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Overexpression of transcription factor *SlNAC35* enhances the chilling tolerance of transgenic tomato

G.-D. WANG[1], Q. LIU[1], X.-T. SHANG[1], C. CHEN[1], N. XU[1], J. GUAN[1]*, and Q.-W. MENG[2]*

School of Biological Science, Jining Medical University, Ri'zhao, Shandong 276800, P.R. China[1]
College of Life Science, State Key Laboratory of Crop Biology, Shandong Agricultural University, Tai'an, Shandong 271018, P.R. China[2]

Abstract

The NAC (NAM, ATAF1/2, and CUC2) transcription factor family participates in responses to various kinds of environmental stimuli in plants. However, the roles of NAC protein in cold resistance, especially in the cold resistance of tomatoes, are not completely understood. This study examined the roles of a tomato (*Solanum lycopersicum*) NAC transcription factor (SlNAC35) in resisting chilling using transgenic tomatoes. GUS staining and expression analysis revealed that *SlNAC35* expression was induced at 4 °C, thereby suggesting its involvement in plant responses to chilling stress. Moreover, transgenic lines over-expressing *SlNAC35* exhibited high chlorophyll content, fresh mass, and low accumulation of reactive oxygen species and membrane damage under chilling stress. These results indicated that *SlNAC35* overexpression enhanced the chilling tolerance of transgenic tomatoes. High expressions of cold tolerance markers *SlCOR518* and *SlCOR413IM1* were observed under chilling stress in transgenic lines. This observation suggested that *SlNAC35* overexpression enhanced the chilling tolerance of transgenic lines by involving the c-repeat binding factor-cold stress response (CBF-COR) signaling pathway and by regulating *SlCOR* expression.

Additional key words: *Arabidopsis thaliana*, catalase, chlorophyll, malondialdehyde, reactive oxygen species, *Solanum lycopersicum*, superoxid dismutase.

Introduction

The metabolic activity of plant is severely affected by cold stress, either directly or indirectly by induction of osmotic stress (chilling inhibit the water uptake by plants, and freezing results in dehydration of plant cells) and oxidative stress. As a result of the existence of cold stress tolerance genes, plants can be well adapted to low temperatures. A series of low temperature-related transcription factors, such as DREB/CBF (dehydration response factor/c-repeat binding factor), NAC (NAM, ATAF1/2), and many others exhibit certain functions under chilling stress (Aslam *et al*. 2010) including regulation of cold signal transduction. In addition, a cold resistance of plants is regulated by a variety of specific genes. Consequently, improving the cold tolerance of plants is difficult. Recent studies showed that transgenic plants overexpressing cold resistance transcription factors can show high freezing tolerance (Shan *et al*. 2014, Shi *et al*. 2015).

A growing number of studies showed that NAC transcription factors play an important role in plant

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Abbreviations: ATAF1/2 - *Arabidopsis thaliana* activating factor 1/2; CaMV35 S - cauliflower mosaic virus 35 S; CAT - catalase; CBF - c-repeat binding factor; COR - cold stress responsive; CUC2 - cup-shaped cotyledon; DAB - 3,3′-diaminobenzidine; GUS - β-glucuronidase; ICE - inducer of CBF expression; MDA - malondialdehyde; MS - Murashige and Skoog; NAC - NAM, ATAF1/2, and CUC2; NAM - no apical meristem; NBT - nitroblue tetrazolium; $O_2^{\cdot-}$ - superoxide anion radical; PR - pathogenesis response; qPCR - quantitative polymerase chain reaction; REC - relative electric conductivity; ROS - reactive oxygen species; *SlNAC35* - *Solanum lycopersicum NAC 35*; SOD - superoxide dismutase; TF - transcription factor; WT - wild-type.
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* Corresponding authors; fax: (+86) 538 8226399, e-mails: qwmeng@sdau.edu.cn, guanjing1616@sina.com

growth and development and in the abiotic and biotic stress resistance. *NAC* is a large gene family, which is found only in plants (Puranik *et al*. 2012). NAC proteins contain a highly conserved N-terminal domain and a variable C-terminal domain (Olsen *et al*. 2005). NAC proteins are also involved in organ development, cell division and expansion, secondary wall synthesis, lateral root development, flowering time, iron homeostasis, and senescence (Zhong *et al*. 2010, Bollhöner *et al*. 2012, Nakashima *et al*. 2012, Hussey *et al*. 2013, Ricachenevsky *et al*. 2013, Christiansen and Gregersen 2014). These proteins also participate in plant responses to abiotic stresses. *NAC* gene overexpression enhances drought, salt, and/or cold stress resistance in rice (Zheng *et al*. 2009, Jeong *et al*. 2010, Song *et al*. 2011). Similarly, *VvNAC1* overexpression in *Arabidopsis* enhances its tolerance to osmotic, salt, and cold stresses (Hénanff *et al*. 2013). A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species (ROS) in rice (Fang *et al*. 2015). In addition, a sweet potato NAC transcription factor IbNAC1 plays an important role in protection against mechanical wounding and herbivore attack (Chen *et al*. 2016a, 2016b). NAC proteins also participate in regulation of the defense response against pathogens. For instance, the *Arabidopsis thaliana* activating factor 1/2 (ATAF2) acts as a repressor of pathogenesis response (*PR*) gene expression in *Arabidopsis* (Delessert *et al*. 2005). ATAF1 negatively regulates the defense response to necrotrophic fungi and bacterial pathogens (Wang *et al*. 2009). Furthermore, the *Arabidopsis* NAC factors ANAC019 and ANAC055 are involved in regulating the jasmonic acid (JA) signaling in defense responses of *Arabidopsis* (Bu *et al*. 2008). OsNAC6 and OsNAC19 are induced in rice upon challenge with the rice blast fungus *M. grisea*, and *OsNAC6* overexpression results in increased resistance toward rice blast (Lin *et al*. 2007). *ZmNAC41* and *ZmNAC100* are also induced in response to *Colletotrichum graminicola* infection (Voitsik *et al*. 2013).

