Isolation and Characterization of *OsNAC6* cDNA from Rice (*Oryza sativa* L.) cv. Nipponbare, Batutegi, and Rojolele

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Abstract

Transcription factors have an important function in regulating gene expression and plant responses to stresses. The *ERF*, bZIP, *WRKY*, *MYB*, and *NAC* are stress inducible transcription factors. The *OsNAC6* is a member of the *NAC* transcription factor family in rice and its expression is induced by abiotic stresses, wounding and blast disease. Characterization of *OsNAC6* gene sequences would give a better undersanding on how *OsNAC* gene functions biologically. The objectives of this research are to isolate the *OsNAC6* cDNA from Nipponbare, Batutegi, and Rojolele cultivars, to characterize their DNA sequences, and to compare their sequences to other *NAC* genes from other plants available in GenBank DNA databases. Isolated cDNA and sequencing of the fragments resulted in a 912 bp DNA sequences. Translation of the sequences yielded a protein consisted of 303 amino acid residue. Blast analysis of amino acid sequences indicated identity of isolated cDNA from three Indonesian rice cultivars are the *OsNAC6* gene. Deduced amino acid residues from amplified cDNAs of Nipponbare, Batutegi, and Rojolele cultivars shared 100% sequence identities to rice *OsNAC6* (Acc. # BAA89800), 71-100% sequence identity to a number of *OsNAC* protein from *Oryza sativa* and 63-83% sequence identity to NAC protein from other plants.

Key words: transcription factor, stress responsive gene, abiotic stress, NAC

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Introduction

Transcription factors are proteins that involved in the regulation of gene expressions. It binds to the promoter region of the gene and can assist or inhibit the transcription of the gene (Nuruzzaman *et al*., 2013). Transcription factors are master regulators that control gene clusters. A single transcription factor can control the expression of many target genes through specific binding of the transcription factors to the *cis*-acting element in the promoters of respective target genes (Nakashima *et al*., 2007). Plants have many families of transcription factors, such as protein *ERF*, bZIP, *WRKY*, *MYB* and *NAC.*

The *NAC* gene family is one of the transcription factor family found only in plants (Riechmann *et al*., 2000). There are at least 151 members of *NAC* genes in rice and 117 members in *Arabidopsi*s (Nuruzzaman *et al*., 2013). The name of *NAC* is derived from the three transcription factors: (i) ***N****AM* from *Petunia*, (ii) ***A****TAF1*-2 from *Arabidopsis* and (iii*)* ***C****UC2* from *Arabidopsis* (Souer *et al*., 1996; Aida *et al*., 1997). The characteristic of *NAC* protein is indicated by its consensus sequence known as an *NAC* domain which is located in the N-terminal region and in the highly variable C-terminal domain.

The *NAC* transcription factors appear to control biochemical and molecular pathways that can protect plants from different stress conditions. The *NAC* transcription factors are multi-functional proteins with various roles in the plant life cycle, such as maintenance of the shoot apical meristem (Souer *et al*., 1996; Kim *et al*., 2007b), cotyledon development (Aida *et*

al., 1997), lateral root development (He *et al*., *et al*., 2005), flower formation (Sablowski & Meyerowitz, 1998), hormone signaling (Greve *et al*., 2003), response to pathogen infection (Xie *et al*., 1999; Ren *et al*., 2000; Olsen *et al*., 2005; Nakashima *et al*., 2007), plant organ senescence (Liu *et al*., 2009), embryo development (Duval *et al*., 2002), response to different abiotic stresses (Tran *et al*., 2004; He *et al*., 2005; Hu *et al*., 2006; Bhatnagar-Mathur *et al*., 2007; Nakashima *et al*., 2007; Yoo *et al*., 2007), formation of secondary walls (Zhong *et al*., 2007), cell division (Kim *et al*., 2006), fiber development (Ko *et al*., 2007), seed development (Sperotto *et al*., 2009), and senescence (Uauy *et al*., 2006).

The *NAC* domain are divided into five subdomains, i.e. Subdomain A, B, C, D, and E (Ooka *et al*., 2003). The NAC domain is required for DNA-binding ability. Meanwhile, a highly variable C-terminal domain played a major role in the regulation of transcription (Olsen *et al*., 2005). This variable C-terminal domain of *NAC* proteins generally operates as a functional domain and acts as a transcriptional activator or repressor (Tran *et al*., 2004; Hu *et al*., 2006; Kim *et al*., 2007a). This variable domain is very large and has protein binding activity. Kim *et al.* (2007b) reported that the C-terminal domain of calmodulin-binding *NAC* can bind with calmodulin proteins.

