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EXPRESSION OF *ZMNAC3* RESPONSIVE TO VARIOUS ABIOTIC STRESSES IN MAIZE (*ZEA MAYS* L.)

XIULAN LI* AND YUESHUI JIANG

School of Life Sciences, Qufu Normal University, Qufu, 273165, China

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Abstract

NAC proteins are plant-specific transcription factors that have a variety of crucial roles in plant growth, development and response to stress. More than 100 members of NAC genes have been identified in maize genome, but almost all of them are not known for their expression profile and functions in plants. In this study, a NAC gene *ZmNAC3* from maize was cloned using rapid amplification of cDNA ends. *ZmNAC3* encodes a nucleus-targeted protein that has an extremely conserved NAC domain in the N-terminus. Expression of *ZmNAC3* was greatly up-regulated by high salinity and low temperature, down-regulated by drought, but not responsive to exogenous abscisic acid (ABA). Furthermore, several *cis*-acting elements in response to stress were found in *ZmNAC3* promoter region. These results suggest that *ZmNAC3* encodes a NAC-domain protein which may function in the response of maize to abiotic stress.

Introduction

Transcription factors (TFs) are regulatory proteins that activate or repress gene transcription through interacting with specific and conserved DNA sequence in promoter of target genes. The NAC named from the initials of NAM (no apical meristem), ATAF (Arabidopsis transcription activation factor) and CUC (cup-shaped cotyledon) TFs are one of the largest families of transcriptional regulators. NAC genes specifically exist in plant, and more than 100 members have been predicted in monocotyledonous and dicotyledonous plants. NAC proteins are characterized by an extremely conserved N-terminal NAC domain and a greatly diverse C-terminus. NAC domain is commonly composed of about 150 amino acids that are divided into A to E sub-domain and function in DNA-binding and protein-protein interactions for NAC protein dimerization (Welner *et al*. 2012). The C-terminal fragments of NAC proteins are variable in both length and amino acid sequence and usually serve as a transcriptional activator or repressor.

NAC genes appear to play significant roles in multiple plant development and biotic stress response, such as secondary wall formation (Nakano *et al.* 2015) and pathogen infection (Kaneda *et al*. 2009). Recently, some NAC genes were reported to regulate plant responses to a wide range of abiotic stresses, including salinity, drought and ABA (Jeong *et al*. 2010). Arabidopsis *ANAC055*, *ANAC072* and *ANAC109* could be induced by drought stress, and overexpression of them improved drought tolerance of transgenic Arabidopsis. *ANAC078* was responsive to a combination of high light and heat stress (Morishita *et al*. 2009). Overexpression of rice *OsNAC6*, *OsNAC10* (Jeong *et al.* 2010) and *OsNAC14* (Shim *et al.* 2018) enhanced drought and salinity tolerance of transgenic lines. Soybean *GmNAC11* and *GmNAC20* were conferred to improve salt and freezing tolerance of transgenic Arabidopsis lines (Hao *et al.* 2011). Wheat *TaNAC2* participated in response to salt, drought and low temperature treatment, and its overexpression enhanced tolerances of Arabidopsis to these stresses (Mao *et al*. 2012).

Maize (*Zea mays*) is a vital food and feed grain in worldwide. The productivity of maize is significantly limited by abiotic stresses which affect plant growth and development. Thus, identifi-

*Author for correspondence: <lxljewel@163.com>.

cation and characterization of abiotic stress-induced NAC genes is one of the useful approach to understand molecular mechanisms of abiotic stress tolerance and increase abiotic stress tolerance in maize. To date, 148 non-redundant NAC genes have been identified by genome-wide transcription profiling in maize (Peng *et al*. 2015), study on the expression profile and possible function of them in the abiotic stress response are limited. Thus, the identifying of stress-related NAC genes in maize will be an effective approach to improve the stress tolerance of maize. In this work, a NAC gene *ZmNAC3* from maize was cloned, its exprssion profile was characterized, and its response to various abiotic stresses in maize was studied.

Materials and Methods

Seedlings of maize W22 were cultivated in a Hoagland nutrient solution in a growth chamber with a day/night temperature of 28/22°C and a light/dark photoperiod of 14/10 hrs.

For ABA, salinity and PEG experiments, two-week-old seedlings were transferred into the Hoagland nutrient solution supplemented with NaCl (200 mM), PEG 6000 (w/v, 20%) and ABA (100 μM) for 0, 1, 2, 4, 8, 12 and 24 hrs. For low temperature experiment, the seedlings incubated in the Hoagland nutrient solution were moved to 4°C for 0, 1, 2, 4, 8, 12 and 24 hrs. Following the treatment, the leaves were collected at different time periods. All samples were quickly treated by liquid nitrogen and kept at –80°C until required. In each assay, three independent biological repeats were used. Each sample for each replicate contained combined material from three individual plants.