The physiological damage of plants under chilling stress is mainly shown as damage on cell membranes, proteins, chloroplasts, and mitochondria. Nevertheless, plants often activate series of cold stress responsive genes (*CORs*), which encode the antifreezing proteins, which help to protect plants (Thomashow 1999). Currently, the most well-known cold acclimation signaling pathway is the inducer of c-repeat binding factor-cold stress response (ICE-CBF-COR) transcription cascade pathway (Thomashow 1999, Chinnusamy *et al*. 2007, Knight and Knight 2012). In this pathway, the CBFs and DREBs can be quickly induced during chilling stress. They are combined with the *COR* gene promoter region to activate the expression of these genes and further responses to chilling stress (Stockinger *et al*. 1997, Liu *et al*. 1998). NAC transcription factors can be involved in the chilling stress signal transduction. For instance, the over-expression of soybean *GmNAC20* improves the freezing tolerance of transgenic *Arabidopsis* plants probably through activation of the DREB/CBF-COR pathway (Hao *et al*. 2011). Moreover, the cold-responsive *MaNAC1* may be involved in the cold tolerance of banana fruits through its interaction with ICE1-CBF cold signaling pathway (Shan *et al*. 2014). Nonetheless, it is not fully understand yet whether tomato NAC transcription factors can be also involved in ICE-CBF-COR transcriptional pathway under chilling stress. Previously, we found that tomato NAC transcription factor (SlNAC35) is an active transcription factor and it is localized in the nucleus. The *SlNAC35* overexpression in tobacco also enhances the tolerance to drought and salt stresses and renders resistance against *Pseudomonas solanacearum* (Wang *et al*. 2016).

In the present study, the *SlNAC35* expression was induced by chilling stress. The cold tolerance index of transgenic plants was compared to that in wild-type (WT) plants to determine if the *SlNAC35* overexpression in tomato can enhance tolerance to chilling stress.

Materials and methods

Plants and treatments: The wild-type (WT) tomato (*Solanum lycopersicum* L. cv. Zhongshu 6) plants and three transgenic plants of the T_2 generation (OE2, OE5, and OE8) were grown in quartz sands irrigated with Hoagland's nutrient solution once every two days in a growth chamber with a 16-h photoperiod, a photon flux density (PFD) of 200 μmol m^{-2} s^{-1}, day/night temperatures of 25/20 °C, and a relative humidity of 50 - 60 %. When the sixth leaf was fully expanded, the plants were transferred to an incubation chamber *GXZ-500C* (*Jiangnan Instruments*, Ningbo, China) and acclimated for 2 d before chilling stress treatments at 4 °C and PFD of 200 μmol m^{-2} s^{-1} for 0, 3, 6, 9, 12, 24, and 48 h. All measurements of physiological and biochemical parameters were conducted on the youngest fully expanded leaves.

Acquisition and identification of transgenic tomato plants: The coding sequence of *SlNAC35* was subcloned into the ProkII expression vector under the control of CaMV 35S promoter. The recombinant plastid *SlNAC35*-ProkII was introduced into *Agrobacterium tumefaciens* strain LBA4404. T_0 kanamycin-resistant transgenic tomato plants were generated by the *A. tumefaciens*-mediated leaf disk method. DNA was extracted from the WT and T_0 kanamycin-resistant plants. Afterward, PCR

was used to detect these transgenic plants with a specific 35S forward primer (5'-TACGCAGCAGGTCTC ATCAAGACGAT-3') and a *SlNAC35* reverse primer (5'-TTACTGAAATTGATAGGATG-3'). Plants that produced 100 % kanamycin resistance in T_2 generation progeny were considered homozygous and used in the following experiments.

Real-time quantitative (q) PCR analysis: Total RNA isolation and reverse transcription were performed according to the manufacturer's instructions using the RNeasy plant mini kit (*Tiangen*, Beijing, China) and the cDNA synthesis kit (*Fermentas*, Glen Burnie, MD, USA). Subsequently, a *CFX96TM* real-time PCR system (*Bio-Rad*, Hercules, USA) was used to carry out the qPCR by using *TransStart Green qPCR SuperMix* (*Transgen Biotech*, Beijing, China). The PCR thermal cycle procedure was as follows: denaturation at 94 °C for 60 s and 40 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 15 s. *EF-1α* (GenBank accession No. LOC544055) was used as a reference according to the method of Vandesompele *et al*. (2002). Template-free, negative, and single primer controls were established prior to the examination. Results represent three biological replicates (each with three technical replicates). The qPCR primers are listed in Table 1 Suppl.

Protein extraction and Western blot analysis: The total proteins were extracted from WT and transgenic plant leaves as described by Kong *et al*. (2014). For Western blot analysis, firstly the 10 % (m/v) sodium dodecylsulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate the total protein sample (30 μg per lane). Afterward, the semidry electroblotting method was used to transfer protein samples to polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with sealing liquid for 2.5 h, anti-hemagglutinin antibody (1:10 000 dilution; *Sigma*, San Francisco, USA) for 2.5 h, and secondary antibody for 2.5 h. The secondary antibody was peroxidase-conjugated *AffiniPure* sheep antimouse IgG diluted at 1:5000 in phosphate-buffered saline (PBS). The staining reaction was composed of 3,3'-diaminobenzidine (DAB, *Sigma*, San Francisco, USA), methanol, 0.03 % (v/v) hydrogen peroxide, and PBS. Protein content was measured as described by Bradford (1976) with bovine serum albumin as a standard.

SlNAC35 promoter amplification and GUS staining analysis: Total genomic DNA was extracted from six-week-old WT tomato plant leaves using the cetyl-trimethyl-ammonium bromide method and used as a template to amplify the *SlNAC35* promoter region. The main putative *cis*-acting elements in the *SlNAC35* promoter region are listed in Table 2 Suppl. For the GUS staining analysis, the recombinant plasmid *SlNAC35*pro-GUS was transformed into *Agrobacterium tumefaciens* strain GV3101 and the transformation of *Arabidopsis* (Col-0) was performed using the floral dip method (Clough and Bent 1998). Subsequently, transgenic *Arabidopsis* plants were grown under 22 °C, 75 % relative humidity, and a 10-h photoperiod-cycle and 1, 3, 5, 7, 10, and 60-d-old transgenic *Arabidopsis* lines expressing *SlNAC35*pro-GUS were used. The 10-d-old transgenic *Arabidopsis* seedlings were subjected to above mentioned conditions (controls) or chilling stress (4 °C for 12 h). GUS staining was performed as described by Horsch *et al*. (1985), with slight modifications. Briefly, transformed *Arabidopsis* seedlings were incubated in GUS staining solution containing 0.1 mM $K_3[Fe(CN)_6]$, 0.5 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 5 mM $K_4[Fe(CN)_6]$, 10 mM EDTA, 100 mM Na_3PO4, and 0.1 % (v/v) *Triton X-100*, pH 7.0, at 37 °C in darkness for 12 h. After being sufficiently decolorized with 75 % (v/v) ethanol, the plant tissues were photographed with a camera.

