Molecular cloning and characterization of NAC genes from four foxtail millet genotypes

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Abstract. Transcription factor gene family of NAC (NAC, ATAF, CUC) is tightly involved in plant development and in the response to stresses. In this study, we reported the isolation and the characterization of NAC gene homolog from four foxtail millet genotypes. Band with approximately 1300 bp size was successfully amplified from the genomic DNA of four foxtail millet genotypes (ICERI-4, ICERI-5, ICERI-6 and ICERI-10) using gene specific primer. The fragment was designated as *SiNAC065* after showing high similarity with NAC gene homologs in the GenBank. Sequence analysis results showed that the *SiNAC065* genes isolated from the four genotypes were 1265 bp in length with one intron and two exons. The two exons encode 325 amino acids with the conserved domain located between amino acid 19-325. The *SiNAC065* protein identified in this study have 8 conserved motives in the conserved region which categorized them as SNAC (stress responsive NACs) orthologs that are involved in the abiotic stress responses. Different features of *SiNAC065* isolated from the tolerant- and the sensitive-genotypes should provide information of the gene's role in salinity tolerance mechanism of foxtail millet.

Keywords: abiotic stress, gene cloning, *Setaria italica*, stress-responsive NAC, transcription factor

INTRODUCTION

Foxtail millet (*Setaria italica* L. Beauv) is carbohydrate producing crop with high nutritional value (Amadou *et al.*, 2013). This crop ranks second in total millet production globally (Yang *et al.*, 2012) and became an essential food crop particularly in areas with drought and saline conditions due to its tolerance to those conditions (Kafi *et al.*, 2009; Karyudi and Fletcher, 2003; Panaud 2006). Several studies have been conducted to reveal the tolerance mechanism of foxtail millet to abiotic stresses, particularly to

drought (Lata *et al.*, 2010) and salinity (Islam *et al.*, 2011) stresses.

Extensive studies in plant abiotic response showed that the genes involved in abiotic stress tolerance mechanisms were evolutionary conserved among plant species (Akpınar *et al.*, 2012). Generally, stress related genes are classified into down-stream genes (functional genes) and up-stream genes (regulatory genes). Functional genes encode enzymes and metabolic proteins which directly function to protect cells from

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stress, such as detoxification enzymes, ion transporters, late embryogenesis abundant (LEA) proteins, and heat shock proteins (HSP). Regulatory genes encode various regulatory proteins which regulate the signal transduction and gene expression in response to the stress, such as protein kinases and transcription factors (TFs) (Hirayama and Shinozaki, 2010; Akpinar *et al.*, 2012). The NAM, ATAF1,2 and CUC2 (NAC) proteins constitute a major plant specific transcription factor (TF) family which have been shown to play wide-range of roles in various developmental programs as well as in abiotic and biotic stress responses (Olsen *et al.*, 2005; Nakashima *et al.*, 2012; Puranik *et al.*, 2012). Several studies reported that the expressions NAC TF members were upregulated by salinity stress in many plants, including cereals such as foxtail millet (Puranik *et al.*, 2013), wheat (Huang *et al.*, 2015), rice (Hong *et al.*, 2016), and pearl millet (Shinde *et al.*, 2019). These studies indicated the pivotal role of NAC TF in salinity stress responses. In this study, we reported the isolation of NAC-containing domain genes from four foxtail millet genotypes. Ardie *et al.* (2015) has reported the salinity tolerance variation between the four foxtail millet genotypes, hence characterization of NAC TF from these genotypes would provide useful information in the breeding strategy of foxtail millet.