Cloning of 5′ and 3′ termini of *ZmNAC3* transcript were carried out using the forward primer 5′-ACAGTTTCCAGACGCACGACTCG-3′ and reverse primer 5′-GCCTTGCCAGAGTAGAA CACCAGC-3′ according to Li (Li *et al*. 2019).

Full-length *ZmNAC3* transcript lacking stop codon was amplified from W22 cDNA with the forward primer 5′-CACCATGGCAATGGTGGCGG-3′ and reverse primer 5′-GAACGGA GGCAAGATTGTCTGC-3′, inserted into pENTR/D-TOPO, and then fused to GFP in pGWB5 vector using LR reaction. The resulting plasmids were transformed into *Agrobacterium* strain EHA105. The transient expression and signal detection followed the method described by Li (Li *et al*. 2019). 4′,6-diamidino-2-phenylindole (DAPI) was used as the nuclei marker.

The putative amino acid sequence of maize *ZmNAC3* and sequences of representative NAC proteins of other species were obtained from NCBI. The phylogenetic tree was constructed by the ME method.

The extraction of total RNA and the synthesis of cDNA were performed according to Li (Li *et al*. 2019). Transcript-level of *ZmNAC3* was tested by qRT-PCR with the forward primer 5′-AAGACGGACTGGATTATGC-3′ and reverse primer 5′-TCGTGCGTCTGGAAACTG-3′using the Bio-Rad MyiQTM Real-time PCR Detection System. The mRNA level of *ZmNAC3* was normalized against *ZmActin* (GRMZM2G126010). The $2^{-\Delta\Delta Ct}$ method was carried out for analysis of gene expression. For each sample, data were obtained from three independent biological and three technical repeats.

Results and Discussion

NCBI database appears that the full-length ORF of *ZmNAC3* (GRMZM2G312201) has 4404 bp nucleotides and encodes a protein of 1467 amino acids. To confirm ends of *ZmNAC3* transcript, 5′ and 3′ RACE was carried out. Results showed that transcripts of *ZmNAC3* have different initiation or termination sites, yet the same coding region (Fig. 1A,B). Alignment of cDNA sequence with gDNA sequence indicated that *ZmNAC3* gDNA is 2170 bp in length and composed by 2 introns and 3 exons (Fig. 1C).

The coding region of *ZmNAC3* is 948 bp in length and encodes a 315-amino acid protein. NAC domain is an essential characteric of the NAC transcription factor family (Puranik *et al*. 2012). Sequence analysis revealed that the N-terminal region of ZmNAC3 protein contains a typical NAC conserved domain, and that can be divided into A to E sub-domain (Fig. 2A). Meanwhile, phylogenetic analysis clearly placed *ZmNAC3* protein into the same branch with ZmNAC1 and SNAC1, NAC proteins in maize and rice respectively (Fig. 2B) (Liu *et al*. 2009, Chen *et al*. 2011). These data indicated that *ZmNAC3* is a member of the NAC gene family in maize.

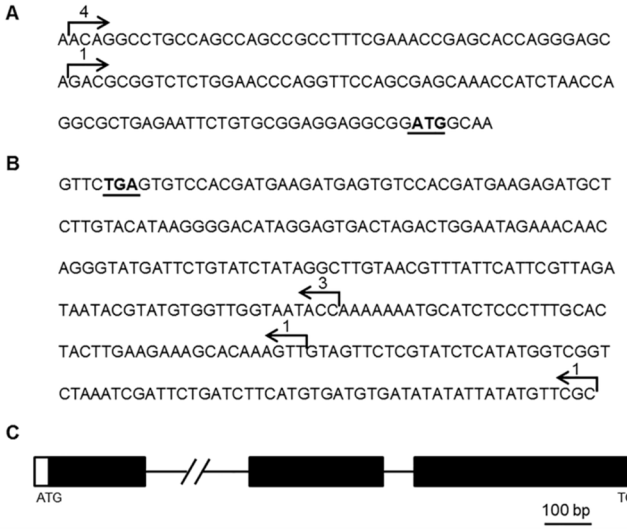

Fig. 1. Transcription ends and gene structure of *ZmNAC3*. A and B: 5′ and 3′ ends of transcripts produced in *ZmNAC3*. Numbers of sequence reads is indicated with arrow. C: *ZmNAC3* gene structure.