Measurements of cold resistance index: WT and transgenic plants (OE2, OE5, and OE8) seeds were grown in quartz sand irrigated with Hoagland's nutrient solution once every two days under conditions mentioned in the first paragraph. When the seedlings had four leaves, the plants were transferred to an incubation chamber and subjected to chilling stress at 4 °C under dark for 48 h. Chilling injury and cold resistance index calculations were as follows: level 0, leaves were normal and without chilling injury; level 1, only a few of leaf edges showed a slight puckering wilting; level 2, less than half of the leaves were dead, but the main stem was living; level 3, more than half of the leaves were dead; and level 4, all plants were killed. The chilling injury index formula is as follows: $\sum Xa/\sum X= (X_1a_1 + X_2a_2 + \cdots + X_na_n)/T$, where X_1, X_2, X_3, … and X_n are numbers of tomato plants at all chilling injury levels; a_1, a_2, a_3, … and a_n are the chilling injury levels; and T is the total number of tomato plants. The cold resistance index is the reciprocal of chilling injury index.

Measurements of physiological parameters and antioxidant enzyme activities: After the plants had been grown for 2 weeks, half of the plants were transferred into another incubation chamber with the same conditions but at a temperature of 4 °C. The remaining plants were allowed to grow in the incubation chamber at 25 °C as controls. After 2 weeks, their fresh mass was quantified and growth inhibition was calculated: growth inhibition = [(FM at 25 °C - FM at 4 °C)/FM at 25 °C] ×100. Six-week-old WT and transgenic plants were treated at 4 °C for 48 h. For the control group, equal number of plants was simultaneously placed at 25 °C for 48 h. Chlorophyll was extracted from the leaves by 80 % (v/v) acetone for 48 h, and the content was measured as described by Kong *et al*. (2014) using a UV-visible spectrophotometer (*Thermo Scientific*, Massachusetts,

USA). Malondialdehyde (MDA) content and relative electrical conductivity (REC) were also measured in the leaves, as described by Kong *et al*. (2014). Leaves (0.5 g) were rapidly ground with 5 cm^3 of ice-cold extraction buffer [50 mM potassium phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 % (m/v) polyvinylpyrrolidone]. After the homogenate was centrifuged at 12 000 *g* and at 4 °C for 20 min, the supernatant was immediately used as crude enzyme for measurement of antioxidant enzyme activities. The superoxide dismutase (SOD) (EC 1.12.1.11) and catalase (CAT) (EC1.11.1.6) activities were measured as described previously (Zong *et al*. 2009). These experiments were repeated at least three times.

Histochemical staining and measurements of H_2O_2 and $O_2^{\bullet-}$: Six-week-old WT and transgenic tomato plants were treated at 4 °C for 24 h. For the control group, equal number of plants was simultaneously placed at 25 °C for 24 h. H_2O_2 was stained with DAB using the method described by Giacomelli *et al*. (2007). Superoxide radical ($O_2^{\bullet-}$) was stained using nitroblue tetrazolium (NBT) according to the method of Rao and Davis (1999). The degree of cell membrane damage was evaluated by trypan blue staining according to the method described by Choi *et al*. (2007). The H_2O_2 and $O_2^{\bullet-}$ content in six-week-old transgenic and WT lines were measured according to the method of Kong *et al*. (2014).

Statistical analyses: Ten plants were usually treated for each line. The experiments were repeated three times. Statistical analyses were performed using *SIGMAPLOT v. 12.5* and *SPSS v. 13.0*. Differences between control and treated plants were analyzed by two-way *ANOVA*, taking $P < 0.05$ as significant, according to Duncan's test.

Results

Previous qPCR studies showed that the *SlNAC35* expression is highest in tomato leaves (Wang *et al*. 2016). To verify this result, GUS staining analysis was carried out in transgenic *Arabidopsis thaliana* plants expressing *SlNAC35*pro-GUS gene. Fig. 1*A* shows that the *SlNAC35* expression was also highest in leaves of transgenic *A. thaliana*. In agreement with previous results (Wang *et al*. 2016), *SlNAC35* expression was remarkably induced at 4 °C (Fig. 1*B*). To elucidate this observation, real-time qPCR was used to evaluate the expression of *SlNAC35* under 4 °C treatment (Fig. 1*C*) and the result was consistent with that of GUS staining.

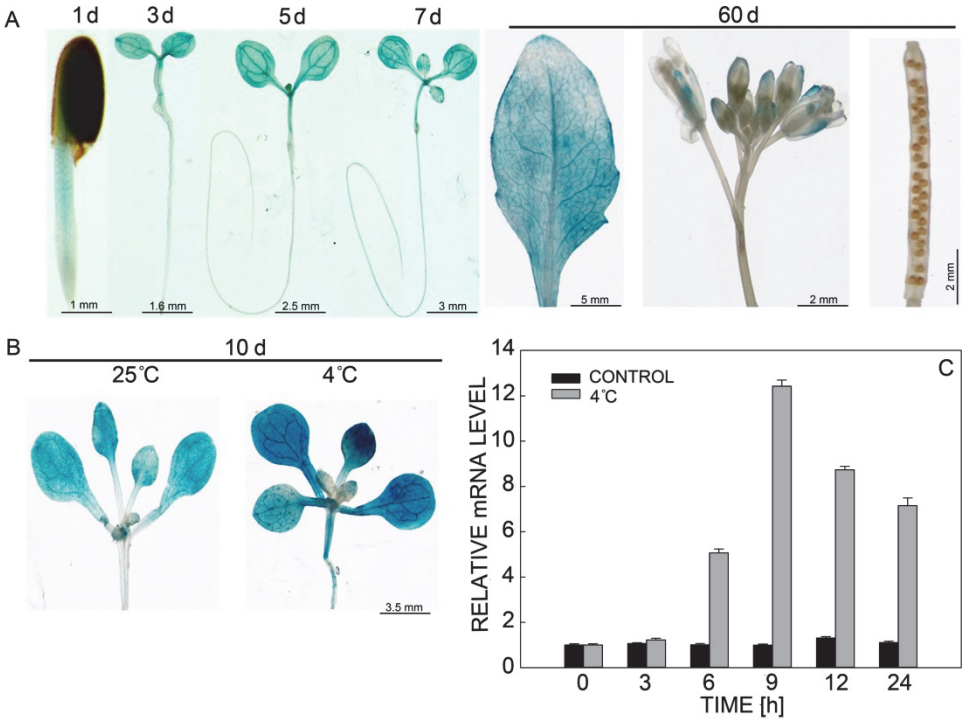

Fig. 1. GUS staining and *SlNAC35* expression analysis in transgenic *Arabidopsis thaliana* plants. *A* - GUS staining (blue colour) in roots, stems, leaves, flowers, and capsules of seedlings grown 1 - 60 d in growth chamber at 25 °C. *B* - GUS staining analysis in leaves of 10-d-old seedlings treated at 25 °C or 4 °C for 12 h. *C* - *SlNAC35* expression under 4 °C for 0 - 24 h as revealed by real time qPCR.

