet 107:972–979

Chen QJ, Zhou HM, Chen J, Wang XC (2006) Using a modified TA cloning method to create entry clones. Anal Biochem 358:120–125

Chen YJ, Perera V, Christiansen MW, Holme IB, Gregersen PL, Grant MR, Collinge DB, Lyngkjaer MF (2013) The barley HvNAC6 transcription factor affects ABA accumulation and promotes basal resistance against powdery mildew. Plant Mol Biol 83:577–590

Christianson JA, Wilson IW, Llewellyn DJ, Dennis ES (2009) The low-oxygen-induced NAC domain transcription factor ANAC102 affects viability of Arabidopsis seeds following low-oxygen treatment. Plant Physiol 149:1724–1738

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

Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J 45:616–629

Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE (1999) Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J 18:4689–4699

Fang Y, You J, Xie K, Xie W, Xiong L (2008) Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics 280:547–563

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 A, Peng R, Jin X, Xu J, Zhu B, Chen J, Yao Q (2010) OsNAC52, a rice NAC transcription factor, potentially responds to ABA and confers drought tolerance in transgenic plants. Plant Cell Tiss Organ Cult 100:255–262

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601–612

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

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J 44:903–916

Hu H, Dai M, Yao J, Xiao B, Li X, Zhang Q, Xiong L (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 103:12987–12992

Hu H, You J, Fang Y, Zhu X, Qi Z, Xiong L (2008) Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. Plant Mol Biol 67:169–181

Hu R, Qi G, Kong Y, Kong D, Gao Q, Zhou G (2010) Comprehensive analysis of NAC domain transcription factor gene family in *Populus trichocarpa*. BMC Plant Biol 10:145

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377–403

Jensen MK, Rung JH, Gregersen PL, Gjetting T, Fuglsang AT, Hansen M, Joehnk N, Lyngkjaer MF, Collinge DB (2007) The HvNAC6 transcription factor: a positive regulator of penetration resistance in barley and Arabidopsis. Plant Mol Biol 65:137–150

Jensen MK, Hagedorn PH, de Torres-Zabala M, Grant MR, Rung JH, Collinge DB, Lyngkjaer MF (2008) Transcriptional regulation by an NAC (NAM-ATAF1,2-CUC2) transcription factor attenuates ABA signalling for efficient basal defence towards Blumeria graminis f. sp. hordei in Arabidopsis. Plant J 56:867–880

Jensen MK, Lindemose S, de Masi F, Reimer JJ, Nielsen M, Perera V, Workman CT, Turck F, Grant MR, Mundy J, Petersen M, Skriver K (2013) ATAF1 transcription factor directly regulates

abscisic acid biosynthetic gene NCED3 in *Arabidopsis thaliana*. FEBS Open Bio 3:321–327

Jeong JS, Kim YS, Baek KH, Jung H, Ha SH, Do Choi Y, 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

Ji L, Hu R, Jiang J, Qi G, Yang X, Zhu M, Fu C, Zhou G, Yi Z (2014) Molecular cloning and expression analysis of 13 NAC transcription factors in *Miscanthus lutarioriparius*. Plant Cell Rep 33:2077–2092

Le DT, Nishiyama R, 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 Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10:1391–1406

Lu PL, Chen NZ, An R, Su Z, Qi BS, Ren F, Chen J, Wang XC (2007) A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. Plant Mol Biol 63:289–305

Lu M, Ying S, Zhang DF, Shi YS, Song YC, Wang TY, Li Y (2012) A maize stress-responsive NAC transcription factor, ZmSNAC1, confers enhanced tolerance to dehydration in transgenic Arabidopsis. Plant Cell Rep 31:1701–1711

Mao X, Zhang H, Qian X, Li A, Zhao G, Jing R (2012) TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. J Exp Bot 63:2933–2946

Mao X, Chen S, Li A, Zhai C, Jing R (2014) Novel NAC transcription factor TaNAC67 confers enhanced multi-abiotic stress tolerances in Arabidopsis. PLoS One 9:e84359

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell 17:2993–3006

Mukhopadhyay A, Vij S, Tyagi AK (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc Natl Acad Sci USA 101:6309–6314

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Nuruzzaman M, Sharoni AM, Kikuchi S (2013) Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front Microbiol 4:248

Olsen AN, Ernst HA, Lo Leggio L, Skriver K (2005) DNA-binding specificity and molecular functions of NAC transcription factors. Plant Sci 169:785–797

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S (2003) Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Res 10:239–247

Peng H, Cheng HY, Yu XW, Shi QH, Zhang H, Li JG, Ma H (2009) Characterization of a chickpea (Cicer arietinum L.) NAC family gene, CarNAC5, which is both developmentally- and stress-regulated. Plant Physiol Biochem 47:1037–1045

Puranik S, Sahu PP, Srivastava PS, Prasad M (2012) NAC proteins: regulation and role in stress tolerance. Trends Plant Sci 17:369–381

Sablowski RW, Meyerowitz EM (1998) A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell 92:93–103

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 Genomics 284:173–183

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinform Chapter 2 Unit:2–3

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 1 promoter. Plant Cell 16:2481–2498

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314:1298–1301

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci USA 97:11632–11637

Wang GH, Xiao JH, Xiong TL, Li Z, Murphy RW, Huang DW (2013) High-efficiency thermal asymmetric interlaced PCR (hiTAIL-PCR) for determination of a highly degenerated prophage WO genome in a Wolbachia strain infecting a fig wasp species. Appl Environ Microbiol 79:7476–7481

Wu Y, Deng Z, Lai J, Zhang Y, Yang C, Yin B, Zhao Q, Zhang L, Li Y, Xie Q (2009) Dual function of Arabidopsis ATAF1 in abiotic and biotic stress responses. Cell Res 19:1279–1290

Xie Q, Frugis G, Colgan D, Chua NH (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14:3024–3036

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

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