Family of *ATAF* gene is responsive to drought (Ooka *et al*., 2003). The *ATAF1* and *ATAF2* genes from *Arabidopsis* are both induced by wounding (Collinge *et al*., 2001). Expression of *OsNAC6* is induced by abiotic stresses, including cold, drought and high salinity (Nakashima *et al*., 2007; Ohnisi *et al*., 2005). In transgenic rice, the *OsNAC6* and *OsNAC10* genes were found to enhance drought and salt tolerance (Nakashima *et al*., 2009; Jeong *et al*., 2010). Overexpression of *OsNAC6* transgene in rice cv. Ciherang improved the growth of the transgenic lines under PEG induced stress and salinity treatments. Moreover, it also enhances the expression of other transcription factor-related drought and salinity stress responses, such as Zincfinger protein, MYB and AP2 (Rachmat *et al*., 2014). The *OsNAC6* gene, a member of the *NAC* transcription factor gene family in rice, is a member of the *ATAF* gene family.

Characterization of the genes associated with important traits in plants can be used to evaluate the nucleotide sequence heterogeneity of the genes. To evaluate the nucleotide sequence heterogeneity of *NAC6* gene of Japonica and Tropical Japonica rice cultivars, the isolation and sequence characterization of *OsNAC6* from those varieties are required. Therefore, the objectives of this study are to characterize the diversity of the *OsNAC6* gene sequences derived from Niponbare (Japonica rice), Batutegi, and Rojolele rice cultivars (Javanica rice).

Materials and Methods

Plant materials, RNA isolation and cDNA synthesis. The total RNA was isolated from the leaves of 2 month-old of Nipponbare, Batutegi, and Rojolele using TRizol (Invitrogen). The total RNA was further used to synthesize the cDNA library using cDNA sintesis kit with oligo-d(T) primer (Invitrogen).

Primer design. Primer pairs used to amplify the target cDNA were designed based on DNA sequences of Nipponbare *OsNAC6* available in NCBI GenBank DNA database (http//www. ncbi.nlm.nih.gov). The nucleotide sequences of forward primer were 5'-TAGAGATCTGATGAGCGGCGGTCAGGA CCTGCA-3' and those of the reverse primer were 5'-AGGGTCACCTAGAATGGCTTGC CCCAGTACATGAG-3'.

Cloning and sequencing. The RT-PCR amplification products were purified using Gel/PCR DNA Fragments Extraction Kit. Purified products were cloned into pGEM-T Easy Vector Systems (Promega) and then transformed into DH-5α competent cells by heat shock method (Sambrook *et al*., 2001). Bacterial cells carrying the recombinant plasmid were selected on an LB plate containing Ampicillin (50 µg/ml), X-gal (50 mg/l) and IPTG (0.1 M). The cultures were incubated at 37°C overnight. Single white colony was picked and inoculated into liquid LB medium containing ampicillin and grown overnight with vigorous shaking. Recombinant plasmids were extracted and purified using High-Speed Plasmid Mini Kit (Geneaid, Taiwan) and sent to Eijckman Institute for DNA sequencing.

The sequences of *OsNAC6* genes. The *OsNAC6* gene sequences isolated from rice cv. Nipponbare, Batutegi, and Rojolele determined in this research were evaluated against those of the *NAC* sequences available in the NCBI GenBank DNA database (http://www.ncbi.nlm.nih.gov). Identification of the NAC accessions in the DNA database was done using BLAST program.

Alignment and phylogenetic analysis. Vector sequences were removed manually from the raw sequence data. The sequences were compared with the available sequences in the NCBI GenBank DNA database using BLASTp (Altschul *et al*., 1997). Multiple sequence allignment (MSA) of the deduced amino acid translated from the determined DNA sequences in this research and those from database were done using ClustalW (Larkin *et al*., 2007). The multiple alignments were graphically displayed using GeneDoc (Nicholas *et al*., 1997). A neighbor-joining (NJ) phylogenetic tree, drawn with MEGA 5.0 (Tamura *et al*., 2011) was subsequently generated based on the MSA to reveal relationship among the compared sequences. Bootstrap analysis was conducted using 1,000 replicates.

Results and Discussions

Amplification of *OsNAC6* gene using *OsNAC6* specific primers and rice cDNA libraries of Rojolele, Nipponbare, and Batutegi cultivars as templates in the RT-PCR reaction yielded an amplicon of approximately 900 bp in all three tested cultivars (Figure 1). PCR product was used for further analysis.

