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Cloning, expression and characterization of drought responsive NAC transcription factor from *Ziziphus nummularia*

Jasdeep Chatrath Padaria*, Radha Yadav, Akhilesh Kumar and Kundan Kumar

ABSTRACT: *The NAC gene family encodes plant-specific transcription factors which are involved in plant developmental processes as well as in response to environmental stresses. In the present study, a drought stress-inducible NAC gene, from Ziziphus nummularia named ZnNAC, was isolated, cloned, characterized and its expression analysis was performed in prokaryotic system. Real-time qPCR expression analysis showed up-regulation of ZnNAC in Ziziphus nummularia under drought condition induced by 30% PEG treatment. Drought stress resulted in 4.83 and 74.02 fold increase in ZnNAC transcript levels after 6 and 48 hours treatment respectively. The involvement of ZnNAC in drought stress tolerance was further investigated by cloning ZnNAC gene in bacterial expression vector pET28a and transformation in Escherichia coli BL21 (DH3). The apparent molecular weight of recombinant protein was found 44.1 kDa as evident from SDS-PAGE analysis. The functional validation of ZnNAC was performed in Escherichia coli by growth curve analysis. The E coli cells transformed with pET28a::ZnNAC was showed better survival and higher growth rates under water stress (10% PEG) conditions when compared to control cells (BL21/ pET28a). Functional validation of ZnNAC protein for drought tolerance in E coli cells suggests its suitability as candidate gene for development of abiotic stress tolerant transgenics.*

Keywords: *Abiotic stress, ZnNAC, qRT-PCR, Bacterial expression.*

INTRODUCTION

Plants are always exposed to several biotic (bacteria, fungi, virus, insect, pest etc.) and abiotic stresses (heat, drought, cold and salinity) during their whole life cycle. Both kind of stresses adversely affect plant's developmental growth and agriculture productivity, and cause more than 50% of worldwide yield loss for major crops every year [1, 2]. In response to abiotic stresses, plants activate number of defense mechanisms which provide them tolerance to the adverse conditions imposed by such stresses [3]. This inducible adaptation or acclimation process evolved throughout the plant life cycle and is critical for plant existence. Foremost step in response to stresses is the perception and transduction of stress signals through signaling component, which results in the activation of numerous stress related genes [4]. Abiotic stress doesn't not only weaken the defense mechanism of plants but also increase their susceptibility to pathogen infection [5, 6]. To date, a large array of stress responsive genes have been identified in many plant system, which can be classified in two group; one is functional genes encoding important enzymes and metabolic proteins (functional protein), such as detoxification enzyme, water channel, late embryogenesis abundant (LEA) protein, which directly function to protect cells from stresses. The second is regulatory genes encoding various protein kinase, which regulate signal transduction and gene expression under stress condition. In the signal transduction processes, transcriptional factors (TFs) play important roles in the conversion of stress signal perception to stress responsive gene expression. The expression of stress related genes is largely regulated by specific transcription factors. In plants, many families of transcription factors, including AP2/ERF. MYB, MYC, bZIP, HSF, NAC, WRKY and zinc-finger TFs play important role in eliciting stress response by regulating the expression of stress-responsive genes under different stress conditions [7, 8]. TFs and their interacting cis-elements function in the promoter region of different stress related genes acting as molecular switches for gene expression [9].

Among the transcription factors, NAC family proteins are one of the largest family containing plant

* (Principal Scientist), National Research Centre on Plant Biotechnology, New Delhi – 110012, India, *E-mail: jasdeep_kaur64@yahoo.co.in*

specific transcription factors. The NAC acronym is derived from three earliest characterized proteins with a particular domain (NAC domain) from petunia NAM (no apical meristem), *Arabidopsis* ATAF1/2 (*Arabidopsis* transcription activation factor) and CUC2 (cup-shaped cotyledon). Large numbers of putative NAC genes have been identified in many sequenced species at genome-wide scale, such as 117 in *Arabidopsis*, 151 in rice, 152 in soyabean and maize, 201 in chinese cabbage and so on. The NAC family has been found to function in various processes including shoot apical meristem [10], flower development [11], cell division [12], leaf senescence [13], formation of secondary walls [14], and biotic and abiotic stress responses [15-18]. However, the evidence for the direct involvement of *Ziziphus* NAC transcription factor under abiotic stresses is not well studied. Therefore, it is important to determine the biological functions of novel ZnNAC protein which may impart abiotic stress tolerance in crops.

