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Isolation and identification of a salt-inducible gene encoding a putative NAC-binding transcription factor from *Poa pratensis*

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Abstract

NAC proteins play essential role in regulating various metabolic processes including molecular signal transduction in plants. A new NAC-binding protein gene *PpNAC* encoding an NAC-type transcription factor was isolated by RACE-PCR from *Poa pratensis* seedlings. Its ORF cDNA was 837bp long, from which a protein with 278 amino acid residues was deduced with a predicted molecular mass of 31.43kDa and a pI of 5.30. Clustering analysis showed that PpNAC is a nuclear protein, which belongs to OsNAC3 subfamily of the NAC protein family. PpNAC exhibited the highest identity to SNAC1, and the lowest identity to NAC1. The expression of *PpNAC* was promoted significantly by 4°C, 37 °C, 250mM NaCl, 100« M ABA and 10mM SA, and inhibited by 20% PEG, 100« M MeJA and 100« M ET. *PpNAC* seems to participates in regulatory processes involved in defense responses and stress resistance in *Poa pratensis*.

Key words: NAC; Transcription factors; RACE; Quantitative RT-PCR

Introduction

Plants grow in a complex and dynamic environment, with factors including drought, salinity, cold and biotic stresses frequently imposing constraints on their growth and development. Among all kinds of stress which plants frequently encounter, high temperatures and osmotic pressures can lead to plant dehydration or salt stress. Drought is among the most important environmental factors influencing plant growth and development. Plants have evolved various survival mechanisms including stress-signal transduction, which leads to various physiological and metabolic responses such as stress responsive gene expression.

NAC(NAM, ATAF1,2 and CUC2) proteins constitute one of the largest families of plant-specific transcription factors existing in a wide range of plants. More than a hundred NAC proteins are reported to exist in Arabidopsis, however, >90% of these are yet to be functionally characterized. These proteins share a common structure consisting of a conserved N-terminal region (the NAC domain) and a highly variable C terminus. The conserved N-terminus may function as the NAC DNA-binding region [1] whereas the C-terminus may serve as a transcriptional activation region [2-3]. The NAC domain was originally characterized from the NAM (No Apical Meristem) sequence from petunia and the ATAF1, ATAF2 and CUC2 (cup-shaped cotyledon) genes from Arabidopsis thaliana [1-4]. NAC proteins are not only involved in many processes of plant, but also in the biological processes and abiotic stress response. For example, Arabidopsis thaliana *RD26* cDNA, isolated from dehydrated plants, encodes a NAC protein. Expression of the *RD26* was also induced by abscisic acid (ABA) and high salinity. ABA- and stress-inducible genes are upregulated in the *RD26*-overexpressed plants and repressed in the *RD26*-repressed plants [5]. Expression of the Arabidopsis NAC domain genes, *ANAC019*, *ANAC055* and *ANAC072*, were reported to be induced by drought, high salinity and ABA [6]. Furthermore, the products of these genes were found to bind specifically to the CATGTG motif within the 63-bp promoter region of the *ERD1*. Transgenic plants overexpressing *ANAC19*, *ANAC055* or *ANAC072* exhibited increased drought tolerance [6]. In particular, *ANAC072* (*RD26*) is believed to be involved in a novel ABA-dependent stress-signaling pathway [5].

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Kentucky bluegrass (*Poa pratensis*) is a cool-season species widely used as forage and turf grass, that can form an attractive, durable, persistent turf [7]. Kentucky bluegrass is a widely used cool-season turfgrass that is considered sensitive to moderate levels of drought and salinity [8]. In early studies, natural mutant screening [9], general parental hybridization [10-11] and other conventional means were often used to improve Kentucky bluegrass, which is expected to be the ideal New germplasm. However, Facultative apomictic reproductive way to Kentucky bluegrass conventional breeding has brought great difficulties [12]. Therefore, the objective of this study is to isolate a NAC transcription factor gene (*PpNAC*) from Kentucky bluegrass and to analyze its properties and expression patterns under different conditions.