MATERIALS AND METHODS

NAC gene homolog isolation from genomic DNA. Four foxtail millet genotypes from Indonesian Cereal Research Institute (ICERI), namely ICERI-4, ICERI-5, ICERI-6 and ICERI-10, were used in this study due to their contrasting tolerance to salinity. ICERI-5 and ICERI-6 genotypes were reported to have better tolerance to salinity compared to ICERI-4 and ICERI-10 (Ardie *et al.*, 2015; Lapuimakuni *et al.*, 2018). Genomic DNA was isolated from leaves of 10 days-old foxtail millet seedlings using CTAB method (Murray and Thompson 1980). Each PCR reaction mixture of 30 μ L consisted of 5 μ L (12 ng/ μ L) genomic DNA, 15 μ L GoTaq® Green Master Mix (Promega, USA), 50 pmol of each

forward (5'-ATGGGAGAAGCAGAAGCATC AGC-3') and reverse primer (5'-CTAGAACATT TCGTCGGCCTCCG-3'), and 5 μ L ddH₂O. The PCR cycle consisted of initial denaturing at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 58°C annealing for 60 s, and extension at 72°C for 30 s. The final extension step was done at 72°C for 7 min, then the PCR products were stored at 4°C. PCR product was gel-purified using FastGene™ Gel/PCR Extraction Kit (Nippon Genetics Europe GmbH, Germany) following the manufacturer instruction and directly sequenced to Eurofin (<https://eurofinngenomics.jp/>). BLASTn algorithm (<http://blast.ncbi.nlm>) analysis was performed to ensure the homology of the fragment with the NAC genes stored in the GenBank. Sequenced fragments were then inserted into T-vector pMD20 (TaKaRa, Japan) with the ligation reaction of ± 80 ng/ μ L PCR product, 1 μ L plasmid pMD20 (50 ng/ μ L), and 1x volume of DNA Ligation Kit Mighty Mix (TaKaRa, Japan) at 16°C for 45 mins. Ligation products were transformed into *E. coli* strain DH5 α , purified using FastGene™ Plasmid MiniKit (Nippon Genetics Europe GmbH, Germany), and sequenced to Macrogen (<http://www.macrogen-japan.co.jp/>).

Sequence analysis of NAC gene homolog from foxtail millet. The NAC nucleotide sequences obtained were aligned and translated into amino acid sequence using Geneious software version 8.1.6 (Biomatters Ltd). Amino acid sequences were aligned using web-based program ClustalW2 (Larkin *et al.*, 2007). Phylogenetic tree was developed by MEGA7 software (Kumar *et al.*, 2016) using Neighbor-Joining method at 1000 replication bootstrap. Conserved domain analysis was conducted using BLAST on Plant Transcription Factor Database v. 4.0 (<http://planttfdb.cbi.pku.edu.cn/blast.php>). Visualization on conserved domain was done using WebLogo program (Crooks *et al.*, 2004).

RESULTS AND DISCUSSION

Fragment with the targeted size (1265 bp) was amplified by using gene specific primer (Figure 1). The gene specific primer was deduced from *SiNAC065* sequence deposited in the plant genome database Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>). Sequence alignment analysis between the four amplified fragments with the reference sequence in Phytozome database (Phytozome identifier: Si012319m) showed that the fragments shared 99% identity with the reference sequence, thus the fragments isolated in this study were designated as *SiNAC065* followed by the foxtail millet genotype name. The fragments were deposited in the GenBank as *SiNAC065_ICERI4* (Accession no. KY404102), *SiNAC065_ICERI5* (Accession no. KY404103), *SiNAC065_ICERI6* (Accession no. KY404104), and *SiNAC065_ICERI10* (Accession no. KY404105). The *SiNAC065* genes isolated have one intron and two exons. The first exon spans between base 1-196, the second exon spans between base 487-1265, while the intron spans between base 197-486. The two exons encode 325 amino acids that showed high homology to similar sequences from other species in GenBank, such as *Oryza brachyantha*, *Zea mays*, *Sorghum bicolor*, *Panicum virgatum*, *Brachypodium distachyon*, *Selaginella moellendorffii*, *Bambusa emeiensis*, *Hordeum vulgare*, *Aegilops tauschii*, and *Triticum aestivum* (Figure 2).

Figure 1. Amplified *SiNAC065* fragment from four foxtail millet genotypes. Bands with targeted gene size (approximately 1300 bp) that are shown in white box were purified for further analyses.

