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ORIGINAL PAPER

Expression Analysis of *TaNAC69-1* and *TtNAMB-2*, Wheat NAC Family Transcription Factor Genes Under Abiotic Stress Conditions in Durum Wheat (*Triticum turgidum*)

Mehmet Cengiz Baloglu · Mehmet Tufan Oz · Huseyin Avni Oktem · Meral Yucel

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Abstract NAC-type plant-specific transcription factor genes encode proteins that play important roles in abiotic stress responses, as well as regulation of plant development. In the current study, expression profiles of wheat NAC-type transcription factor genes, *TaNAC69-1* and *TtNAMB-2*, were examined under drought, salt, cold, and heat stress conditions in wheat. Based on reverse transcription quantitative PCR results, *TaNAC69-1* was strongly expressed under drought, salinity, and high-temperature stress conditions. Compared to control samples, a quick response at the transcription level of *TaNAC69-1* was observed after 3 h of salt treatment with a ninefold upregulation. Highest level of expression was observed at 24 and 48 h posttreatment under heat and salinity treatments, respectively. Meanwhile, expression of *TtNAMB-2* was significantly induced by salt and low-temperature stresses. Salt treatment induced expression of *TtNAMB-2* and caused a 13-fold increase in transcript copy numbers at 48 h posttreatment. Examination of expression changes under abiotic stresses may provide important information for understanding roles of *TaNAC69-1* and *TtNAMB-2* genes which might be involved in response to environmental stresses.

Keywords NAC-type transcription factors · Wheat · Gene expression · RT-qPCR

M. C. Baloglu · M. T. Oz · H. A. Oktem · M. Yucel (✉)
Department of Biological Sciences,
Middle East Technical University,
Üniversiteler Mah. Dumlupınar Blv. No. 1,
06800 Ankara, Turkey
e-mail: meraly@metu.edu.tr

M. C. Baloglu
Department of Biology, Kastamonu University,
37100, Kuzeykent,
Kastamonu, Turkey

Introduction

Wheat is one of the most important and widely grown cereal grain crops in the world. About 40 % of the world population is nourished by wheat products which provide 20 % of total food calories and protein in human diet (Gupta et al. 2008). In order to meet the growing need for wheat, production should be raised to an annual rate of 2 %, without any additional land (Patnaik 2001). It is also predicted that average wheat production will be 760 and 900 million tons in 2020 and 2050, respectively (Rosegrant et al. 2001). Recently, studies associated with genetic improvement in wheat have therefore concentrated on increasing the grain yield, quality characteristics and minimizing crop loss because of biotic and abiotic stress conditions.

Plants are constantly threatened by biotic and abiotic stresses and have evolved complex response mechanisms including genes encoding important metabolic or regulatory proteins like transcription factors (Xia et al. 2010a, b). Certain members of plant transcription factor families have been well characterized in terms of their regulatory roles in response to stress (Guo et al. 2004; Buchanan-Wollaston et al. 2005; Agarwal et al. 2010). The NAC-type transcription factors constitute one of the largest families and are involved in diverse roles in plant development and in response to stimuli from biotic and abiotic factors (Olsen et al. 2005). Proteins of this family contain NAC domain which was originally characterized from consensus sequences of petunia NAM (no apical meristem) and Arabidopsis ATAF1, ATAF2, and CUC2 (cup-shaped cotyledon) (Aida et al. 1997). The NAC family has 149 and 106 predicted members in *Oryza sativa* and *Arabidopsis thaliana* genomes, respectively (Gong et al. 2004; Xiong et al. 2005).