Material and methods

Plant culture and treatments

Kentucky bluegrass 'NASSU' were seeded in plastic pots with sandy soil medium and in a greenhouse at 28^{o}C and 60% relative humidity (RH) and 12h/12h (light/dark) photoperiod and white light illumination (0.1mol/m^2/s). Sodium chloride (NaCl) solution was added to the pots at 250mM at the four-leaf stage (four weeks later). The seedlings were harvested after 6h treatment, then quickly frozen in liquid nitrogen and stored at –80^{o}C for RNA extraction.

Isolation of cDNA encoding PpNAC protein

Total RNA was extracted from the seedlings after the treatment with 250mM NaCl for 8h with TRIZOL® Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from the total RNA by the ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's instructions. The two degenerate primers (Dp) for cloning *PpNAC* cDNA intermediate fragment were as follow, PpNAC-Dp-forward: 5'-G(A/G/C)(A/C)-GGACCAAGTT(C/G/T) (A/C)(A/G)(G/C)GAGAC-3 and PpNAC-Dp-reverse: 5'-CTCAACTTCGCCGA(T/C)T(T/C)CGC-3'. A 498-bp fragment was sequenced, showing 40%-50% homology with those of other NAC genes. To obtain the full length of the gene, The 3'-RACE and the 5'-RACE cDNA synthesis was performed with SMART RACE cDNA Synthesis Kit (clontech, US). Two gene-specific primers for amplifying a overall length (Ol) fragment containing the open reading frame were as follows: PpNAC-Ol-forward: 5'-TCACCAGAGCAACCCA CAAGC-3' and PpNAC-Ol-reverse: 5'-TCTGCGTCTT CCCATATTTGG-3'.

Sequence analysis and prediction of secondary structure of PpNAC

Homology searches were performed using blastn with default parameters at NCBI http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment of NAC proteins was performed using DNAMAN. Total 12 NAC protein sequences for constructing phylogenetic tree were retrieved from the GenBank database and depicted by ClustalX program (version 1.83) with default parameters. Phylogenetic analysis was based on the Neighbour-Joining (NJ) using MEGA (version 4.1) with 1000 bootstrap replications. The sequences of 12 NAC proteins are as follows: ANAC019 (NP_175697), ANAC055 (NP_188169), RD26 (NP_567773), CUC1 (NP_188135), NAC1 (AAF21437), NTM1 (NP_001078343), TIP (NP_197847), ATAF1 (NP_171677), SNAC2 (CBX55846), NAM (NP_175696), NAP (NP_564966), SNAC1 (ABD52007). The secondary structure of PpNAC was analyzed by SOMPA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl? page=npsa_ sopma.html).

Subcellular localization of PpNAC-GFP proteins

The *PpNAC-GFP* cDNAs were individually subcloned into pBI construct with 35S promoter and the green fluorescent protein (GFP) reporter gene. For generating the *PpNAC-GFP* fusion construct, the *PpNAC* ORF was amplified with a 5'-terminal *Xba* I-adapted primer (5'-CTCTAGAGACACAACAACGCTCAAAGAA-3') and a 3'-terminal *Kpn* I-adapted primer (5'-GGGTACCAGGAGAGTCAGAGCGCAGAG-3').

The resulting PCR product was cloned to the pMD-T 19 Simple Vector (TaKaRa, Japan) and sequenced. The *PpNAC* fragment released after digestion with *Xba* I and *Kpn* I was further subcloned into pBI-GFP vector. The resulting *pBI-PpNAC-GFP* construct contained an in-frame fusion between PpNAC and GFP gene. The recombinant plasmid *pBI-PpNAC-GFP* and control vector *pBI-GFP* were transformed into onion epidermal cells by the particle delivery method with a PDS1000/He (Bio-Rad, Hercules, CA). Transformed cells were incubated for 12h at 22^{o}C in the dark, and green fluorescence was monitored under a fluorescence microscope, ECLIPSE TE300 (Nikon, Tokyo, Japan). To visualize the nucleus, 1mg/ml 4,6-diamidino-2-phenylindole (DAPI) solution was dropped on the onion epidermal cells and blue fluorescence was monitored.