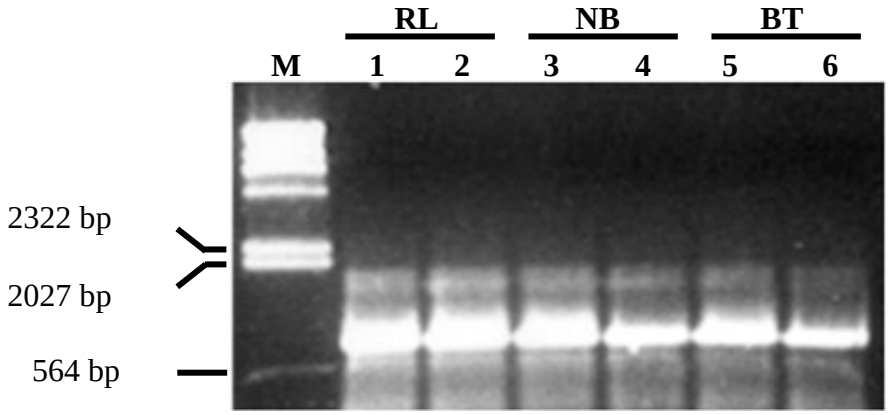

Figure 1. Amplification products obtained by RT-PCR using *OsNAC6* specific primer and rice cDNA of three rice cultivars as templates. Lane M: λ*Hind*III DNA marker, Lane 1-2: Rojolele (RL), 3-4: Nipponbare (NB), and 5-6: Batutegi (BT).

Sequence analysis

Based on the presence of five conserved motifs in deduced amino acid residues located in the N-terminal region, the cDNA isolated from Rojolele, Batutegi, and Nipponbare were proven as the fragments of *NAC*6 gene (Figure 2). BLASTp analysis revealed that those shared significant amino acid residue identities to a number of NAC proteins from other plants deposited in NCBI GenBank database. The deduced amino acid isolated from cDNA of Nipponbare, Batutegi, and Rojolele shared 100 % sequence identity to rice *OsNAC6* (BAA89800), 71-99% sequence identity to OsNAC protein from *Oryza sativa* (Table 1) and 63-83% sequence identity to NAC protein from other plants (Table 2).

Multiple Sequence Alignment analysis of the deduced amino acid sequences of cDNA fragments isolated from Nipponbare, Batutegi, and Rojolele cultivars and 9 *NAC* proteins from other plants deposited in NCBI GenBank database revealed that the presence of conserved domains located in the N-terminal region were higher than in C-terminal region (Ernst *et al*., 2004). Differences of amino acid residues in the C-terminal region produced differences in the NAC protein length. The N-terminal regions are highly conserved with around 150-160 of amino acids. These regions was divided into five subdomains (A-E) and served as a DNA-binding domain (Kikuchi *et al*., 2000) (Figure 3).