In recent years, the expression of NAC-type transcription factors has been well studied in various plant species under

different abiotic stress conditions. *CarNAC1*, *CarNAC3*, and *CarNAC5* were isolated from cDNA libraries constructed from the leaves of PEG-treated chickpea seedlings and a spatial expression was observed for *CarNAC1* and *CarNAC3* genes (Peng et al. 2009a, b, 2010). In soybean, 31 unigenes containing the complete open reading frames that encode GmNAC proteins were defined and cloned. *GmNAC* genes were differentially expressed in different organs. In addition to dehydration stress, the responses of *GmNAC* genes to other stresses such as high salinity, cold, and treatment of abscisic acid (ABA) were screened by reverse transcription quantitative PCR (Tran et al. 2009). Six full-length (*GhNAC1–GhNAC6*), putative transcription factor genes were isolated from *Gossypium hirsutum* L. All *GhNAC* genes were highly expressed in leaves while they had little to no expression in stems, roots, and 7-day post-anthesis fibers. According to RT-qPCR results, the genes showed differential expression patterns under drought, high salt, cold and/or ABA conditions (Meng et al. 2009). A rice *NAC* gene, *ONAC045*, was functionally characterized in response to abiotic stress conditions. Expression analysis of *ONAC045* revealed that drought, high-salt and low-temperature stresses, and ABA treatment altered gene expression level in leaves. Moreover, transgenic rice overexpressing *ONAC045* gene showed enhanced tolerance to drought and salt treatments (Zheng et al. 2009). Novel wheat *NAC* genes, designated as *TaNAC4* and *TaNAC8* were isolated from *Triticum aestivum* using RACE method. The expression level of *NAC* genes differed in different tissues of wheat seedling and higher level of gene expression was observed in roots rather than leaves and stem. Abiotic stresses arising from high salinity, wounding, and low-temperature also caused induction of *TaNAC4* and *TaNAC8* gene expression (Xia et al. 2010a, b). *CsNAC* was isolated from peel pitting of citrus fruit and belongs to ATAF subfamily, which plays an important role in response to stress stimuli. Its expression level was affected from low (4 °C) and high temperature (40 °C) and exposure to ethylene (Fan et al. 2007). In *Triticum turgidum* fine mapping and sequencing of a quantitative trait locus for grain protein content on chromosome arm 6BS revealed a gene encoding a NAC domain protein, *TtNAM-B1*. Quantitative PCR results showed that expression levels of three *TtNAM* genes were low in flag leaves before anthesis, but increased significantly toward grain maturity (Uauy et al. 2006). In addition to responses of NAC-type transcription factors to different abiotic stress conditions, they are also highly related to development and senescence. A citrus NAC-like gene (*CitNAC*) isolated from fruit tissues of *Citrus sinensis* has the conserved region at the N terminus of the NAC domains. Expression analysis of *CitNAC* indicated that its mRNA level was detected in fruit peel and pulp during fruit ripening or senescence stage (Liu et al. 2009). The changes in expression levels of *PtNAC068* and *PtNAC154* genes suggested that they are associated with vascular tissue development in poplar (Han et al. 2011).

Environmental stresses including drought, salinity, and extreme temperatures cause adverse effects on the growth and development of wheat plant, resulting in crop loss and reduced average yields. Abiotic stress responses of plants are mainly characterized by regulation of transcription factors and stress-related genes. Identification, characterization, and expression profiling of these genes have become an important tool to understand response of wheat plant against severe environmental changes and to develop stress resistant transgenic wheat plant. To identify effects of salinity, drought, cold, and heat stresses on expression profiles of *TaNAC69-1* and *TtNAM-B2* genes, reverse transcription quantitative PCR was used in this study. Monitoring transcript level changes under abiotic stresses may provide important information for understanding roles of NAC-type transcription factors which might be potential targets for improving wheat plant against abiotic stresses.

Materials and Methods

Growth of Plants and Stress Applications

Wheat (*T. turgidum* ssp. durum.) cultivar Kızıltan-91, which is winter sown and resistant to drought and cold, was used for gene expression studies including reverse transcription quantitative PCR. The seeds were obtained from Central Research Institute for Field Crops, Ankara (Turkey). Seeds were surface sterilized with 10 % NaOCl for 10 min and then washed three times with sterile distilled water. They were germinated and hydroponically grown in half-strength Hoagland's Solution (Hoagland and Arnon 1950) for 10 days in a growth chamber at 24±2 °C with 16-h light and 8-h dark photoperiod at a light intensity of 400 μmol m^{-2} s^{-1}.