Expression of PpNAC following treatments with abiotic stresses and phytohormones

The expression patterns of *PpNAC* were elucidated under stresses such as drought (20%PEG6000), high salinity, high temperature, low temperature.Drought stress was applied by 20%PEG6000 solution and leaves were sampled. For high salinity treatment, sodium chloride (NaCl) solution was added with a final concentration of about 250mM. Seedlings were picked out randomly and grown at 4°C and 37°C for cold and heat treatment, respectively.

In order to examine the transcript level of *PpNAC* gene under various phytohormones (ABA, SA, ET, or MeJA). Abscisic acid, ET or MeJA (10mM stock solution in ethanol) was diluted to 100μM with surfactant (0.02% Tween-20) water and sprayed onto the leaves of seedlings, respectively. And the SA solution was added with a final concentration of about 10mM.

The control one was only filled with water. The stress-treated and control seedlings were harvested at various times (0.5h, 1h, 3h, 6h, 12h, 24h, 48h), then quickly frozen in liquid nitrogen and stored at –80°C for use.

Total RNA was extracted from young leaves of *P. pratensis* plants subjected to various treatments, and then subjected to quantitative realtime-PCR (qRT-PCR) for *PpNAC* expression. These treatments included first growing the plant at normal situation for 4d, subsequently treating at various treatments for 0.5h, 1h, 3h, 6h, 12h, 24h and 48h, then quickly frozen in liquid nitrogen and stored at –80°C for use. qRT-PCR using a One Step PCR Kit (Takara, Japan) were performed at 95°C for 30s followed by 45 cycles of amplification (95°C for 3s, 60°C for 34s), and qRT-PCR analyses with a pair of primers (NAC-F: 5'-AGCTGGATAACGACGACTGG-3' and NAC-R: 5'-AGAGCGCAGAGGTCAGAAGA-3') amplifying a 149-bp fragment. The elongation factor (eEF-1α) gene was used as the internal reference for the qRT-PCR analyses, and the primer sequences were as follows: eEF-1α-F: 5'-AGCACGCTCTTCTTGCTTTC-3', eEF-1α-R: 5'-GGGACCTTGTCAGGGTTGTA-3', (amplifying a 163-bp fragment).

Results and discussion

Isolation of the PpNAC-cDNA

According to homologous regions of the NAC genes, we successfully isolated a putative NAC gene from Kentucky bluegrass by using the RACE-PCR method and designated it as *PpNAC* (Fig. 1). Its cDNA is 1146 bp in length and it contained an open reading frame (ORF) of 837 bp, a 5'-untranslated region (UTR) of 91 bp, and a 3'-UTR of 220 bp. *PpNAC* encoded a deduced protein of 278 amino acid residues with a predicted molecular mass of 31.43kDa and a pI of 5.30.The N-terminal region of PpNAC had a NAC domain (amino acid 16-172) according to Ooka [13] whereas the C-terminal region showed no significant similarity to that of any other members of NAC family. Clustering analysis showed that PpNAC belongs to OsNAC3 subfamily of the NAC protein family [13]. PpNAC exhibited the highest identity to SNAC1, and the lowest identity to NAC1 (Fig. 2). All these data suggested that PpNAC is a member of NAC protein family.

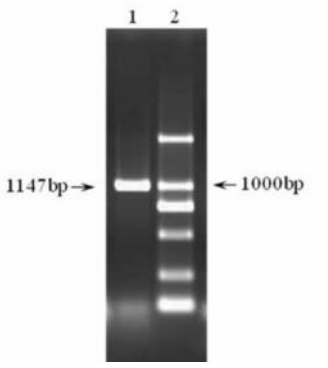

Fig. 1. Amplification of PpNAC overall length. Notice: lane 1, amplification of overall length of PpNAC; lane 2, DL2000 DNA Ladder