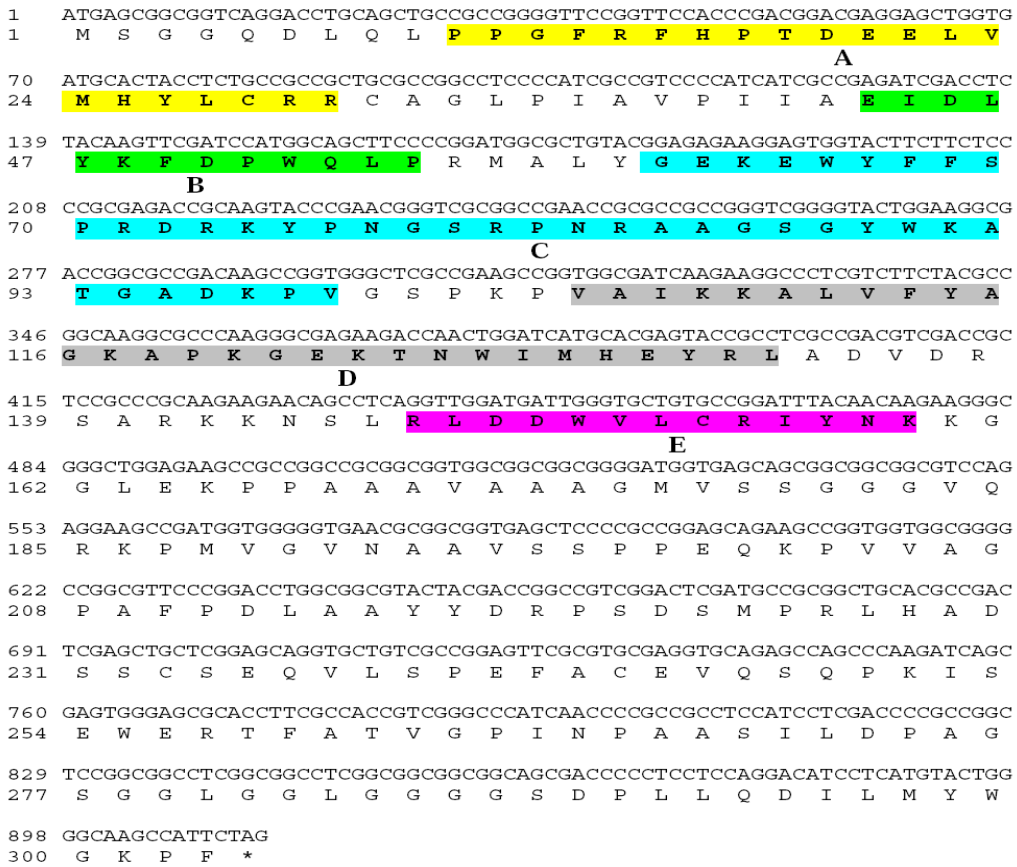

Figure 2. Nucleotide sequences and predicted amino acid residues of a PCR amplified product of rice cv. Rojolele using *NAC6* specific primers. PCR amplified products from rice cv. Rojolele, Nipponbare, and Batutegi also have the same nucleotide sequences. A typical conserved domain associated with NAC protein at the N-terminal are highlighted. There are five subdomains (A-E) within the conserved region as indicated by different colors.

Table 1. Results of BLASTp analysis to find the similarity among amino acid residue of the PCR amplified product to those of available protein from rice in the GenBank DNA database

Accession	Description	Query coverage (%)	E value	Identity (%)
BAA89800	OsNAC6 protein [*Oryza sativa*]	100	0	100
AP003561.2	*Oryza sativa* Japonica Group genomic DNA, chromosome 1, BAC clone:B1065E10	100	0.0	100
EU846993.1	*Oryza sativa* Japonica Group clone KCS089G05 NAC6 protein mRNA, complete cds	100	0.0	99
NM_001072451.1	*Oryza sativa* Japonica Group Os11g0184900 (Os11g0184900) mRNA, complete cds	44	4e-131	88
AK102475.1	*Oryza sativa* Japonica Group cDNA clone:J033094I01, full insert sequence	44	4e-131	88
AK063399.1	*Oryza sativa* Japonica Group cDNA clone:001-114-H12, full insert sequence	44	4e-131	88
AC124836.2	*Oryza sativa* Japonica Group chromosome 5 clone OSJNBb0092E21, complete sequence	97	5e-121	96
NM_001051152.2	*Oryza sativa* Japonica Group Os01g0816100 (Os01g0816100) mRNA, complete cds	51	1e-101	81
AY986504.1	*Oryza sativa* (japonica cultivar-group) NAC protein mRNA, complete cds	51	1e-101	81
AK064292.1	*Oryza sativa* Japonica Group cDNA clone:002-105-H07, full insert sequence	25	8e-84	91
JN634071.1	*Oryza sativa* Japonica Group secondary wall NAC transcription factor 2 mRNA, complete cds	41	9e-14	71%

Table 2. Results of BLASTp analysis to find the similarity among amino acid residue of the PCR amplified product to those of available NAC protein from various plants in the GenBank DNA database

Accession	Description	Query coverage (%)	E value	Identity (%)
AAW62955	*NAC*23 [*Saccharum officinarum*]	99	1e-170	83
ABY67929	*NAC*-like protein [*Zea mays*]	100	1e-166	82
NP_001266607	*NAC* domain-containing protein 48 [*Zea mays*]	92	2e-151	81
ADE59453	*NAC* transcription factor 6B [*Triticum aestivum*]	100	8e-164	79
CAM57978	*NAC* transcription factor [*Hordeum vulgare* subsp. vulgare]	100	2e-165	75
EXB60108	*NAC* domain-containing protein 2 [*Morus notabilis*]	98	2e-129	66
NP_001240958	*NAC* domain-containing protein 2-like [*Glycine max*]	99	2e-129	64
AGL39681	*NAC* transcription factor 025 [*Jatropha curcas*]	100	7e-125	63
AGJ76628	*NAC* domain protein *NAC*3 [*Medicago sativa*]	98	2e-120	63

Meanwhile, the C-terminal region which is acts as a transcriptional activation regions (TARS) have various motifs depend on the *NAC* subfamily (Olsen *et al*., 2005; Tran *et al*., 2004; Hu *et al*., 2006; Kim *et al*., 2007b; Xie *et al*., 1999; Duval *et al*., 2002). The results also showed that the cDNA fragment obtained from this study have EVQSQPK motifs in the TARS region (Figure 3).

The *NAC* family is divided into three of major subfamily, namely *NAM* (non-apical meristems), *CUC* (cup-shaped cotyledons) and *ATAF* (*Arabidopsis* Transcription Activation Factor) (Souer *et al*., 1996; Aida *et al*., 1997). The NAM determines the position of the apical meristem and the *CUC* determine the formation of cotyledons, sepals, petals and meristem formation (Kikuchi *et al*., 2000; Olsen *et al*., 2005). Meanwhile, those in the *ATAF* subfamily are associated with the responses to biotic and abiotic stresses (Seki *et al*., 2003). *OsNAC6* is a member of the *ATAF* gene family.