All stress treatments were initiated after 10 days of normal growth. Stress treatments were applied using half-strength Hoagland's Solution containing 20 % polyethylene glycol 6000 (PEG-6000) and 250 mM of sodium chloride (NaCl) for drought and salinity, respectively. Both treated and untreated (control) plants were kept in the growth chamber under same physical conditions. For application of cold and heat stresses, 10-day-old plants were transferred to growth chambers adjusted to either 4 or 40 °C for low- and high-temperature stresses. Leaf samples from treated and control plants were harvested after 1, 3, 6, 12, 24, and 48 h of stress application and immediately frozen in liquid nitrogen. Each set of experiment was repeated three times and samples from each set were used as biological replicates.

Total RNA Isolation and Characterization

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the method of Chomczynski (1995). DNA contamination in samples was removed using DNase I (Fermentas, Thermo Scientific) according to the manufacturer's instructions. After DNase treatment, precipitation with ethanol and sodium acetate was carried out to concentrate samples. Quant-iT™ RiboGreen® RNA assay kit (Invitrogen), which employs a sensitive fluorescent dye, was used for quantitating RNA in solution. Diluted RNA samples were incubated with Quant-iT RiboGreen RNA reagent for 5 min at room temperature and the fluorescence was recorded with NanoDrop 3300 Fluorospectrometer (Thermo Scientific). RNA concentrations of the samples were determined according to the standard curve generated using at least five different dilutions (1–10 μg/mL) of ribosomal RNA standard. Quality and integrity of the total RNA was checked using agarose gel electrophoresis and with the Agilent 2100 Bioanalyzer.

Measurements of *TaNAC69-1* and *TtNAMB-2* Gene Expression Level in RT-qPCR

Changes in expression levels of *TaNAC69-1* (GenBank accession no. AY625682) and *TtNAMB-2* (GenBank accession no: DQ869676) under the abiotic stresses applied were measured on Corbett Rotor-Gene 6000 (Qiagen, Valencia, CA) using QuantiTect SYBR Green RT-PCR Kit (Qiagen) which employs one-step reverse transcriptase (RT) PCR method. One-step RT-PCR method provides cDNA synthesis and PCR reactions to occur in a single tube. RT-qPCR reactions were carried out in a total volume of 20 μL containing 0.5 μM of each forward and reverse primers, 1× QuantiTect SYBR Green RT-PCR Master Mix, 0.2 μL of QuantiTect RT Mix and 100 ng of total RNA from the leaf tissues of wheat seedlings. Nucleotide sequences of oligonucleotide primers used for real-time PCR amplification of *TaNAC69-1* and *TtNAMB-2* genes and their product sizes were shown in Table 1. Thermal cycling was 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C.

For determination of expression levels, absolute quantification method was performed using external standards of plasmid DNA which contain fragments of *TaNAC69-1* and *TtNAMB-2*. The fragments were amplified in a conventional PCR reaction using same primer pairs and cloned into pENTR™/D-TOPO® cloning vector (Invitrogen). Sequences were confirmed using universal M13 forward (−20) and M13 reverse primers. Six different tenfold dilutions (10^7 and 10^2 copies) of the plasmids were used in triplicates to generate standard curves. The threshold cycle (C_T value) was plotted against the $\log_{10}$ of initial amount of standard. After construction of standard curves, each run of RT-qPCR included two standards (10^5 and 10^3 copies) from the standard curve. These two concentrations of plasmids provided normalization. For primer pairs used in this study, the standard curves were linear with a slope between −3.6 and –3.3 and an R^2 value above 0.98. No-template control reactions which contained all PCR components except the RNA template was included in each run of RT-qPCR. Specificity of primer pairs were confirmed with melting curve analysis performed after each run. Quantitative real-time RT-PCR was performed in triple technical replicates of each RNA samples from three biological replicates. The C_T values of reactions including RNA samples obtained from control and stressed wheat plants were compared with the standard curve to determine the copy number of transcripts of *TaNAC69-1* and *TtNAMB-2* in the samples.