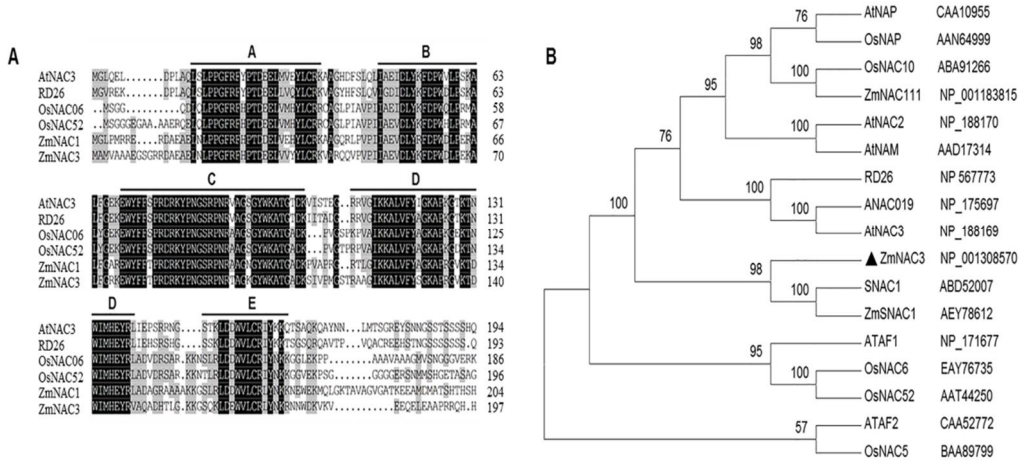

Fig. 2. Sequence alignment and phylogeny of ZmNAC3. A: Sequence alignment of NAC domain in ZmNAC3 and other NAC proteins. B: Phylogenetic tree of ZmNAC3 and other NAC proteins.

To assess *ZmNAC3* expression in maize tissues, qRT-PCR was carried out. As shown in Fig. 3A, *ZmNAC3* expressed in all tested tissues, with relatively higher level in leaves, stems, silk, and lower in roots, pollen, developing kernels, suggesting *ZmNAC3* is widely expressed in maize.

NAC proteins are usually targeted to nucleus. For *ZmNAC3*, no putative bipartite nuclear localization signal was predicted by the NLS mapper website. To determine its subcellular localization, ZmNAC3 was fused to GFP and transiently expressed in tobacco leaf epidermal cells. Under the confocal microscope, GFP signals were observed in dots that overlapped with DAPI-labelling of nuclei (Fig. 3B), indicating that ZmNAC3 is located in the nucleus.

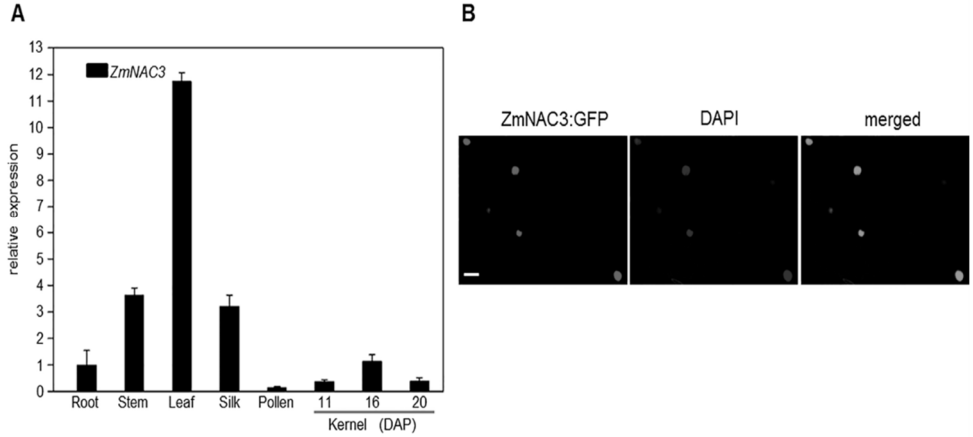

Fig. 3. Expression of *ZmNAC3* gene and subcellular localization of ZmNAC3 protein**.** A: Expression of Z*mNAC3* in maize. DAP, days after pollination. B: Subcellular localization of ZmNAC3 protein. Scale bar, 20 μm.