The *OsNAC6* gene isolated from monocots is clustered into different clade to those from dicots. The results of this evaluation is in line with that finding since the *NAC* genes from rice cv. Rojo Lele, Nipponbare, and Batutegi belongs to the same clade as the NAC6 from other monocots (*Hordeum vulgare*, *Triticum aestivum*, *Saccharum officinarum* and *Zea Mays*) and in separate clade to ones isolated from dicots (Figure 4).

To date, over 150 fragments of the transcription factor genes were found from rice and had been registered in the NCBI GenBank database and the Plant Transcription Factor Database.The phylogenetic analysis of three predicted amino acid *OsNAC6* gene fragment (*OsNAC6*_RL, *OsNAC6*_BT, *OsNAC*_NB) compared to 114 of *NAC* protein from rice, *Triticum aestivum*, *H. vulgare*, and *Tamarix hispida* shown in Figure 5. The dendogram depicted that all of the *OsNAC6* obtained during this study are originally sub-clustered with rice (Os01g66120, Os05g34830), *T. aestivum*, *H. vulgare* with identity around 73.7-100%. The dendogram in Figure 5 also showed that *OsNAC6* has a conserved motif of sub family *ATAF*, along with 15 other proteins including *OsNAC4* and *OsNAC5* which incorporated in subgroups of *SNAC* (STRESS-responsive NAC) (Nuruzzaman, 2013).

The *NAC* genes from rice, sugarcane, *Arabidopsis*, and wheat were included in the sub group of *SNAC* in general which contribute to respond to the abiotic stresses (Fang *et al*., 2008). Research by Nuruzzaman *et al.* (2010) showed that some members of the subgroup *SNAC* also respond to the presence of rice virus infection. Study by Hegedus *et al.* (2003), Nakashima *et al.* (2007) and Oh *et al.* (2005) also proved that the genes in the subgroup of *SNAC* also play an important role toward biotic and abiotic stresses. In general, these genes are marked by a common amino acid motif that is downstream of the five conserved domains (A-E) of the *NAC*, which is WVLCR (Figure 6).

Studies have shown that a transcription factor can have several different signaling pathways function (Nakashima *et al*., 2009). Transcription factors and *cis*-elements function in the promoter region of different stress-related genes, and the overexpression or suppression of these genes may improve the plant's tolerance to biotic and abiotic stresses (Nuruzzaman *et al.*, 2013). For example, The *OsNAC6* was induced by jasmonic acid (JA), a plant hormone activating the defense

mechanisms against biotic stress, such as: herbivores and pathogens (Ohnishi *et al*., 2005). In addition, the OsNAC6 expression was also induced by abiotic stresses such as drought, high soil acidity, and low temperature stresses (Takasaki *et al*., 2010; Shindu *et al*., 2008).