A total of 18 kanamycin-resistant tomato transgenic lines were harvested from the tissue culture. Nine T_2 lines were selected for real-time qPCR analysis. The relative *SlNAC35* mRNA content in the transgenic lines was increased by 12.7-, 9.6-, 14.6-, 17.8-, 44.5-, 29.2-, 17.9-, 30.4-, and 21.3-fold compared with that of the WT (Fig. 2*A*). From these lines, OE2, OE5, and OE8 were selected for Western blot analysis. The change pattern in the SlNAC35 protein content was similar to that of the mRNA content (Fig. 2*B*). Thus, OE2, OE5, and OE8 were selected for subsequent physiological experiments.

To investigate the chilling tolerance of transgenic plants, two-week-old seedlings were placed in an incubation chamber at 4 °C for 2 weeks. The growth inhibition extent of plants was also calculated (Table 1). Moreover, we investigated the cold resistance index of WT and transgenic tomato plants under chilling stress (Table 1). These results indicated that *SlNAC35*-overexpressing tomato showed less chilling stress.

Plants produce a large amount of ROS under chilling stress. Therefore, the intracellular levels of H_2O_2 and $O_2^{\bullet-}$were analyzed by DAB and NBT staining, respectively. Under 25 °C, both H_2O_2 and $O_2^{\bullet-}$ accumulation was low, with no significant difference between WT and transgenic plants. After 24 h at 4 °C treatment, amount of blue polymerization products caused by the presence of H_2O_2 and $O_2^{\bullet-}$ was higher in WT than in transgenic plants (Fig. 4*A,B*). Quantitative analysis of H_2O_2 and $O_2^{\bullet-}$ content revealed a similar results (Fig. 4*C,D*). These results suggested that *SlNAC35* overexpression alleviated the accumulation of these ROS under chilling stress.

Plants maintain cellular ROS homeostasis through scavenging the excess ROS by antioxidants (Cai *et al.* 2014). Therefore, the SOD and CAT activities were assayed. Under 25 °C, no significant difference was observed in the activities of these two antioxidant enzymes (Table 1). By contrast, their activities were increased under chilling stress; the increase was considerably more apparent in the *SlNAC35*-over-expressing plants than in control plants. To determine the reason for the high enzyme activities of transgenic plants, qPCR was used to assess the expression of respective genes (*SlCnZnSOD* and *SlCAT*) (Table 2). The chilling stress induced a considerably higher expression of these genes in the transgenic plants than in control plants. These results indicated that *SlNAC35* overexpression possibly alleviated the ROS accumulation in transgenic plants by activating the antioxidant system.

To investigate whether *SlNAC35*-overexpressing mature tomato also exhibited tolerance to chilling stress, 6-week-old WT and transgenic plants were treated at 4 °C for 48 h. Their responses to chilling stress were also observed. Under 25 °C, both transgenic and WT plants grew well and showed no significant difference in phenotype and physiological indices (Fig. 3). After chilling treatment, the plant leaves exhibited different degrees of withering. Leaf withering was less severe,

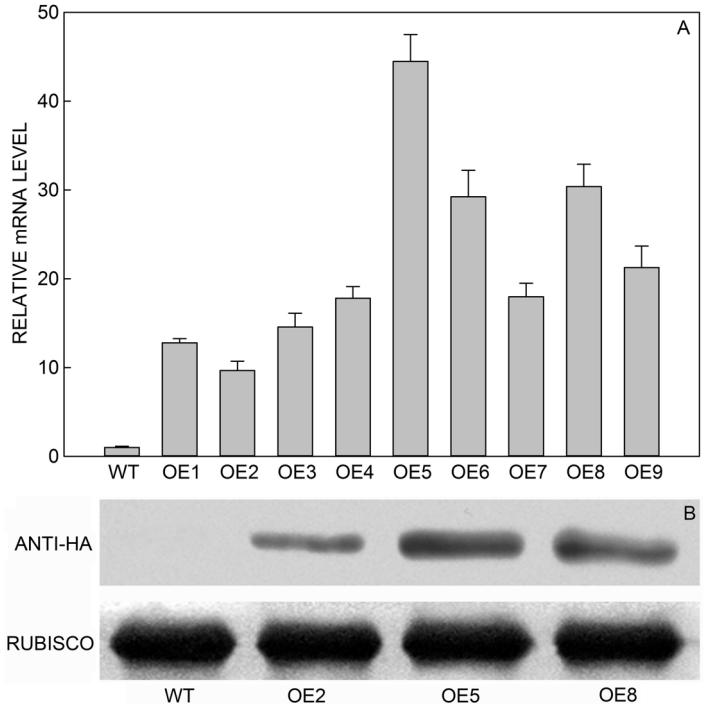

Fig. 2. Identification of *SlNAC35*-overexpressing plants by real-time qPCR and Western blot analysis. *A* - Amount of transcripts of *SlNAC35* in wild-type (WT) and different SlNAC35-overexpressing lines (OE1 - OE9). *B* - Determination of the SlNAC35 protein in WT and selected SlNAC35-overexpressing lines. The large subunit of RUBISCO is shown as a loading control.

Table 1. Effect of chilling stress on extent of growth inhibition [%], cold resistance index, superoxide dismutase (SOD) activity [U g^{-1}(f.m.)], and catalase (CAT) activity [U g^{-1}(f.m.)] in WT and transgenic tomato plants. One U corresponds to the amount of enzyme transforming 1 μmol of substrate per minute under certain conditions. Means ± SDs, $n = 3$. Means within a row followed by different letters indicate significant differences at $P < 0.05$ according to Duncan's test.