Figure 2. Unrooted minimum-evolution tree of NAC-domain containing proteins. The translated sequences of known NAC-domain containing genes were aligned by the ClustalW program and the tree was constructed using the MEGA 7 program (Kumar *et al.*, 2016). Scale bar indicates 0.05 substitutions per site. 'Yugu 1' genotype of *S. italica* (XM_004971405.1), *Oryza brachyantha* (XM_006645364.1), *Zea mays* (JN673545.1), *Sorghum bicolor* (XM_002463358.1), *Panicum virgatum* (XM_008657306.1), *Brachypodium distachyon* (XM_003559734.3), *Selaginella moellendorffii* (XM_002987191.1), *Bambusa emeiensis* (HM626401.1), *Hordeum vulgare* (KT779954.1), *Aegilops tauschii* (DQ869677.1), *Triticum aestivum* (HQ630372.1).

Multiple sequence alignment analysis of the deduced amino acid sequences of *SiNAC065* fragments isolated from the four foxtail millet genotypes and the deduced amino acid sequence of *SiNAC065* from *S. italica* inbred 'Yugu1' reported by Bennetzen *et al.* (2012) deposited in NCBI GenBank database and PHYTOZOME v8.0 database (www.phytozome.net/) revealed the presence of conserved domains located in the N-terminal region (Figure 3). Domain identification analyses conducted by Puranik *et al.* (2013) showed that the N-terminal region of *SiNAC065* share the general structure of NAC proteins. In general, the N-terminal NAC-domain (~150 aa) can be divided into five sub-domains, A-E, which each possess particular function (Nakashima *et al.*, 2012; Puranik *et al.*, 2012). The DNA binding ability is reported to be possessed by the highly conserved positively charged C and D sub-domains, whereas the A sub-domain may be involved in the functional dimer formation,

and the B and E sub-domains may be responsible for the functional diversity of NAC genes (Jensen *et al.*, 2010; Chen *et al.*, 2011; Puranik *et al.*, 2012). The SiNAC065 proteins isolated in this study also have the well conserved N-terminal NAC-domain. Based on the conserved motif determination of NAC TF family member in foxtail millet by Puranik *et al.* (2013), the N-terminal NAC-domain of SiNAC065 can be divided into 8 conserved motifs (Table 1). Two putative Nuclear Localization Signal (NLS) domains were found within the NAC domain in SiNAC65 protein, which is previously reported by Puranik *et al.* 2011 (Figure 2). The presence of NLS domains suggest that the SiNAC65 protein was directed into the nucleus. NLS is a short peptide motif that able to mediate the import of proteins into nuclear by binding to their receptors (Kosugi *et al.*, 2009; Sakuma *et al.* 2006). It is no surprise if NLS domains are present in SiNAC65 proteins since this protein has a DNA binding ability as a transcription factor. Meanwhile, the C-terminal region of NAC TF is greatly varied among the family member and it serves as a potential transcriptional activation regions (TARs) which has either activator or repressor function and some of them possess protein binding activity (Olsen *et al.*, 2005; Puranik *et al.*, 2012).

