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Effect of Temperature and ABA Exposure on *LlaNAC* Expression in Transformed Tobacco Plants

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Research Article

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Abstract

NAC is one of the largest TF families in plants, with more than 100 genes per plant. NAC proteins are involved in a panorama of functions in a cell, virtually controlling the growth and development of the plants, as well as, protecting the plants against variety of stresses. We have assessed *LlaNAC* over-expressor (transgenic) tobacco lines (NC2, NC7b, NC10 and NC18), which were earlier displayed enhanced cold tolerance, biomass, growth and early maturity [1] in comparison to the WT. Here we report, enhanced level of relative transcript abundance in *LlaNAC* transformed plants to cope with heat stress together with the activation of antioxidant enzymes. The transgenic plants showed maximum of nearly two fold elevations in *LlaNAC* expression when exposed to temperature stress of 40°C. Exposure to ABA (10 µM) caused up-regulation of *LlaNAC* gene with an elevation of upto 1.45 fold while, higher concentration of ABA (50 µM) renders transgenic plants hypersentive to ABA treatments as compared to wild type plants (WT).

Keywords: ABA stress; Temperature stress; Hypersensitive*; LlaNAC*

Introduction

Plants are often targeted by variety of biotic and abiotic stress including salinity drought and temperature. Many genes are induced in response to these unfavorable conditions and transcription factors (TFs) are one of them [2]. These are one such gene family that upon exposure to various environmental stresses get induced. The NAC (NAM, ATAF1/2, and CUC) super family is one of the largest TF families with more than 100 copies per plant [3] participates in a number of vital processes in a plant's life cycle like development processes [4-6], stress tolerance [7], lignin synthesis [8], leaf senescence, phytohormone homeostasis [9,10] etc. NAC family TFs contain a highly conserved N-terminal DNA domain and a diversified C-terminal domain that generally regulates transcriptional activation [11,9]. In this study, *LlaNAC* gene was identified and cloned using a cold stress induced cDNA library of *Lepidium latifolium* L. [12] in expression vector pBinAR, under the influence of promoter CaMV35S and the construct also contained *npt*II gene as a selectable marker. The construct was introduced in *Agrobacterium tumefaciens* LBA4404 and transferred to in vitro cultures of *N. tabacum* by previous workers in the laboratory [13]. T2 seeds of four transgenic lines, viz., NC2, NC7b, NC10 and NC18 were thus available for this study. Gene expression pattern analysis demonstrated that *LlaNAC* transcripts was increased and followed similar pattern under heat stress and lower abscissic acid ABA (10 µM) among transgenic lines.

Materials and Methods

Plant Material and Growth Conditions

Nicotiana tabacum (tobacco) plants over-expressing *LlaNAC* gene, and co-transformed *npt*II gene, (four transgenic lines-NC2, NC7b, NC10 and NC18 in

generations T2) along with wild-type (WT) plants were maintained in containment under controlled conditions of temperature (25 ± 2°C) and light (16/8 h photoperiod), and grown in soilrite. The plants were nourished with MS basal medium twice a week, and were watered (distilled water) on daily basis. Each individual plant was tested for its genetic stability based on herbicide tolerance assay (150 ppm paromomycin) following PCR assay with an initial denaturation at 94°C for 5 min., followed by 30 cycles of 94°C for 30 s and 55°C for 30 s and 72°C for 1.5 min. and a final extension of 10 min. at 72°C. Amplicons thus obtained was run on 1% agarose gel to check the presence or absence of *LlaNAC* gene. For whole sets of experiments, six technical replicates were used. The native growth conditions, i.e., temperature (25 ± 2°C) and carbon dioxide (400 ppm) were considered as the control conditions.