Studies of whole-genome expression profiles in Arabidopsis and rice have found NAC genes to be mediated by at least one type of abiotic stress like salinity, cold, drought or ABA (Fujita *et al.* 2004). To investigate the functional relevance of *ZmNAC3* to abiotic stress response, its expression profile in leaves under salt, drought, low temperature and ABA stress were detected by qRT-PCR. As indicated in Fig. 4, *ZmNAC3* transcript was strongly induced by salt and low temperature treatment. Under salt treatment, the level of *ZmNAC3* transcript increased and peaked at 12 hrs showing 11-fold higher than the control subjects (Fig. 4A). *ZmNAC3* expression was induced rapidly and peaked at 3 hrs after low temperature treatment (Fig. 4B). On the contrary, the level of *ZmNAC3* transcript was greatly suppressed under the drought stress (Fig. 4D). These data demonstrated that ZmNAC3 may play roles in response to these stresses and may act as a positive regulator of low temperature and salt response in maize, a negative regulator in drought stress response. This is in agreement with previous reports that NAC genes are often induced or suppressed by various abiotic stresses like high salinity, cold, drought (Nakashima *et al*. 2012). In maize, expression of *ZmSNAC1* (Lu *et al.* 2012) and *ZmNAC55* (Mao *et al*. 2016) were significantly induced by cold, high-salinity, ABA and drought stresses, and their overexpression strongly enhanced stress tolerance in Arabidopsis.

Transcription of stress-responsive NAC genes are proposed to be regulated by some *cis*-acting elements contained in the promoter-regulatory sequence (Nakashima *et al.* 2012). To investigate the putative *cis*-acting regulatory elements, 1.5-kb gDNA sequence upstream from ATG codon of *ZmNAC3* was analyzed by PlantCARE database. Two binding sites for MYB (MBS) and one low-temperature (LTR) responsive element were detected (Fig. 4E). MBS and LTR elements have been reported to be involved in drought and cold (Pandey *et al.* 2015) stress response, respectively, which at least partially explained the responsive expression of *ZmNAC3* under drought and cold treatment.

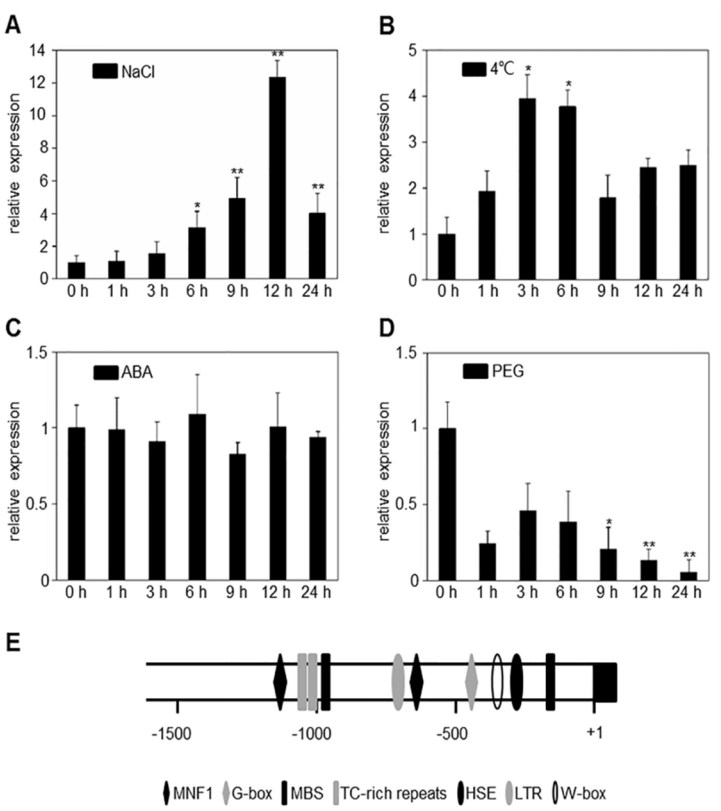

Fig. 4. Expression of *ZmNAC3* under abiotic stress**.** A-D: *ZmNAC3* expression in maize leaves upon treatment. *,** represent significant difference $p < 0.05$ and $P < 0.01$, respectively. E: Putative *cis*-elements related to abiotic or biotic stress within the *ZmNAC3* promoter.

The *ZmNAC3* promoter also includes one HSE, two MNF1, and one G-box elements. These *cis*-acting elements are predicted to function in plant response to heat or light stress (Lee *et al.* 2014). Additionally, *ZmNAC3* promoter has one TC-rich repeats and one W-box which are reported to be associated with defense responses. However, functions of these elements in stress response of *ZmNAC3* need to be investigated further. These data indicated that *ZmNAC3* may play regulatory roles in both abiotic and biotic stresses.

This work suggests that *ZmNAC3* may regulate abiotic stress response and provides insights into the function of NAC transcription factors in mediating abiotic stress tolerance in maize.

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