	1	10	20	30	40	50	60
ICERI-4	MGEAEASAGGSLVFRGCOLPPGFRFPQTDQEIIVCYLKKKIDAATAVTSIIADVDIYKFD						
ICERI-5	MGEAEASAGGSLVFRGCOLPPGFRFPQTDQEIIVCYLKKKIDAATAVTSIIADVDIYKFD						
ICERI-6	MGEAEASAGGSLVFRGCOLPPGFRFPQTDQEIIVCYLKKKIDAATAVTSIIADVDIYKFD						
ICERI-10	MGEAEASAGGSLVFRGCOLPPGFRFPQTDQEIIVCYLKKKIDAATAVTSIIADVDIYKFD						
'Yugul'	MGEAEASAGGSLVFRGCOLPPGFRFPQTDQEIIVCYLKKKIDAATAVTSIIADVDIYKFD						
ICERI-4	PWDLFDKAMFGDGEWFFFSFDRKYPNGARNRAGSGYWKATGTDKPIILASGRCLGVK						
ICERI-5	PWDLFDKAMFGDGEWFFFSFDRKYPNGARNRAGSGYWKATGTDKPIILASGRCLGVK						
ICERI-6	PWDLFDKAMFGDGEWFFFSFDRKYPNGARNRAGSGYWKATGTDKPIILASGRCLGVK						
ICERI-10	PWDLFDKAMFGDGEWFFFSFDRKYPNGARNRAGSGYWKATGTDKPIILASGRCLGVK						
'Yugul'	PWDLFDKAMFGDGEWFFFSFDRKYPNGARNRAGSGYWKATGTDKPIILASGRCLGVK						
ICERI-4	KALVFYQGRSPKGTKTHVMHEYRLDAPMSSNSMRLLDQVLCRVNRKQDQLLPVDHGY						
ICERI-5	KALVFYQGRSPKGTKTHVMHEYRLDAPMSSNSMRLLDQVLCRVNRKQDQLLPVDHGY						
ICERI-6	KALVFYQGRSPKGTKTHVMHEYRLDAPMSSNSMRLLDQVLCRVNRKQDQLLPVDHGY						
ICERI-10	KALVFYQGRSPKGTKTHVMHEYRLDAPMSSNSMRLLDQVLCRVNRKQDQLLPVDHGY						
'Yugul'	KALVFYQGRSPKGTKTHVMHEYRLDAPMSSNSMRLLDQVLCRVNRKQDQLLPVDHGY						
ICERI-4	SSSSSEPTTAAADIVSSSSSEVLPDSSSAFADIHWNDDHLLRYLIGGGSGNPFSTA						
ICERI-5	SSSSSEPTTAAADIVSSSSSEVLPDSSSAFADIHWNDDHLLRYLIGGGSGNPFSTA						
ICERI-6	SSSSSEPTTAAADIVSSSSSEVLPDSSSAFADIHWNDDHLLRYLIGGGSGNPFSTA						
ICERI-10	SSSSSEPTTAAADIVSSSSSEVLPDSSSAFADIHWNDDHLLRYLIGGGSGNPFSTA						
'Yugul'	SSSSSEPTTAAADIVSSSSSEVLPDSSSAFADIHWNDDHLLRYLIGGGSGNPFSTA						
ICERI-4	SASAVAAGHDNYAPPHPHALSVLESIKRNLFSQAIDELYLLQPPSKRANCIAAAGD						
ICERI-5	SASAVAAGHDNYAPPHPHALSVLESIKRNLFSQAIDELYLLQPPSKRANCIAAAGD						
ICERI-6	SASAVAAGHDNYAPPHPHALSVLESIKRNLFSQAIDELYLLQPPSKRANCIAAAGD						
ICERI-10	SASAVAAGHDNYAPPHPHALSVLESIKRNLFSQAIDELYLLQPPSKRANCIAAAGD						
'Yugul'	SASAVAAGHDNYAPPHPHALSVLESIKRNLFSQAIDELYLLQPPSKRANCIAAAGD						
ICERI-4	DDDDHIQILSPTSFSISEADEMF						
ICERI-5	DDDDHIQILSPTSFSISEADEMF						
ICERI-6	DDDDHIQILSPTSFSISEADEMF						
ICERI-10	DDDDHIQILSPTSFSISEADEMF						
'Yugul'	DDDDHIQILSPTSFSISEADEMF						

Figure 3. Identification of conserved domain found in SiNAC65 proteins. The NAC domain located in the N-terminal was boxed and the shaded amino acid sequences was predicted as Nuclear Localization Signal.