Stress Treatments and Gene Expression Analysis

Plants of 10 week old were provided with 10 µM and 50 µM of ABA (Sinha et al. 2014). Regularly watered plants under optimum conditions of temperature (25 ± 2°C) and light (16/8 h photoperiod) for 1 week was considered as control. Heat stress was induced by exposing the 10 week old plants to elevated temperature of 40°C and light (16/8 h photoperiod) in plant growth chamber for 24 h following Pospisilova et al. (2011) [14], Tan et al. (2011) [15], Mackova et al. (2013) [16]. Plants under optimum conditions of temperature (25 ± 2°C) and light (16/8 h photoperiod) for 24 h were considered as control for both the extremes in temperature stress. Expressions of *LlaNAC* gene was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was isolated from young leaf tissues of control and heat exposed (40°C)/ ABA treated (10 and 50 µM). Equal amount (1.0 µg) of the RNA quantified using Qubit fluorometer (Invitrogen, USA) was used for first-strand cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen, Germany) following manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, USA) kit according to the manufacturer's instructions. Normalization reaction was carried out with an internal control actin gene using forward (5'-AGGGTTTGCTGGAGATGATG-3') and reverse (5'-ACCACGTTTG GATTGAGCTT-3') primers. The PCR amplification was carried out using the primer pair *LlaNAC-Tq*F (5'-ACA GTG GTA AAC CTC CAA AAG G-3') and *LlaNAC-Tq*R (5'-CGA AGA GAG TTC TTG TTG ACG A-3') at an initial denaturation at 94°C for 10 min., followed by 40 cycles of 94°C for 30 s and 55°C for 30 s and 72°C for 30 s. Experiments were performed in triplicate for each set of primers. The transcript was quantified based on ΔΔCt method [17] and presented as fold change over control.

Result and Discussion

About 90% germination could be seen in transgenic lines grown on antibiotic medium. In the present communication, we have initiated transcriptional monitoring of stress-inducible *LlaNAC* gene in response to 40°C of heat exposure and 10 and 50µM of ABA application. *LlaNAC* expression levels were examined in leaf tissues. PCR based validations of plants were carried out using the primer pair *LlaNAC-Tq*F (5'-ACA GTG GTA AAC CTC CAA AAG G-3') and *LlaNAC-Tq*R (5'-CGA AGA GAG TTC TTG TTG ACG A-3') to obtain a band of size 122 bp (Figure 1).

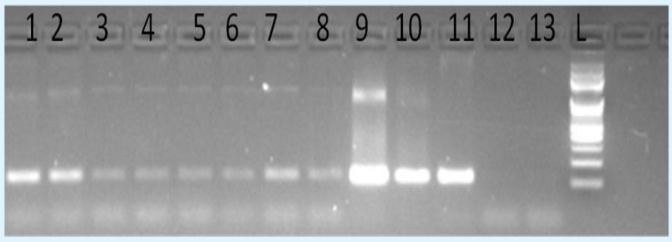

Figure 1: Representative gel picture showing amplicons (122 bp) obtained after PCR amplification using *LlaNAC* specific primers. Lane 1-8 (NAC lines), lane 9-11 (Vector, lepidium as positive control), lane 12-13 (WT as negative control), L (GeneRuler™100 bp DNA ladder, Fermentas USA)

Transgenic plants found positive for the presence of *LlaNAC* gene along with WT were subjected to heat and ABA stress.

Expression of *LlaNAC* Transcript under Heat Stress

Heat stress poses a serious threat to global crop production. Plants cope with adverse temperature stress by altering molecular mechanisms and elevated antioxidants activities that decrease levels of stress inducible reactive oxygen species (ROS). Induction of NAC transcription factors in response to stresses activates expression of various stress inducible genes thereby participating in improving stress tolerance [18]. In the present study, the expression of *LlaNAC* gene over control was noteworthy upon heat exposure to 40°C. Though, the amount of transcript abundance was varied among different lines. *NAC* Transcript expression in transgenic lines (NC10, NC7b, NC18, NC2) when exposed to

temperature stress of 40°C was found significantly increased with a maximum of 1.9 fold in NC7b line, followed by an elevation of 1.4 fold in NC10 line and 1.3 fold in NC18 line over the control (Figure. 2).

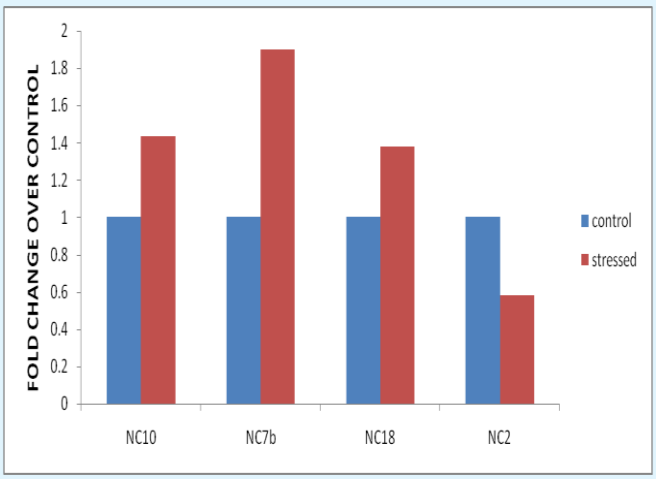

Figure 2: *LlaNAC* gene expression in terms of fold change over control in response to heat stress (40°C) for 24 h against control.