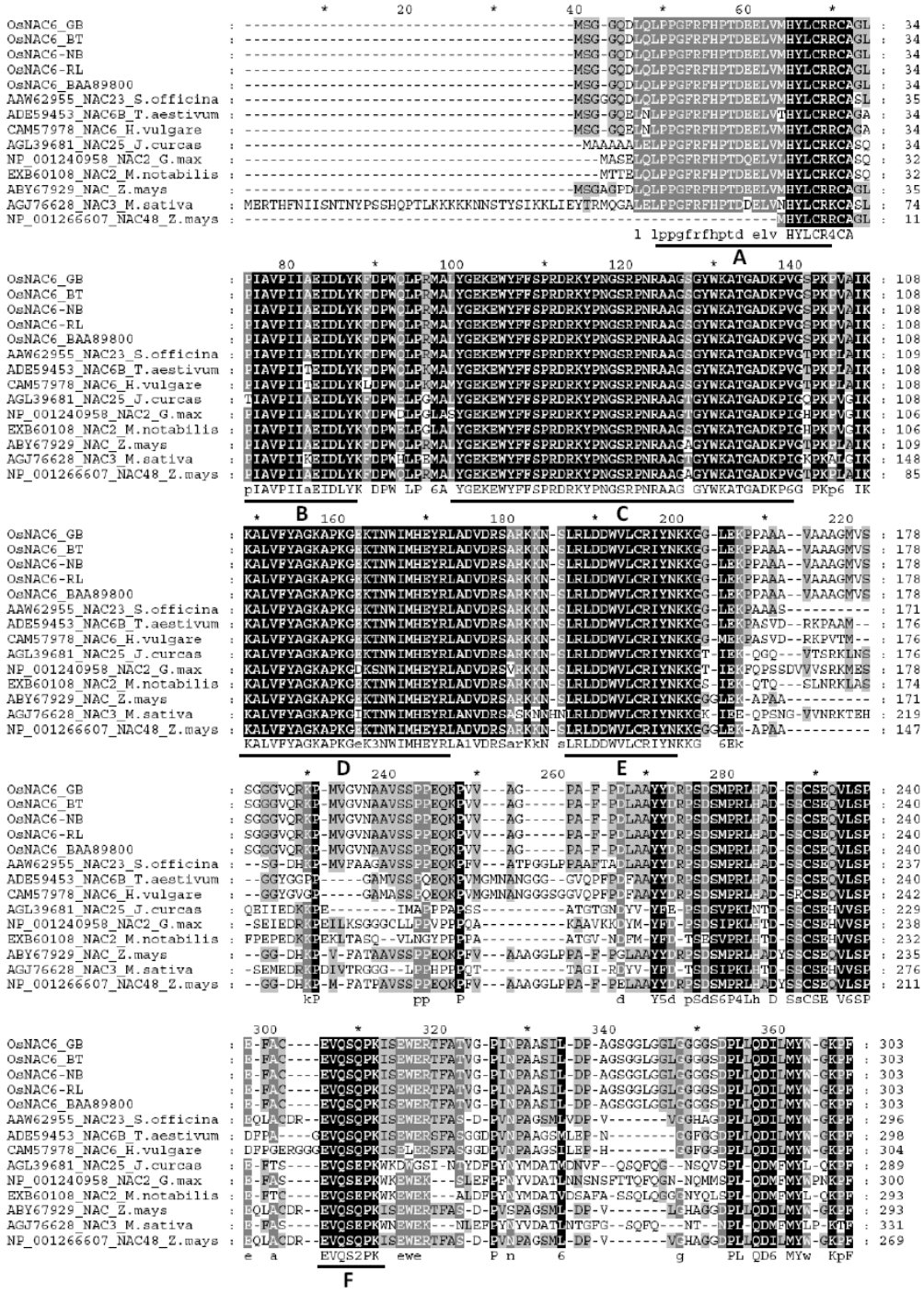

Figure 3. Alignment analysis of cDNA fragment from Rojolele (*OsNAC6*_RL), Batutenggi (*OsNAC6*_BT), Nipponbare (*OsNAC6*_NB) compared to *OsNAC6* and *NAC* gene deposited in the NCBI GenBank database. The conserved domain of the *NAC* gene are designated by A, B, C, D and E, while the transcription activating region is indicated by F.