Parameter	Temperature [°C]	WT	OE2	OE5	OE8
Growth inhibition	4	0.360±0.025a	0.280±0.026b	0.250±0.019b	0.260±0.025b
Cold tolerance index	4	0.370±0.029b	0.750±0.057a	0.820±0.069a	0.780±0.046a
SOD activity	25	92.435±5.896a	93.514±7.564a	94.057±7.889a	92.276±5.875a
	4	122.538±5.788b	162.766±9.566a	166.970±9.775a	161.725±7.897a
CAT activity	25	4.735±0.589a	4.851±0.756a	4.905±0.788a	4.727±0.587a
	4	12.538±0.578b	17.766±0.956a	18.697±0.977a	17.258±0.789a

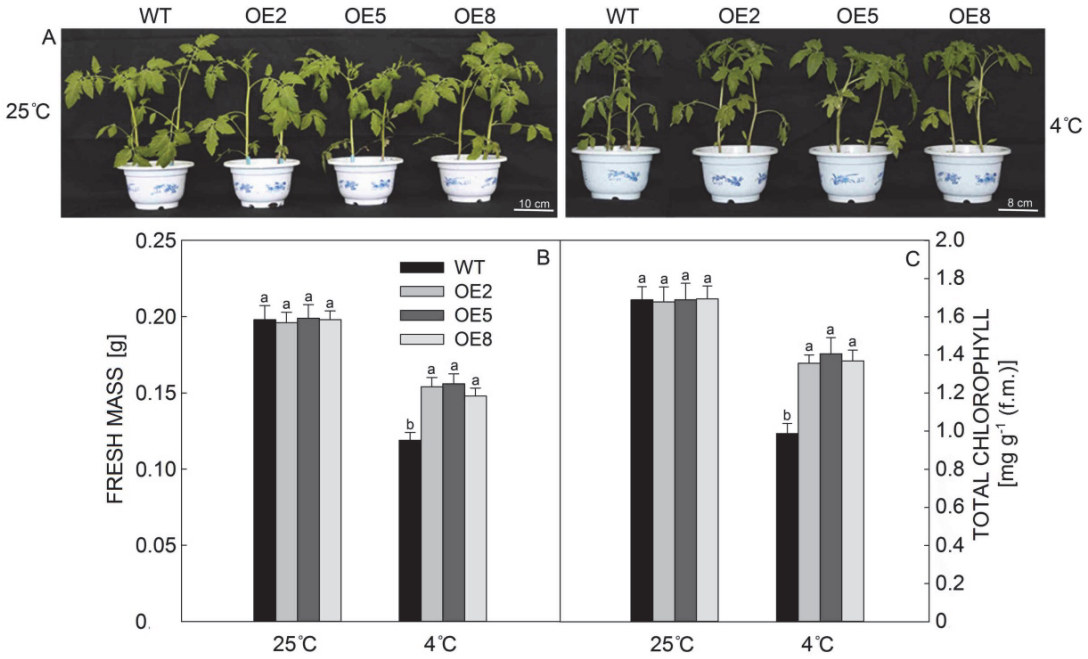

Fig. 3. Response to chilling stress of six-week-old WT tomato plants and transgenic lines OE2, OE5, and OE8. *A* - Phenotype of the plants grown at 25 °C (*left panel*) or at 4 °C for 24 h (*right panel*). *B, C* - fresh mass per plant and total chlorophyll content in WT and transgenic plants OE2, OE5, and OE8). Means ± SDs, $n = 3$. Means followed by different letters indicate significant differences at $P < 0.05$ according to Duncan's test.

and the average chlorophyll content was higher in *SlNAC35*-overexpressing plants than that in the WT (Fig. 3*B,C*). These results suggested that the *SlNAC35* overexpression in mature tomato conferred the tolerance to chilling stress, which was similar to that of the seedlings. As the primary target of chilling stress, membranes are particularly susceptible to ROS-initiated lipid peroxidation reactions. This possibility was investigated by trypan blue staining. Six-week-old plants grown at 25 °C were treated at 4 °C for 24 h. Transgenic plants exhibited lighter blue staining than WT plants at 4 °C (Fig. 5*A*). However, all plants showed similar blue staining at 25 °C. To confirm these results, MDA accumulation and REC, which are cell damage indicators, were determined. Under 25 °C, no significant differences in MDA accumulation and REC were observed between WT and transgenic plants. After chilling stress treatment, both MDA accumulation and REC increased, and their increase was more evident in WT plants than in transgenic ones (Fig. 5*B*,*C*). These results suggested that *SlNAC35* overexpression protected the cells from chilling-induced damage.

To investigate how *SlNAC35* enhanced the cold tolerance of transgenic plants, real-time qPCR was used to detect the expression of two CBF genes (*SlCBF1* and *SlCBF2*). The expression of *SlCBF1* and *SlCBF2* displayed no evident difference between WT and transgenic plants before and after chilling stress treatment. We also detected the expression of *COR* genes, namely, *SlCOR413IM1* and *SlCOR518*. The *SlNAC35*-

overexpressing plants showed higher expressions of *SlCOR413IM1* and *SlCOR518* in response to chilling stress than control plants (Table 2). These results indicated that SlNAC35 may enhance the plant chilling tolerance by upregulating the expression of *SlCOR413IM1* and *SlCOR518*.