Ziziphus nummularia is a xerophytic plant which can survive even at 45 °C of threshold temperature and widely cultivated in arid and semi arid region. This crop comes up reasonably well in dry land areas with receding soil moisture condition and poor soil where other crops fail to grow. In a previous study in our laboratory, drought stress responsive transcriptome profile of *Z. nummularia* genotype from Jaisalmer (CIAHZ-J) revealed that 283 transcripts were up-regulated and 554 down-regulated (*http://www.ciah.ernet.in/reserch_highlights.php*). Based on the results obtained, a multiple stress inducible NAC (*ZnNAC*) gene from *Z. nummularia* seedlings, which shows significant up regulation under abiotic stress conditions was isolated, characterized and cloned in an expression vector pET28a.The expression of *ZnNAC* in *E. coli* was confirmed by SDS-PAGE analysis.in the present study. The role of *ZnNAC* in response to drought stress under *in vitro* conditions (10% PEG treatment) was evaluated in prokaryotic system by carrying out growth curve analysis. The finding of the present study could potentially be used to develop transgenics crops for improved tolerance to abiotic stresses using approach.

MATERIAL AND METHODS

Seeds of *Z. nummularia* were obtained from Central Institute of Arid Horticulture (CIAH), Bikaner, Rajasthan. Germination of seeds of *Z. nummularia* was standardized at 30 °C with 16 h light/8 h dark photoperiod at National Phytotron Facility, Indian Agricultural Research Institute, New Delhi, India. Hard seed coat of seeds was broken and cotyledons were sown in autoclaved soilrite. One month old seedlings of *Z. nummularia* were subjected to 30% PEG6000 (Polyethylene glycol) (Himedia, India) in ½ MS media (w/v) (Himedia, India) for different time period 0 h, 6 h, 12 h, 24 h, 48 h and 72 h to induce drought stress in plant. The media was supplemented with 1mM MES buffer (2-(N-morpholino) ethanesulfonic acid- Himedia, India) to maintain a pH of 5.6.

Extraction of total RNA and genomic DNA

Total RNA was isolated from control and treated seedlings of *Z. nummularia* for different time period 0 h, 6 h, 12 h, 24 h, 48 h and 72 h respectively, using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA). Concentration and integrity of the RNA were determined by spectrophotometry (Nano Drop 2000, Thermo Scientific, USA) and relative intensity of brightness of Ethidium Bromide (*EtBr*) stained bands resolved on a 1.2% agarose gel respectively.

Full length *ZnNAC* gene isolation by using 5' RACE and 3' RACE

To obtain the open reading frames (ORFs) of the NAC gene, total RNA was used for reverse transcription by using oligo-dT primer, dNTPs and reverse transcriptase enzyme of SMARTer™ RACE cDNA Amplification kit (Clontech, USA) according to manufacturer's instructions. First strand cDNA thus synthesized was used as template for subsequent PCR using Advantage 2 polymerase mix. PCR products were cloned into a pGEMT-easy vector (Promega) and sequenced. Different primer pairs, *ZnNAC*-F1 toF4 and R1 to R4 (Table 1) were used to amplify *ZnNAC* open reading frame (ORF). The full length cDNA sequence of *ZnNAC* along with UTR region was retrieved by comparing and aligning the sequences of 5' and 3' RACE products using BioEdit version 7. 1. 11.

Amplification of the *ZnNAC* gene from genomic DNA

Amplification and cloning of full length CDS region of NAC gene was carried out with cDNA template of *Z. nummularia*. Further gene specific primers *ZnNACgDNA-F* and *ZnNACgDNA-R* (Table 1) were used to amplify *ZnNAC* gene from genomic template by using polymerase chain reaction (PCR). A reaction mixture of volume 25 µl contains 1 X Taq buffer A (Banglore genei), 0.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.2

μM gene specific primers and 1 unit of Taq polymerase was used in polymerase chain reaction (PCR). Reaction mixture was placed in thermocycler with program, one cycle of 94 °C for 3 min (primary denaturation); 35 cycles of 30 sec min at 94 °C (denaturation), 30 sec at 60 °C (annealing) and 1 min 30 sec at 72 °C (extension) and final extension was done at 72 °C for 10 min. Purified PCR product was cloned in pGEMT-easy vector and sequenced.

In silico analysis of ZnNAC protein

Sequences of NAC gene from other plant species were retrieved from the GenBank (NCBI) database for *in silico* analysis (Table 2). Multiple sequence alignments with protein sequences of NAC gene were performed and Phylogenetic tree was constructed using Clustal X. Neighbor joining method was used to study the phylogenetic relationship of these NAC genes. Physiochemical property of a protein like molecular weight, isoelectric point, aliphatic index, instability index and grand average of hydropathicity (GRAVY) provides many significant information about the role of ZnNAC protein [19]. Expasy's ProtParam server was used to analyze these parameters. Sub-cellular localization of the proteins gives an important insight about the protein function. CELLO is a SVM based tool used to predict sub-cellular localization of a given sequence [20].