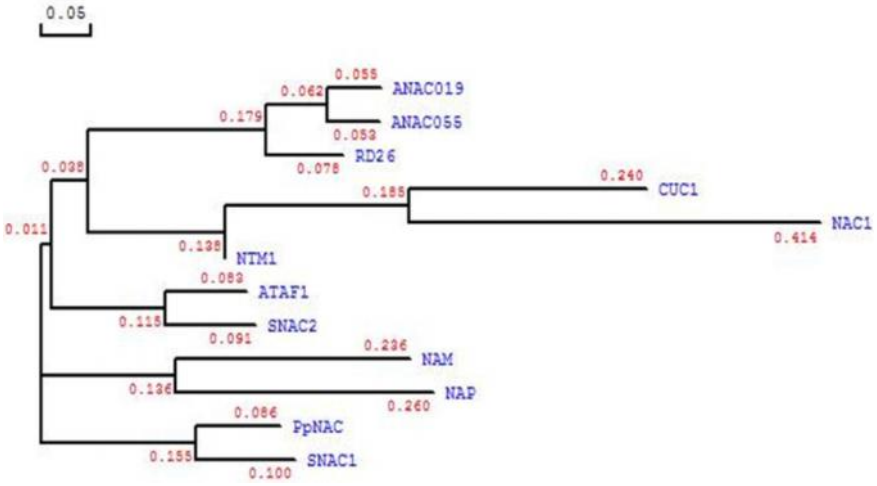

Fig. 2. Phylogenetic analysis of the deduced PpNAC amino acid sequence with other plant NAC protein

The highly homology between PpNAC and rice NAC protein SNAC1 [14] illustrated the similar function. Drought induced *SNAC1* expression [14] in stomatal guard cell, promoted stomatal closing, which has no effect on the photosynthetic rate, resulting in the improvement of the plant drought resistance. Overexpression of *SNAC1* can significantly improve plant tolerance to drought with no phenotypic changes and output decline. *SNAC1* were also induced by cold, whereas did not significantly improve plant cold tolerance [15]. PpNAC may belong to the OsNAC3 subfamily of NAC protein, which contained many abiotic stress related NAC transcription factors.

Sequence analysis and prediction of secondary structure of PpNAC

Based on an alignment of the six NACs (ANAC019, ANAC055, ATAF1, RD26, SNAC1 and SNAC2) (Fig. 3), higher sequence similarities are found in the N-terminus than in the C-terminus. These divergent C-terminal regions result in NACs protein length variation. Like NAC domain proteins from other plants, the conserved NAC domains from the PpNAC contain several distinguishable blocks of heterogeneous amino acids or gaps and are divided into five subdomains (A-E) (Fig. 3). and the conservation order of these domains is: C > D > E > A > B. C-terminal also contains more serine (S), asparagine (E), glutamic acid (G) and proline (P).

According to previous research, C-terminal containing acidic amino acid glutamine rich areas may be involved in transcriptional activation [16]. C-terminus in ANAC019, ANAC055 and ANAC072 protein contains a serine rich activation domain [17]. The PpNAC amino acid sequence revealed rich in glutamate (E), serine (S) and proline (P) in the C-terminal. It speculated that C-terminal regions of PpNAC may be involved in transcriptional activation.

Hao [18] found a transcriptional repression domain of 35 amino acid composition, named NARD (NAC Repression Domain), in DNA binding domain of the D domain in soybean GmNACs. NARD-type sequence contained 17 identical residues comprising the sequence (G**K*LVFY*G*P*G*K*W*MHEYRL) and 12 conserved amino acids (GKLVFYPWMHER) were also found in other NAC family proteins. And D domain of PpNAC contained the similar sequence: GIKKALVFYHGKPPGVKTDWIMHEYRL, LVFY and MHEYRL were the highest conserved motif. Hao [18] speculated that inhibition of NARD function and LVFY motifs were closely related. And the transcriptional activation capacity of NAC proteins depends on interactions between NARD and activation domains. Hao [18] also believed that NAC transcriptional activation relies on the interaction between the activation domain and NARD. If the function of NARD is stronger than the activation domain, NAC protein will be shown as a transcriptional repressor, otherwise exert transcriptional activation function, and when equilibrium is reached between the two effects, the function of NAC protein depends on the environment.