Results and Discussion

Construction of Standard Curves

Two different standard curves were generated for *TaNAC69-1* and *TtNAM-B2* genes expression analysis. The linear range of quantification was determined after serial dilutions of standard plasmids containing both gene fragments. Serial dilutions of pENTR-*TaNAC69-1* and pENTR-*TtNAM-B2* were tested from 1.0×10^7 to 1.0×10^2 and 1.0×10^8 to 1.0×10^2 copies, respectively. The optimal range of RT-qPCR commonly accepted by Taylor et al. (2010) was obtained between dilutions of 10^2 and 10^3 for pENTR-*TaNAC69-1* and pENTR-*TtNAM-B2*, respectively. The efficiency for pENTR-*TaNAC69-1* was of 98.28 % with R^2=0.9984 and for pENTR-*TtNAM-B2* was 96.71 % with R^2=0.9835 (Fig. 1).

Table 1 Nucleotide sequences of oligonucleotide primers used for real-time PCR amplification of genes of interest and their product sizes

Genes	Primer sequences (5′→3′)	Amplicon size (bp)
TaNAC69-1	F:ACTACCAGCTGCCTCCCGAAAACC R:GCCGTAGTCATCTACGCGCGCC	146
TtNAMB-2	F:AACAGGAGCAGAAATGTCGGCAAC R:GGATGACATGCTGTTGATGGTAGG	152

F forward, *R* reverse primers

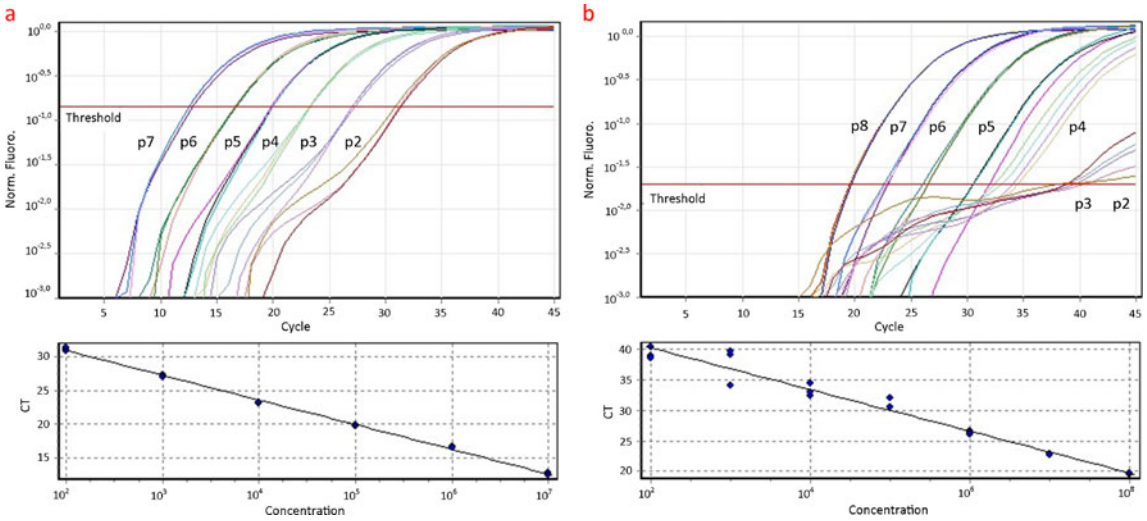

Fig. 1 Construction of standard curves for *TaNAC69-1* and *TtNAM-B2* genes quantification. Amplification curves of different concentrations of **a** pENTR-*TaNAC69-1* plasmid DNA and its standard curve and **b** pENTR-*TtNAM-B2* plasmid DNA and its standard curve. Plasmid dilutions were abbreviated as: *p8*, 10^8; *p7*, 10^7; *p6*, 10^6; *p5*, 10^5; *p4*, 10^4; *p3*, 10^3; and *p2*, 10^2 copies/μL

Expression Analysis of NAC-Type Transcription Factors

To elucidate the expression patterns of NAC-type transcription factor genes, *TaNAC69-1* and *TtNAM-B2*, under salt, drought, cold, and heat stress conditions, RT-qPCR was performed using RNA samples extracted from leaves of wheat seedlings at various time points.