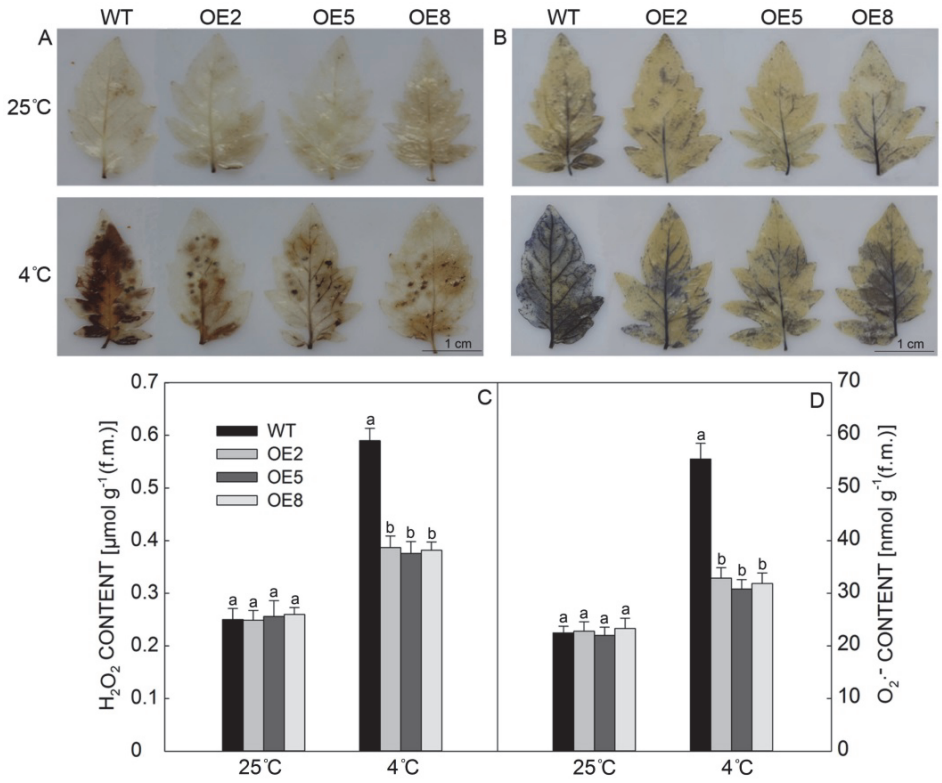

Fig. 4. H_2O_2 and $O_2^{\bullet-}$ analysis in 6-week-old WT and transgenic plants OE2, OE5, and OE8 grown at 25 °C or 4 °C for 12 h: *A* - 3,3'-diaminobenzidine staining for H_2O_2; *B* - nitroblue tetrazolium staining for $O_2^{\bullet-}$ *C* - H_2O_2 content; and *D* - $O_2^{\bullet-}$ content in tomato plants. The experiment was conducted three times with similar results. Means ± SDs, $n = 3$. Means followed by different letters indicate significant differences at $P < 0.05$ according to Duncan's test.

Table 2. Relative expressions of genes encoding antioxidative enzymes (*SlZnCuSOD* and *SlCAT*), c-repeat binding factors (*SlCBF1* and *SlCBF2*), and cold stress response (*SlCOR518* and *SlCOR413IM1*). Means ± SDs, $n = 3$. Means within a row followed by different letters indicate significant differences at $P < 0.05$ based according to Duncan's test.

Gene	Temperature [°C]	WT	OE2	OE5	OE8
SlCnZnSOD	25	1.000±0 .101a	1.188±0.181a	1.246±0.182a	1.099±0.170a
	4	1.666±0.165b	2.602±0.164a	2.950±0.142a	2.835±0.201a
SlCAT	25	1.000±0.102a	1.108±0.151a	1.146±0.185a	1.069±0.162a
	4	1.966±0.164b	4.028±0.162a	4.950±0.146a	3.835±0.201a
SlCBF1	25	1.000±0.193a	1.084±0.087a	1.026±0.099a	1.060±0.197a
	4	5.765±0.345a	5.886±0.478a	5.264±0.389a	5.674±0.456a
SlCBF2	25	1.000±0.094a	0.935±0.098a	1.175±0.167a	0.956±0.085a
	4	2.765±0.189a	2.286±0.220a	1.564±0.251b	2.974±0.289a
SlCOR518	25	1.000±0.193a	0.984±0.087a	1.126±0.099a	1.078±0.197a
	4	2.350±0.375c	5.340±0.220a	4.360±0.302b	2.540±0.198c
SlCOR413IM1	25	1.000±0.094a	1.235±0.098a	1.075±0.167a	0.956±0.085a
	4	1.020±0.095b	9.560±0.345a	12.350±0.623a	10.230±0.589a

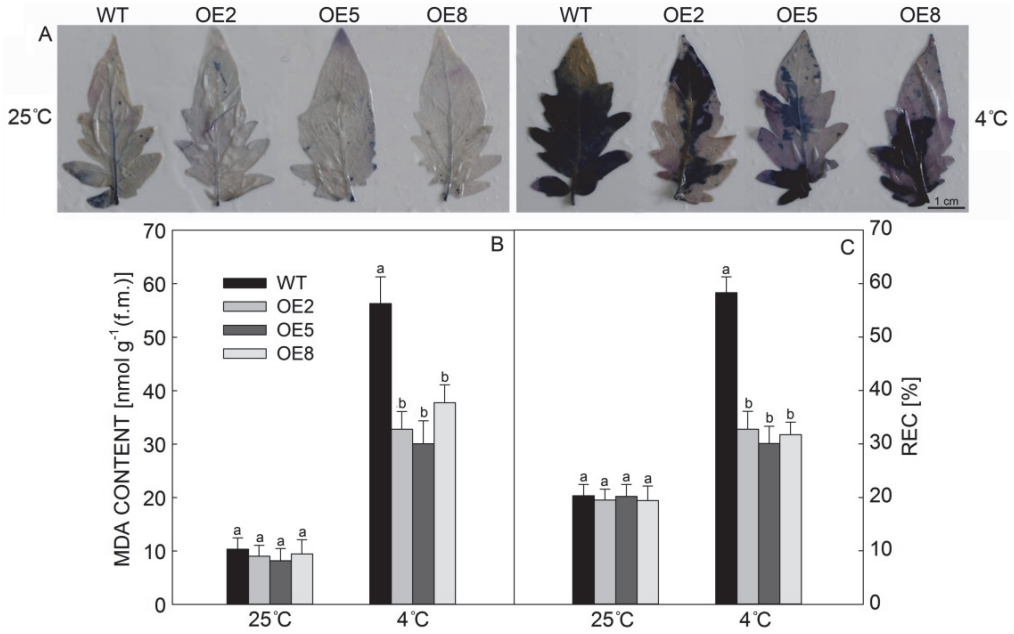

Fig. 5. Cell damage analysis in 6-week-old WT and transgenic plants grown at 25 °C or treated at 4 °C for 12 h: *A* - trypan blue staining; *B* - malondialdehyde (MDA) content; and *C* - relative electrical conductivity (REC). Means ± SDs, $n = 3$. Means followed by different letters indicate significant differences at $P < 0.05$ according to Duncan's test.