PpNAC was predicted to encode a 278-amino acids protein. The deduced PpNAC protein contained a conserved NAC domain (16-172) using ScanProsite analysis (Fig. 4). Protein secondary structure refers to the polypeptide chain with the aid of hydrogen bond array into unique folding or coiling way, main secondary structure are: alpha helix, beta fold, beta Angle and random crimp, etc. According to the results of SOPMA prediction (Fig. 5), it is known that the amino acid sequence of PpNAC protein secondary structure is mainly composed of random crimp (57.55%), alpha helix (29.86%) and beta folding sheet (10.79%) composition.

PpNAC is a nuclear protein

Numerous NACs have been located in the nucleus [5, 19-20]. In this study, protein was predicted to be located in the nucleus by WoLF PSORT (http://wolfpsort.org/) To confirm this prediction, we transiently expressed PpNAC-GFP fusion proteins in the onion epidermal cells. As shown in Fig. 6, free GFPs were distributed uniformly in the whole cell, whereas the PpNAC-GFP fusion proteins were targeted to the nucleus. This result indicated that PpNAC protein is a nuclear protein, supporting that PpNAC functions as a transcriptional factor.

NAC proteins with similar biological functions were more likely to be located in the same subcellular structure. C and D domains of many NAC proteins detected the putative nuclear location sigal (NLS) [6, 21-22]. A NLS of the NAC domain was very necessary for RD26 localized in the nucleus, then the lack of NAC domain of RD26 localized in the cytoplasm and nucleus [5]. ATAF1 was localized in the nucleus because of its NLS in D domain [19]. Hu [15] also found that SNAC2 contained NLS in N-terminal 144 amino acids. Greve [15] determined a degradation of the bipartite NLS, IKKALVFYAG KAPKGEKTN, in researching different NAC proteins. Nogueira [23] found