Expression Level Changes under Salt and Drought Stress Conditions

Expression patterns of *TaNAC69-1* and *TtNAM-B2* under salt and drought stress conditions at certain time points over 2 days are given in Fig. 2. The expression levels of *TaNAC69-1* and *TtNAM-B2* peaked at 48 h posttreatment (hpt) with similar patterns over time under salinity stress. Although expression profiles resembled each other, concentrations of transcripts were different. Transcript concentrations of *TaNAC69-1* and *TtNAM-B2* were approximately 120 and 34 molecules/ng, respectively (Fig. 2). Compared to control samples the transcription level of *TaNAC69-1* significantly upregulated at 3 hpt with ninefold and peaked at 48 hpt with about 64-fold increase. However, change in level of *TtNAM-B2* transcript reached to approximately 13-fold at 48 hpt. Besides tissue-specific expression pattern, expression levels of NAC-type transcription factors also change when plants are exposed to different biotic and abiotic stresses (Meng et al. 2007; Xia et al. 2010a). This suggests an important role for NAC-type transcription factors during growth, development, and responses to biotic and abiotic stresses. In the present study, it was found that *TaNAC69-1* and *TtNAM-B2* genes were strongly induced by salinity at 48 hpt. These results indicated that NAC-type transcription factors might be involved in responses to salt stress. Xia et al. (2010a, b) showed that the expression levels of *TaNAC4* and *TaNAC8*, novel wheat NAC-type transcription factor genes, increased transiently at 3 hpt after treatment with 200 mM NaCl. Similarly it was shown that the transcript level of *CarNAC1* from chickpea (*Cicer arietinum* L.) significantly increased under salt treatment (Peng et al.

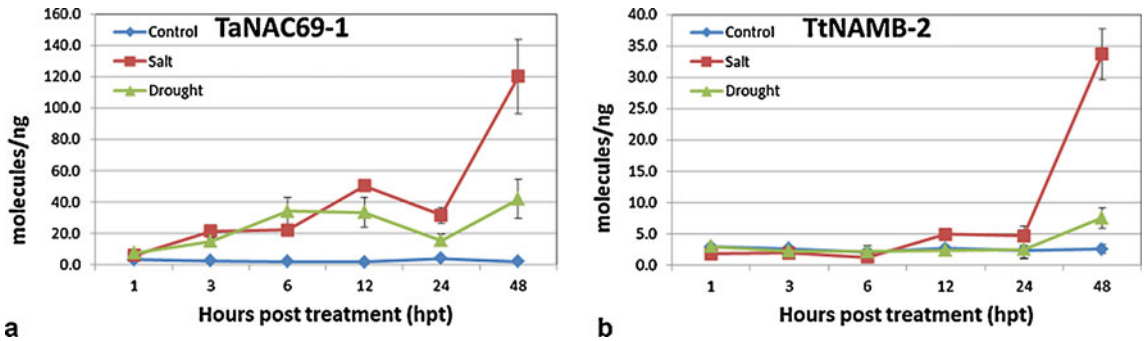

Fig. 2 Expression patterns of *TaNAC69-1* and *TtNAM-B2* genes of wheat under salt and drought stress conditions at different time points

2010). Meng et al. (2007) characterized six novel NAC family genes (*GhNAC*s) from cotton (*G. hirsutum* L.) and showed their responses to abiotic stresses. The expression levels for five of the six *GhNAC*s increased following salt treatments. Overexpression of a *Stress-responsive NAC1* (*SNAC1*) (Hu et al. 2006) and *SNAC2* (Hu et al. 2008) in transgenic rice plants significantly improved drought and salt resistance. Our results further support that NAC-type transcription factor genes play crucial roles in the regulation of response to salt stress in various plant species.