Discussion

The NAC superfamily is one of the largest TF families found only in plants; this superfamily plays diverse roles in plant development and in the response to environmental stimuli (Puranik *et al*. 2012, Ricachenevsky *et al*. 2013, Wang *et al*. 2016). Nevertheless, the roles of NAC proteins in cold resistance, especially in the cold resistance of tomatoes, are not completely understood. In this study, GUS staining and real time qPCR results showed that *SlNAC35* expression was remarkably induced at 4 °C (Fig. 1). After chilling treatment, the phenotype, growth inhibition extent, and cold resistance index in seedlings indicated that *SlNAC35* overexpression enhanced the chilling tolerance in transgenic tomatoes (Table 1). In addition, the chlorophyll content and fresh mass of transgenic plant were higher than in WT plants after chilling treatment (Fig. 3). These results also suggested that *SlNAC35* overexpression enhanced the chilling tolerance of transgenic tomatoes.

Various stresses, including drought, high salinity, heavy metal, and extreme temperature, may give rise to the accumulation of ROS, which can cause damage to plants (Mittler 2002). H_2O_2 and $O_2^{\bullet-}$ were detected after chilling stress, and lower amounts of H_2O_2 and $O_2^{\bullet-}$ were observed in the leaves of transgenic plants than in WT plants (Fig. 4). In the evolution, plants gradually form complex and delicate mechanisms to cope with oxidative stress. NAC proteins are involved in mediating the antioxidative system under stress (Ma *et al*. 2013, Pandurangaiah *et al*. 2014). Furthermore, *SlNAC35*-overexpression alleviated the accumulation of ROS by maintaining high activities of antioxidative enzymes, such as SOD and CAT (Table 1). The increased activity of these enzymes in *SlNAC35* transgenic plants was due to the high expression of *SlZnCuSOD* and *SlCAT* genes (Table 2). As the primary target of chilling stress, membranes are particularly susceptible to ROS-initiated lipid peroxidation. Plant membranes are also converted from the liquid crystal state to the gel state under chilling stress. This change can alter the metabolism and dysfunction in plant cells. After chilling stress for 24 h, *SlNAC35*-overexpressing plants exhibited less membrane damage than WT plants (Fig. 5). These results strongly indicated that *SlNAC35* overexpression can alleviate ROS-induced cell damage under chilling stress.

Hao *et al*. (2011) reported that *GmNAC20* might activate the DREB/CBF-COR pathway to improve the freezing tolerance of transgenic *Arabidopsis* plants. *SlNAC1* enhances the plant chilling tolerance by upregulating the *SlCBF1* expression (Ma *et al*. 2013). The homology of SlNAC1 and SlNAC35 is not remarkably high (only 34.02 %). This study revealed that no significant difference existed between the expression of *SlCBF1* and *SlCBF2* in WT and transgenic plants (Table 2). Nonetheless, *SlCOR518* and *SlCOR413IM1* expressions in response to chilling stress were higher in *SlNAC35*-overexpressing plants than in WT plants (Table 2). These findings showed that SlNAC35 is

possibly involved in CBF-COR signaling pathway and that SlNAC35 overexpression enhances the cold tolerance of transgenic plants by regulating *COR* expression.

In summary, SlNAC35 participated in the responses of the plants to the chilling stress. Additionally, *SlNAC35* overexpression possibly enhanced the cold tolerance in transgenic plants by regulating the *SlCOR* expression. Although these findings broadened our knowledge about the biological roles of *SlNAC35* gene, which enables plants to cope with environmental stresses, a detailed molecular mechanism of these functions requires further research.

References

Aslam, M., Sinha, V.B., Singh, R.K., Anandhan, S., Pande, V., Ahmed, Z.: Isolation of cold stress-responsive genes from *Lepidium latifolium* by suppressive subtraction hybridization. - Acta Physiol. Plant. **32**: 205-210, 2010.

Bollhoner, B., Prestele, J., Tuominen, H.: Xylem cell death: emerging understanding of regulation and function. - J. exp. Bot. **63**: 1081-1094, 2012.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Bu, Q., Jiang, H., Li, C.B., Zhai, Q., Zhang, J., Wu, X., Sun, J., Xie, Q., Li, C.: Role of the *Arabidopsis thaliana* NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. - Cell Res. **18**: 756-767, 2008.

Cai, G.H., Wang, G.D., Wang, L., Pan, J.W., Liu, Y., Li, D.Q.: *ZmMKK1*, a novel group A mitogen-activated protein kinase kinase gene in maize, conferred chilling stress tolerance and was involved in pathogen defense in transgenic tobacco. - Plant Sci. **214**: 57-73, 2014.

Chen, S.P., Kuo, C.H., Lu, H.H., Lo, H.S., Yeh, K.W.: The sweet potato NAC-domain transcription factor IbNAC1 is dynamically coordinated by the activator IbbHLH3 and the repressor IbbHLH4 to reprogram the defense mechanism against wounding. - PloS Genet. **12**: e1006397, 2016b.

Chen, S.P., Lin, I.W., Chen, X.Y., Huang, Y.H., Chang, S.C., Lo, H.S., Lu, H.H., Yeh, K.W.: Sweet potato NAC transcription factor, IbNAC1, upregulates sporamin gene expression by binding the SWRE motif against mechanical wounding and herbivore attack. - Plant J. **86**: 234-248, 2016a.

Chinnusamy, V., Zhu, J., Zhu, J.K.: Cold stress regulation of gene expression in plants. - Trends Plant Sci. **12**: 444-451, 2007.

Choi, H.W., Kim, Y.J., Lee, S.C., Hong, J.K., Hwang, B.K.: Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defense response to bacterial pathogens. - Plant Physiol. **145**: 890-904, 2007.

Christiansen, M.W., Gregersen, P.L.: Members of the barley NAC transcription factor gene family show differential co-regulation with senescence-associated genes during senescence of flag leaves. - J. exp. Bot. **65**: 4009-4022, 2014.

Delessert, C., Kazan, K., Wilson, I.W., Van der Straeten, D., Manners, J., Dennis, E.S., Dolferus, R.: The transcription factor ATAF2 represses the expression of pathogenesis-related genes in *Arabidopsis*. - Plant J. **43**: 745-757, 2005.

Fang, Y.J., Liao, K.F., Du, H., Xu, Y., Song, H.Z., Li, X.H., Xiong, L.Z.: A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. - J. exp. Bot. **66**: 6803-6817, 2015.

Giacomelli, L., Masi, A., Ripoll, D.R., Lee, M.J., Van Wijk, K.J.: *Arabidopsis thaliana* deficient in two chloroplast ascorbate peroxidases shows accelerated light-induced necrosis when levels of cellular ascorbate are low. - Plant mol. Biol. **65**: 627-644, 2007.