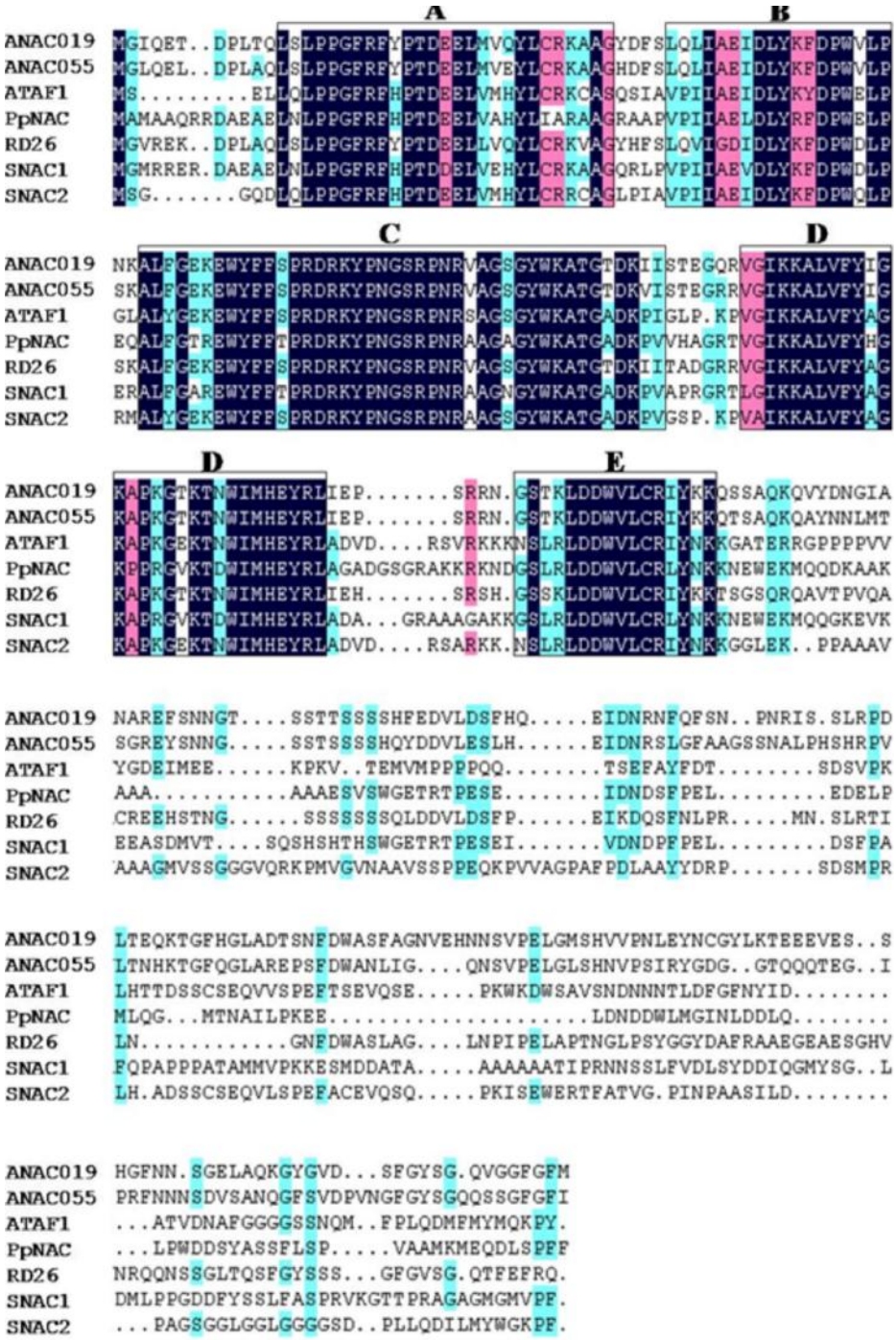

Fig. 3. Alignment of the deduced PpNAC amino acid sequence with other plant NAC

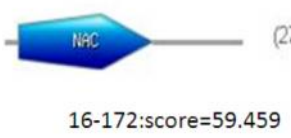

Fig. 4. PpNAC conservative domain using Scan Prosite program

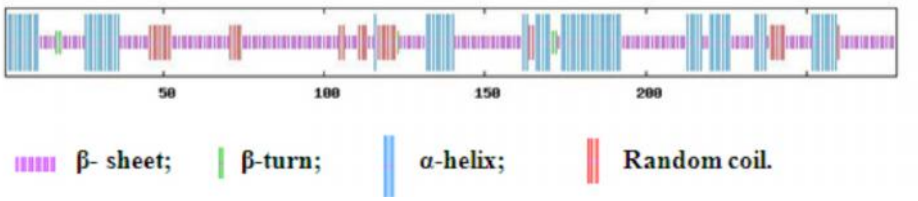

Fig. 5. Prediction of PpNAC secondary structure

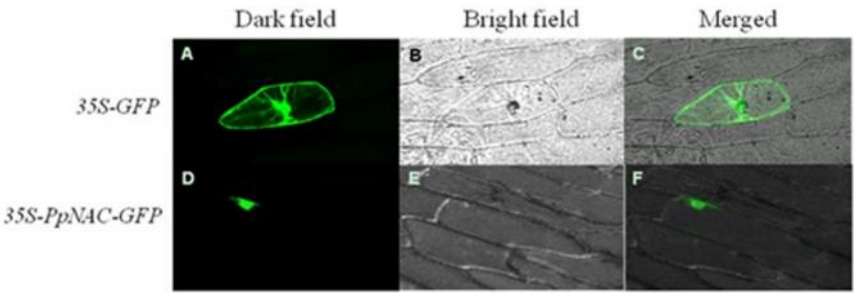

Fig. 6. The localization of GFP-Tagged PpNAC in Onion epidermal Cells. A: Images of green fluorescence of GFP; D: PpNAC-GFP protein in onion cells under the confocal microscope; B, E: Out-look of onion epidermal cells; C, F: Overlaid images of A and B, D and E