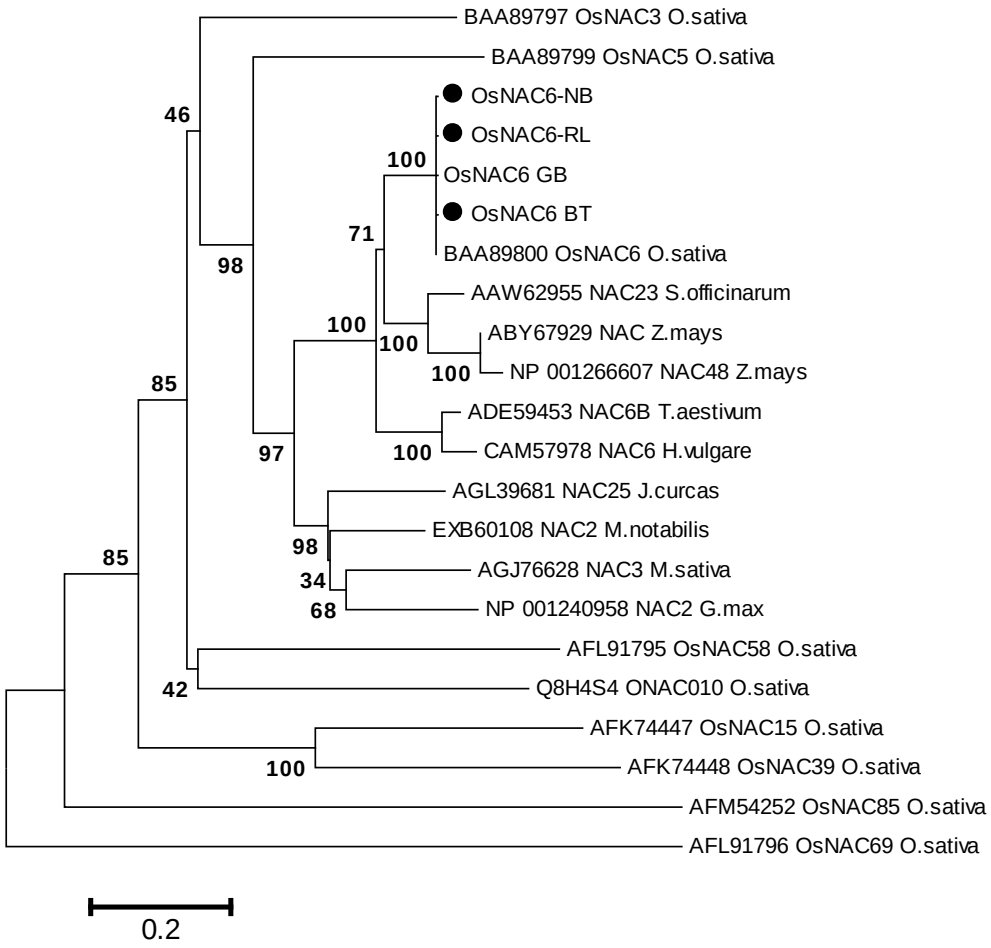

Figure 4. Phylogenetic analysis of deduced amino acids from cDNA fragment of Rojolele (*OsNAC6*_RL), Batutenggi (*OsNAC6*_BT) and Nipponbare (*OsNAC6*_NB) and 19 *NAC* genes available from NCBI GenBank DNA database. The alignment was done using ClustalW and the phylogenetic was constructed using Neighbour Joining methods. Bootstrap values shown in the axis were based on 1000 replicates. The existing scale represents the average of amino acid substitutions per site.

Nilsson *et al*. (2010) study showed Os04g0477300 (one of the *NAC* gene) functions in 3 different processes, namely: defense against pathogens, senescence and response to both phosphate and boron deficiency. Function of the other *NAC* genes in plants is affected by auxin, ethylene (*AtNAC2*) and ABA (*OsNAC*5; Xie *et al*., 1999; He *et al*., 2005; Sperotto *et al*., 2009). Results of those studies indicated that a single NAC gene (Os04g0477300) can function as regulator of several different processes, such as: defense against pathogens, senescence and response to both nutrient deficiency, and that the NAC can mediate cross communication between the signaling processes, such as: auxin, ethylene, and ABA responses.

The *SNAC* group has a highly conserved motif within regions outside the conserved domain (Figure 6). A 28-amino acid motif, RSARKKNSLRLDDWVLCRIYNKKGGLE K in *OsNAC*, is found at the amino-terminal to the conserved DNA-binding domain (WVLCR) in monocots and in dicots (Nuruzzaman *et al*., 2012). We first identified putative conserved motifs outside of the *NAC* domain in rice and compared with those of *T. aestivum* and *H. vulgare*. Outside of the NAC domain, rice specific conserved motifs were detected (Nuruzzaman *et al*., 2012). These conserved motifs most likely to be involved in gene expression activation or perhaps in the control of protein stability (Nuruzzaman *et al*., 2013). It is notable that only some of these motifs are conserved in both dicots and monocots, suggesting that protein function has both diverged and conserved even within this evolutionarily conserved NAC family.