Therefore, we speculated that the rapid increases in *LlaNAC* transcript abundance might be associated with heat tolerance in transgenic plants. However, the transcript accumulation declined in case of NC2 when compared to the control plants. Similarly, Guo et al. [19] reported up-regulation of *TaNAC2L*, promotes heat tolerance in transgenic arabidopsis, Han et al. [20] reported over-expression of NAC transcription factors *CiNAC4* and *CiNAC3* confers salinity tolerance in Arabidopsis, Hong et al. [21] reported the over-expression of *ONAC022* for drought and salt tolerance in rice. Plants produce antioxidant enzymes like glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) to cope up with the stress induced by the increased levels of ROS. Further, we noticed elevated antioxidant enzyme activities ranging between 1.5 to 3 fold (data not shown) indicating up-regulation of *LlaNAC* improves heat tolerance in transgenic plants.

Differential Expression of *LlaNAC* Transcript by ABA Treatments

ABA is an important signaling molecule in various stresses and often considered as the secondary messenger [19]. As an important phytohormone, ABA controls various processes throughout the life cycle of plants, especially in response to external environmental stimuli [9,18]. Several NAC genes are involved in stress responses in an ABA-dependent manner [22], while Fang et al. [23] reported *SNAC3* confers heat and drought tolerance in ABA independent manner. The inducibility by ABA was clear from the gene expression pattern at both the concentrations. Transgenic lines NC7b, NC18 and NC10 showed up-regulation of NAC gene at 10 µM ABA. NAC transcript accumulation of 1.45 fold was observed in line NC7b, followed by fold of 1.37 in case of lines NC18 and NC10 over the control and again the expression of NC2 line was found repressed (Figure 3). This indicates over-expression of *LlaNAC* reduced ABA sensitivity at this concentration and is involved in ABA signaling. However, 50 µM ABA leads to down-regulation of NAC expression in all transgenic lines (Figure 3).

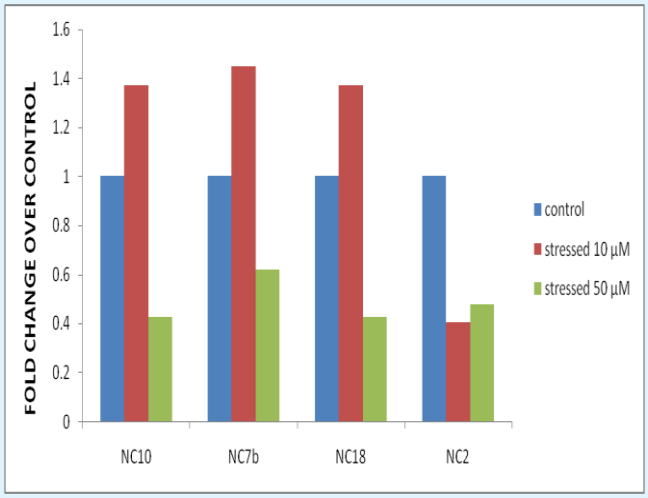

Figure 3: *LlaNAC* gene expression in terms of fold change over control in response to 10 and 50 µM ABA for one week against control.

Our results showed that plants over-expressing *LlaNAC* exhibited hypersensitivity to higher dose of ABA (50 µM) that might be a due to lower transpiration rates and faster stomatal closure in *LlaNAC* over-expression plants, leading to lower water loss and enhanced tolerance. Similarly, Huang et al. [24] reported *TaNAC29*-over expressing arabidopsis plants exhibit ABA-hypersensitive response and its expression confers salt and drought tolerance. A relationship between ABA-hypersensitivity and abiotic stress tolerance was established. Upon treatment to 10 µM ABA, elevated levels of GR (2.6 fold) and SOD (1.5 fold) activities were seen between transgenic and WT (data not shown). Interestingly, treatment of 50 µM ABA caused no appreciable increase among transgenic lines. These results indicate *LlaNAC* transgenic lines exhibited ABA hypersensitivity and were positively regulated by ABA at higher concentrations and provide evidence that *LlaNAC* participates in the ABA

signal pathway, and plays important roles in stress responses and developmental processes.

Conclusion

NAC genes have widely been reported to play crucial roles in growth, development, senescence and stress responses [25] of a plant. We have here demonstrated the differential expression of the *LlaNAC* gene which is a transcription factor and upon binding to their respective target sites regulates the expression of various downstream genes. Over-expression of a novel *NAC* gene, such as *LlaNAC* can further offer insights into the diversity of functions that the products of these genes can play, and suitably be utilized in agricultural biotechnology.

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