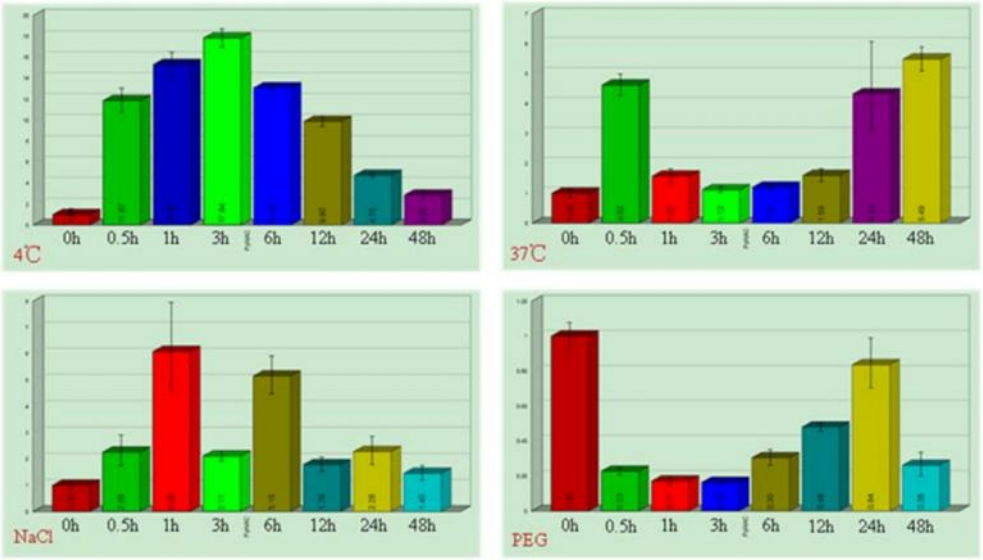

Fig. 7. Expression patterns of *PpNAC* in Kentucky bluegrass leaves after treatment with environmental stimuli, including low-temperature (4°C), high-temperature (37°C), high salinity (250 mM NaCl), and drought (20% PEG6000). Note: Ordinate in picture show the relative value of *PpNAC* against eEF-1- gene intensity show the quantitative increase in the expression levels, and abscissa respects hours of disposition, the 0 h time point served as the control