Expression level of *TaNAC69-1* gene gradually increased from 1 to 12 hpt under drought stress applied. Although there was no significant difference between transcript amounts at 6, 12, and 48 hpt, maximum expression of *TaNAC69-1* was observed at 48 hpt (Fig. 1). Under the drought stress, expression pattern of *TaNAC8* gene (Xia et al. 2010b) was similar to profile of *TaNAC69-1* gene observed in this study. In contrast to increase in expression levels of *TaNAC8* and *TaNAC69-1* genes in wheat, drought stress elicitors had no obvious effect on expression of *TaNAC4* (Xia et al. 2010a). It has been previously shown that the NAC family genes have ability to regulate drought stress response through both ABA-dependent and ABA-independent pathways (Fujita et al. 2004; Tran et al. 2004). Peng et al. (2010) reported that the expression of *CarNAC1* was induced by dehydration, but not by ABA which indicated that this protein may be associated with drought response in an ABA-independent manner. On the other hand, other NAC-type transcription factors, *CarNAC3* and *CarNAC5* from chickpea were significantly upregulated by drought stress and they were proposed to be regulated by the ABA signaling pathway (Peng et al. 2009a, b). Members of NAC family genes, *ANAC047* and *GhNAC5*, have been found to be significantly induced by drought stress and ABA (Seki et al. 2003; Meng et al. 2009). Recently, NAC-type transcription factors from various organisms have been widely used to improve drought tolerance in transgenic plants. Overexpression of three different Arabidopsis NAC family genes (*ANAC019*, *ANAC055*, and *ANAC072*) showed significantly increased drought tolerance (Tran et al. 2004). It was shown that *SNAC1* was induced by drought specifically in rice guard cells. *SNAC1*-overexpressing transgenic plants showed significantly improved drought resistance under field conditions without phenotypic changes or yield penalty (Hu et al. 2006). Gao et al. (2009) isolated and characterized a novel *OsNAC52* from rice, which is ABA-dependent NAC-like gene. Overexpression of *OsNAC52* triggered expression of downstream genes in transgenic Arabidopsis, resulting in enhanced tolerance to drought stress. Another rice NAC gene, *ONAC045* was functionally characterized by Zheng et al. (2009). Drought, high-salt, and low-temperature stresses, and ABA treatment induced *ONAC045* gene expression in rice leaves and roots. They reported that transgenic rice plants over-expressing *ONAC045* showed enhanced tolerance to drought and salt treatments. These studies suggested that NAC-type transcription factors have a potential for improvement of wheat towards tolerance to drought and salt stresses.

NAM gene family plays a central role as transcriptional regulators of multiple processes during leaf senescence, which affects nutrient concentrations in developing wheat grain (Uauy et al. 2006). However, expression patterns of NAM genes under different abiotic stress conditions have not been reported yet. Transcription level changes of *TtNAM-B2* gene under salt and drought stress conditions are displayed in Fig. 1. Gene expression patterns of *TaNAC69-1* and *TtNAM-B2* were similar which suggested that their expression might be stimulated by the same abiotic stimuli or at the same period of treatment (Fig. 1). Like salinity, effect of drought stress on expression of *TtNAMB-2* in wheat was shown in this study for the first time. According to the expression profiles of *TtNAMB-2* gene under drought stress, there was no significant difference between all time points except for 48 hpt, at which expression of *TtNAMB-2* gene was significantly upregulated and reached to a peak. Together with *TtNAMB-2* gene, *TaNAC69-1* gene responded to drought stress after 48 h of PEG-6000 treatment which suggested that they might be acting simultaneously under the drought stress (Fig. 1).

Expression Level Changes under Cold and Heat Stresses

The low-temperature treatment at 4 °C did not have pronouced effects on gene expression level of *TaNAC69-1* in wheat cultivar, Kızıltan-91 (Fig. 3). Although mRNA copy numbers were extremely low, *TtNAM-B2* gene expression level was sharply increased and reached peak level at 12 hpt. Gene expression patterns of *TaNAC4* and *TaNAC8* were strongly induced after 3 h of cold stress treatment (Xia et al. 2010a, b). Low temperature (4 °C) also increased the expression level of *CsNAC* gene from citrus (Fan et al. 2007). *CarNAC1* and *CarNAC3* share common functions in certain processes of physiological metabolism (Peng et al. 2010). However, their expression profiles under the cold stress were different. Although *CarNAC1* showed significant increase under cold stress application (Peng et al. 2010), no obvious effect on the expression of *CarNAC3* (Peng et al. 2009a) was observed in chickpea. NAC genes (*GhNAC*s) from cotton showed different expression patterns under cold stress. All the *GhNAC*s except for *GhNAC1* gene were strongly induced by low-temperature stress. *SNAC2* and *SNAC1* genes, which are highly similar at amino acid level, have similarity in response to various abiotic stresses including salt, drought, and ABA treatment (Ooka et al.