Phylogenetic analyses and gene functional studies have clearly classified the stress-responsive NAC proteins into one group, the stress-responsive NAC (SNAC) group (Fang *et al.*, 2008; You *et al.*, 2015). Experimental evidences are available for some members of the SNAC group that demonstrated their functions in abiotic stress responses. For example, over-expression of *SNAC1* from rice improved drought and salt tolerance in rice (Hu *et al.*, 2006), cotton (Liu *et al.*, 2014), and ramie (An *et al.*, 2015), while the over-expression of *SNAC2* increased salt and cold tolerance in transgenic rice (Hu *et al.*, 2008) and the over-expression of *ATAF1* from *Arabidopsis* also enhanced drought tolerance in transgenic *Arabidopsis* (Wu *et al.*, 2009). Puranik *et al.* (2013) reported the identification of 147 putative NAC domain-encoding genes from foxtail millet and distributed them into 11 distinct sub-families. Phylogeny-based function prediction in that study indicated that the member of sub-family VII probably involved in similar regulatory roles as the SNAC orthologous in other species. *SiNAC065* is one member of the 18 members of sub-family VII which was up-regulated in the early stress of drought and salinity, indicating the role of this gene in drought and salinity stress responses.

A study utilized foxtail millet cultivars with different tolerance to salinity, cv. Prasad (salt-tolerant) and cv. Lepakshi (salt-sensitive), showed that the salt-tolerant cultivar had better salt-induced-oxidative tolerance due to increased total superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity, whereas both enzyme activities decreased in the salt-sensitive cultivar (Sreenivasulu *et al.*, 2001). It is long-known that the increased of SOD and CAT activities correlates with lower oxidative stress induced by various abiotic stress (Dhindsa *et al.*, 1981). The overexpression of *JUB1*, one of NAC TFs, dampened intracellular H_2O_2 levels and enhances tolerance to various abiotic stresses (Wu *et al.*, 2012). Those studies indicate the possible role of NAC TFs in improving tolerance to salt stress by escalating antioxidant components in the tolerant genotypes. Whether or not *SiNAC065* is involved in antioxidant component modulation still need to be further elaborated.

Table 1. Conserved motifs identified in SiNAC065 protein.

Motif No.	Amino acid sequence composition of motif	Amino acid span	NAC sub-domain
1	W[YF]FF[SC]P[RK]DRKYP[TN]GSR[TP]NRAT	76-96	C
2	LPPGFRFHPTDEEL[VI] _x [HYF]	19-32	A
3	P[KR]G _x [KR]T[GD]W-[VI]MHEYRL	130-145	D
4	IA[ED]VD[LI][YN][KR]C[ED]PW[DE]LP[ED]KAKIG	50-68	B
5	[VI] _{xx} GGRL[VI]GM[KR]KTLVFYRGRA	118-130	D
6	xx[DE][DE]WV[LV]C[RK][VI][FY]K[KS]PR	160-171	E
7	[SA]G[YF]WKATGKD[-KR]	98-107	C
8	YL[RK]RK[VA]Agx-[PR][LI]PLD[VI]	36-41	-

The amino acid sequences were aligned with the reference sequence (Phytozome identifier: Si012319m) and conserved motifs were identified according to Puranik *et al.* (2013).

Characterization of genes associated with abiotic stress would be useful to develop efficient breeding strategy for tolerant variety, such as through genetic engineering (Turan *et al.*, 2012) and molecular marker assisted breeding (Ashraf and Foolad, 2013). Previous studies reported transgenic plants overexpressing NAC gene exhibited enhanced tolerance to salinity stress (Huang *et al.*, 2015; Hong *et al.*, 2016; Shinde *et al.*, 2019). Study conducted by Widyawan *et al.* (2018) utilized single nucleotide polymorphism (SNP) at the 558th base of *SiDREB2* gene, a TF gene which tightly associated with abiotic stress responses, to develop SNAP marker related to drought stress in foxtail millet. Similar approaches can be performed utilizing *SiNAC065* gene. As a conclusion, this study has successfully isolated *SiNAC065* genes from foxtail millet genotypes which differ in their salinity tolerance. Different features of *SiNAC065* isolated from the tolerant and the sensitive-genotypes should provide information of the gene's role in salinity tolerance mechanism of foxtail millet.

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