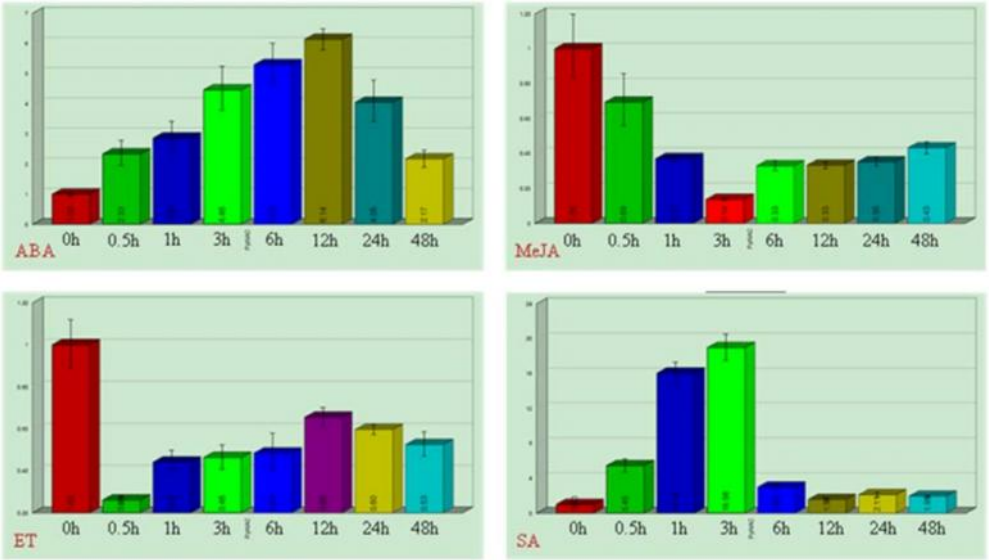

Fig. 8. Expression patterns of *PpNAC* in Kentucky bluegrass leaves after treatment with phytohormone stimuli, including ABA (100μM), MeJA (100μM), ET (100μM), and SA (10mM). Note: Ordinate in picture show the relative value of *PpNAC* against eEF-1a gene intensity show the quantitative increase in the expression levels, and abscissa respects hours of disposition, the 0 h time point served as the control.

that SsNAC23 contained a bipartite NLS. It also possess a similar sequence, IKKALVFYHGK PPGVKTD, in D domain of PpNAC protein. And IKKALVFY was the highly conserved motif, which may be necessary for entering the nucleus.

Expression of the PpNAC in response to various abiotic stresses

The expression patterns of the *PpNAC* under various stresses (cold, heat, high salinity, and PEG) (Fig. 7) and phytohormones treatments (ABA, MeJA, Et, SA) (Fig. 8) conditions were investigated using Realtime Quantitive-PCR analysis.

For the 4^{o}C treatment, the transcript level of *PpNAC* was up-regulated at first and then gradually decreased within 0.5-48h post treatment (hpt), and the transcript showed the maximum expression at 3hpt with 17.84-fold. 37^{o}C promoted the expression of *PpNAC*. The transcript level of *PpNAC* was increasing gradually within 1-48h, and the transcript showed the maximum expression at 48hpt with 5.49-fold. However, there was a transient rise at 0.5hpt with 4.62-fold. In Kentucky bluegrass plants watered with 250mM NaCl, *PpNAC* transcript showed increased expression at first and then gradually decreased within 0.5-48hpt except for 3hpt, and the peak of expression appeared at 1hpt with 6.08-fold. 20% PEG6000 strongly suppressed the expression of *PpNAC* within 0.5-48hpt, and the transcript showed the minimum expression at 3hpt with 0.16-fold.

Some of the phytohormones also influenced the expression of *PpNAC* to various degrees (Fig. 8). *PpNAC* expression was induced by 100μM ABA and 10mM SA treatment. The maximum expression occurred at 12hpt and 1hpt with 6.14 fold and 18.98 fold, respectively. However, when the seedlings were exposed to 100μM MeJA and 100μM ET, the PpNAC transcript was dramatically decreased to 0.5hpt, and the transcription levels of *PpNAC* decreased to a peak at 3hpt and 0.5hpt with 0.14 fold and 0.26 fold, respectively.

In this study, we found that *PpNAC* was induced by high temperature, low temperature, NaCl, ABA and SA stress, which suggested that PpNAC may be involved in a variety of abiotic stress responses, such as ABA signal transduction pathway. ABA is a phytohormone that is extensively involved in responses to abiotic stresses. NAC genes from different plant species were proved to be regulated by these phytohormones [5, 24-25]. Lin *et al*. [24] showed that OsNAC19 transcript was induced by the exogenous application of ET, MeJA, and ABA. The transcription level of *CaNAC1* could be elevated by exogenous SA, ET, and MeJA treatment [25]. In Arabidopsis, the expression of a NAC transcription factor encoding gene RD26 was mainly regulated by ABA [5]. Those previous researches have proposed sound links between the NAC genes and their putative function signaling pathways mediated by SA, ethylene, MeJA, or ABA, whereas our results showed that *PpNAC* was inhibited by 20%PEG6000, ET and MeJA treatment. Thus we speculate that TaNAC4 might be likely implicated in the defense responses mediated by crossed signaling pathways activated by ET, MeJA and ABA.

Taken together, we infer that *PpNAC* may be an important component in defense-signaling pathway and play a crucial role in plant defense against pathogen infection. However, the biological functions of *PpNAC* in Kentucky bluegrass need to be further investigated using over-expression and gene silencing technique like RNAi.

Acknowledgements

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