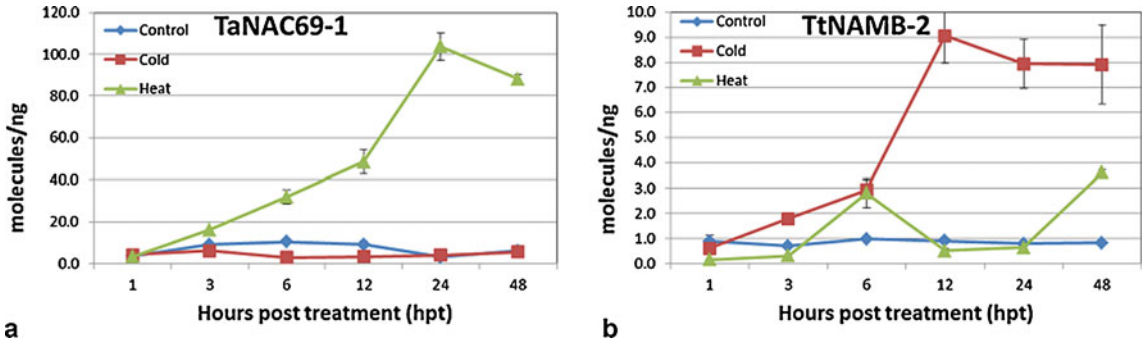

Fig. 3 Expression patterns of *TaNAC69-1* and *TtNAMB-2* genes in wheat cv. Kızıltan-91 under the cold and heat stress treatments at different time points

2003). However, expression profiles of *SNAC1* and *SNAC2* were different under the cold stress application. In spite of the fact that *SNAC1* gene expression was induced by cold, overexpression of *SNAC1* had no significant effect on improving cold tolerance while *SNAC2*-overexpression caused formation of resistance against prolonged low-temperature treatments (Hu et al. 2006, 2008). Among NAC family proteins of wheat, *TaNAC69-1* might not be involved in response to cold stress whereas members of the same family such as *TaNAC4* and *TaNAC8* might have important roles.

TaNAC69-1 gene expression level was gradually increased from the initiation of heat stress treatment and peaked at 24 hpt. A relative fold difference of 34 was observed between mRNA amounts of control and high-temperature-treated samples. Although there was slight decrease in expression level of *TaNAC69-1* at 48 hpt, the relative fold difference was still high with 15-folds. Like under salt stress treatment, the concentration of *TaNAC69-1* transcripts was also high and about 100 molecules/ng. However, no significant changes in expression profiles of *TtNAM-B2* gene was observed under the heat stress condition (Fig. 2). The expression patterns of chickpea NAC-type transcription factors, *CarNAC1* and *CarNAC5*, were evaluated under heat stress (Peng et al. 2009b, 2010). In contrast to cold stress, heat stress did not affect the expression of *CarNAC1*. However, significant increase in gene expression level of *CarNAC5* was detected under high-temperature stress. In contrast to low-temperature stress, high temperature (40 °C) caused suppression of *CsNAC* gene from citrus (Fan et al. 2007).

Expression profiles of NAC-type transcription factors under the severe environmental stresses applied in laboratory conditions were investigated by RT-qPCR in wheat. It was found that *TaNAC69-1* gene was significantly upregulated by salt, drought, and heat stresses, which indicated a possible function in responses to these environmental stimuli. Although transcript copy numbers of *TtNAMB-2* was low, salt and cold stress treatments induced its expression. In conclusion, results from this study indicate that NAC-type transcription factors *TaNAC69-1* and *TtNAMB-2* might be involved in cellular responses to osmotic stress. The link between these transcription factors and their downstream genes should be investigated at transcriptome as well as proteome level for further understanding of their role under abiotic stresses. Recent studies have shown that small protein signaling and regulatory molecules play important for many aspects of plant development. Larue et al. (2009) indicated genetically interaction of NAC1 transcription factor with a DVL/ROT family member, resulting in overexpression of a DVL/ROT family member. Therefore downstream genes of *TaNAC69-1*, *TtNAMB-2* should be also detected and evaluated as candidate genes for improvement of wheat against abiotic stress.

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