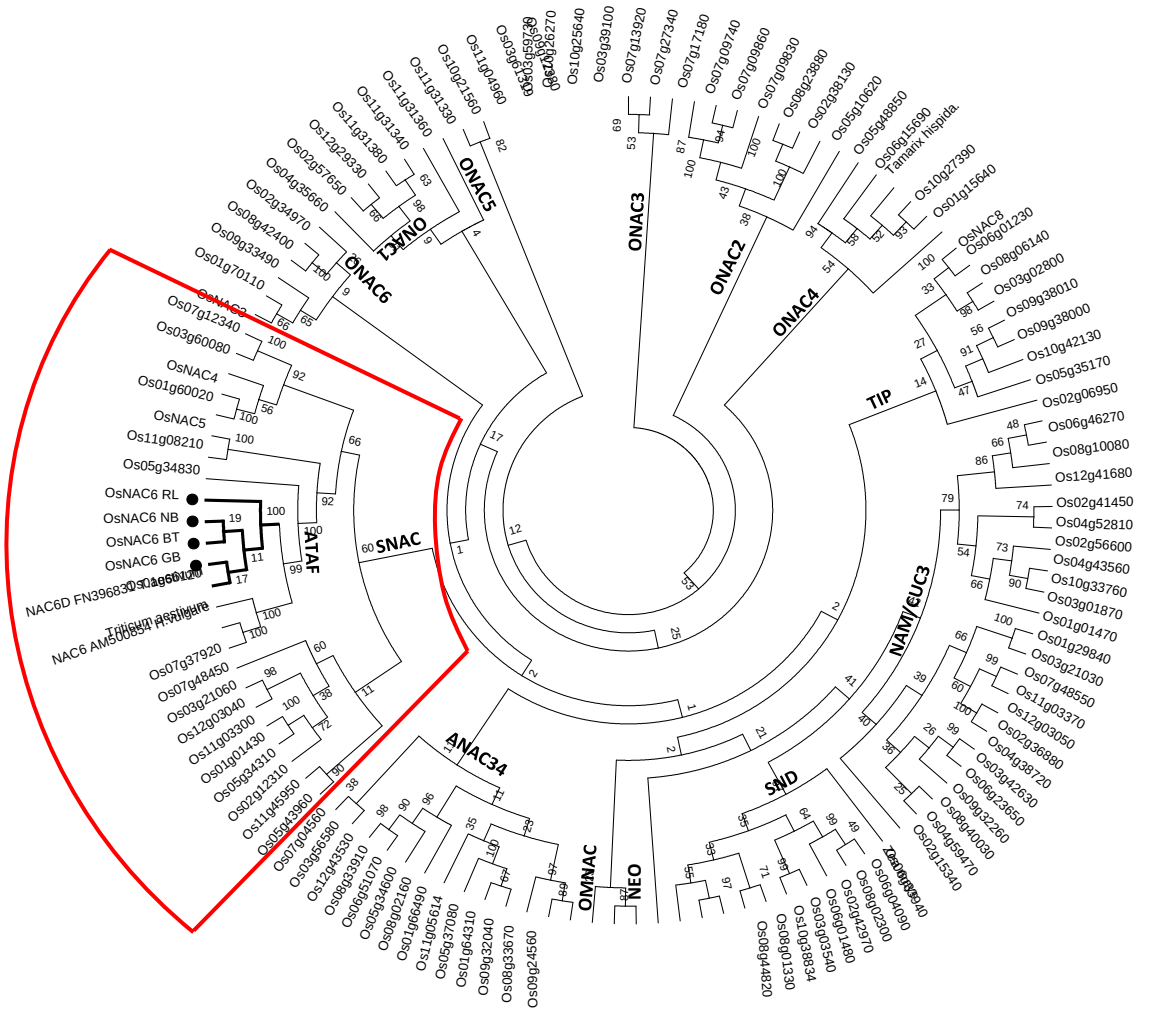

Figure 5. Phylogenetic analysis of predicted amino acids of *OsNAC* isolated and sequence in this experiment along with the *NAC* genes from *Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare* and *Tamarix hispida*. Most of the *NAC* genes used to generate phylogram were either from NCBI Genbank DNA database or Plant Transcription Factor database. The phylogenetic tree was constructed using *unrooted* Neighbour Joining method. Bootstrap values shown in axis were calculated based on 1,000 replicates.

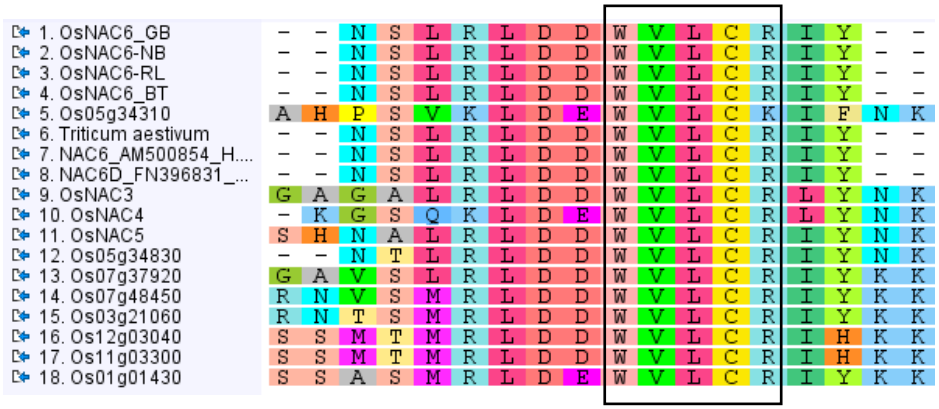

Figure 6. Conserved motif outside the *NAC* domain in the *SNAC* group of *Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare* (*OsNAC6*_NB, *OsNAC*_RL, *OsNAC6*_BT are generated in this study, other NAC sequences are from NCBI DNA database)

Conclusions

We have amplified cDNAs using NAC specific primer pairs from cDNA libraries of three rice cultivars (Nipponbare, Batutegi, and Rojolele). Sequencing of the cDNA fragments resulted in a 912 bp DNA sequences. Translation of the sequences yielded a polipeptide consisted of 303 amino acid residues. Blast analysis of amino acid sequences indicated identity of isolated cDNA is *OsNAC*. Deduced amino acid residues from amplified cDNAs shared 100% sequence identities to rice *OsNAC6* (Acc. # BAA89800), 71-100% sequence identity to a number of *OsNAC* protein from *O. sativa* and 63-83% sequence identity to NAC protein from other plants.

Future Prospects

This bioinformatics study of *OsNAC6* can be useful for genetic engineering of stress-tolerant plants. Furthermore, molecular characterization of *OsNAC6* expression under different stresses will clarify the mechanisms that are controlled by *OsNAC6* proteins in plants. Such understanding could bring economical benefits to agricultural production.

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