Hao, Y.J.,Wei, W., Song, Q.X., Chen, H.W., Zhang, Y.Q.,Wang, F., Chen, S.Y.: Soybean NAC transcription factors promote abiotic stress tolerance and lateral root formation in transgenic plants. - Plant J. **68**: 302-313, 2011.

Hénanff, G.L., Profizi, C., Courteaux, B., Rabenoelina, F., Gérard, C., Clément, C., Baillieul, F., Cordelier, S., Dhondt-Cordelier, S.: Grapevine NAC1 transcription factor as a convergent node in developmental processes, abiotic stresses, and necrotrophic/biotrophic pathogen tolerance. - J. exp. Bot. **64**: 4877-4893, 2013.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science **227**: 1229-1231, 1985.

Hussey, S.G., Mizrachi, E., Creux, N.M., Myburg, A.A.: Navigating the transcriptional roadmap regulating plant secondary cell wall deposition. - Front. Plant Sci. **4**: 325, 2013.

Jeong, J.S., Kim, Y.S., Baek, K.H., Jung, H., Ha, S.H., Do Choi, Y., Kim, M., Reuzeau, C., Kim, J.K.: Root-specific expression of *OsNAC10* improves drought tolerance and grain yield in rice under field drought conditions. - Plant Physiol. **153**: 185-197, 2010.

Knight, M.R., Knight, H.: Low-temperature perception leading to gene expression and cold tolerance in higher plants. - New Phytol. **195**: 737-751, 2012.

Kong, F.Y., Deng, Y.S., Zhou, B., Wang, G.D., Wang, Y., Meng, Q.W.: A chloroplast-targeted DnaJ protein contributes to maintenance of photosystem II under chilling stress. - J. exp. Bot. **65**: 143-158, 2014.

Lin, R., Zhao, W., Meng, X., Wang, M., Peng, Y.: Rice gene *OsNAC19* encodes a novel NAC-domain transcription factor and responds to infection by *Magnaporthe grisea*. - Plant Sci. **172**: 120-130, 2007.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K.: 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, respectively, in *Arabidopsis*. - Plant Cell **10**: 1391-1406, 1998.

Ma, N.N., Zuo, Y.Q., Liang, X.Q., Yin, B., Wang, G.D., Meng, Q.W.: The multiple stress-responsive transcription factor

SlNAC1 improves the chilling tolerance of tomato. - Physiol. Plant. **149**: 474-486, 2013.

Mitter, R.: Oxidative stress, antioxidants and stress tolerance. - Trends Plant Sci. **7**: 405-410, 2002.

Nakashima, K., Takasaki, H., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K.: NAC transcription factors in plant abiotic stress responses. - Biochim. biophys. Acta **1819**: 97-103, 2012.

Olsen, A.N., Ernst, H.A., Leggio, L.L., Skriver, K.: NAC transcription factors: structurally distinct, functionally diverse. - Trends Plant Sci. **10**: 79-87, 2005.

Pandurangaiah, M., Rao, G.L., Sudhakarbabu, O., Nareshkumar, A., Kiranmai, K., Lokesh, U., Thapa, G., Sudhakar, C.: Overexpression of horsegram (*Macrotyloma uniflorum* Lam.Verdc.) NAC transcriptional factor (*Mu*NAC4) in groundnut confers enhanced drought tolerance. - Mol. Biotechnol. **56**: 758-769, 2014.

Puranik, S., Sahu, P.P., Srivastava, P.S., Prasad, M.: NAC proteins: regulation and role in stress tolerance. - Cell **17**: 369-381, 2012.

Ricachenevsky, F.K., Menguer, P.K., Sperotto, R.A.: kNACking on heaven's door: how important are NAC transcription factors for leaf senescence and Fe/Zn remobilization to seeds? - Front. Plant Sci. **4**: 226, 2013.

Rao, M.V., Davis, K.R.: Ozone-induced cell death occurs *via* two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. - Plant J. **17**: 603-614, 1999.

Shan, W., Kuang, J.F., Lu, W.J., Chen, J.Y.: Banana fruit NAC transcription factor MaNAC1 is a direct target of MaICE1 and involved in cold stress through interacting with MaCBF1. - Plant Cell Environ. **37**: 2116-2127, 2014.

Shi, Y.T., Ding, Y.L., Yang, S.H.: Cold signal transduction and its interplay with phytohormones during cold acclimation. - Plant Cell Physiol. **56**: 7-15, 2015.

Song, S.Y., Chen, Y., Chen, J., Dai, X.Y., Zhang, W.H.: Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. - Planta **234**: 331-345, 2011.

Stockinger, E.J., Gilmour, S.J., Thomashow, M.F.: *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcription activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. - Proc. nat. Acad. Sci. USA **94**: 1035-1040, 1997.

Thomashow, M.F.: Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. - Annu. Rev. Plant Biol. **50**: 571-599, 1999.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F.: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. - Genome Biol. **3**: 1-12, 2002.

Voitsik, A.M., Muench, S., Deising, H.B., Voll, L.M.: Two recently duplicated maize NAC transcription factor paralogs are induced in response to *Colletotrichum graminicola* infection. - BMC Plant Biol. **13**: 85, 2013.

Wang, G.D., Zhang, S., Ma, X.C., Wang, Y., Kong, F.Y., Meng, Q.W.: A stress-associated NAC transcription factor (SlNAC35) from tomato plays a positive role in biotic and abiotic stresses. - Physiol. Plant. **158**: 45-64, 2016.

Wang, X., Basnayake, B.M., Zhang, H., Li, G., Li, W., Virk, N., Mengiste, T., Song, F.: The *Arabidopsis* ATAF1, a NAC transcription factor, is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens. - Mol. Plant microbe Interact. **22**: 1227-1238, 2009.

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

Zhong, R., Lee, C., Ye, Z.H.: Evolutionary conservation of the transcriptional network regulating secondary cell wall biosynthesis. - Trends Plant Sci. **15**: 625-632, 2010.

Zong, X.J., Li, D.P., Gu, L.K., Liu, L.X., Hu, X.L., Li, D.Q.: Abscisic acid and hydrogen peroxide induce a novel maize group C MAP kinase gene, *ZmMPK7*, which is responsible for the removal of reactive oxygen species. - Planta **229**: 485-495, 2009.