ZnNAC structure prediction and docking study

Protein homology recognition engine (Phyre 2) server has been used to predict the structure of the protein [21]. A library of known protein structures taken from the Structural Classification of Proteins (SCOP) database and the PDB is used by Phyre to predict the function of the protein. Here, Abscisic-acid-responsive *ANAC* (PDBID: 1UT7) of *Arabidopsis thaliana* was used as template to predict the three dimensional structure of the *Z. nummularia's* ZnNAC Protein Sequence. Validation of stereo-chemical property of the predicted structure by analyzing the overall structure and residue-by-residue geometry was validated using PROCHECK [22] at PDBSum server [23]. A neural network based predictor server ProQ, was used to define the correctness and quality of predicted structures [24].

DP-dock server has been used for docking of predicted model of NAC with DNA [25]. The Docked protein-DNA complexes were analysed considering its energy and Matthews Correlation Coefficient to select specific and best binding pose. Further, molecular visualisation was done with Pymol.

Expression analysis of *ZnNAC* transcript by real-time qPCR

Expression pattern of *ZnNAC* transcripts in one month old seedlings was investigated in response to drought stress induced by PEG treatments (30%) for different time intervals (0, 6, 12, 24, 48 and 72 h) by real time qPCR. Total RNA was isolated and cDNA was synthesized by using SuperScript®III first strand synthesis system (Invitrogen, USA) using oligo (dT) primer. *Ziziphus jujube elongation factor*1 (*Zjef*1) gene (accession no. EU916202) was used as an internal control for real time qPCR analysis. *qZnNAC-F* and *qZnNAC-R* for *ZnNAC,* and *qZjef1-F andqZjef1-R* primers set (Table 1) were used in real time qPCR. Real time qPCR cocktail (10 μl) contained 100 ng template cDNA, 0.5 μM of each primer and 2X KAPA SYBR FAST qPCR master mix buffer (5 μl) (KAPA Biosystems, USA). Reactions were performed in LightCycler® 480 II (Roche, Switzerland). The cycling conditions consisted of 4 min polymerase activation at 94 °C and 40 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec. Experiments were performed on two biological replicates and three technical replicates for samples from each treatment. Melting curves were analyzed to confirm the specificity of the reactions. Ct (threshold cycles) value were calculated by the $2^{--"CT}$ method. Ct values from two replicates were averaged and normalized with the Ct values of the internal control *Zjef1*, and standard deviations and errors were calculated.

Cloning and over expression of *ZnNAC* gene in *E. coli*

Full length *ZnNAC* cDNA was PCR amplified using forward primer *rZnNAC_NdeI-F* and reverse primer *rZnNAC_BamHI-R* (Table 1). The amplified PCR product was double digested with *NdeI and BamHI* restriction enzymes and cloned into pET28a expression vector. *E coli* BL21 (DH3) cells (Novagen, USA) were transformed with recombinant plasmid (pET28a::*ZnNAC*) and pET28a vector alone as negative control.

Expression analysis of *ZnNAC* in *E. coli* by SDS-PAGE

The protein expression was induced by the addition of 1mM IPTG (isopropyl-β-D-thiogalactoside). Two ml aliquots were collected before adding IPTG (negative control) and samples were collected at 1h, 2h, 3h, 4h, 5h, 6h and 16h of time intervals. Total proteins were extracted from the bacterial cells with lysis buffer containing 100 mM Tris-Cl (pH-7.5) (v/

v), 150 mm NaCl (w/v), 1 mM EDTA (pH-8.0) (v/v), 1mM PMSF (w/v) and 20 µg/ml lysozyme (w/v)) by sonication followed by centrifugation. Prior to electrophoresis, β-mercaptoethanol and bromophenol blue at final concentrations of 10% and 100 mg/ml were added to the samples, and the samples were heated in boiling water bath for 10 min. Levels of protein expression were analyzed by resolving total protein on 12% (w/v) polyacrylamide slab gel electrophoresis as per the method of [26], followed by staining with coomassie brilliant blue R250 to confirm the molecular weight of the expressed protein.

Western blot analysis of ZnNAC protein was performed with total ZnNAC protein eluted from the gel piece after SDS-PAGE electrophoresis. The protein eluted was electro-blotted onto PVDF membrane using Mini Trans-Blot® electrophoretic transfer cell system (BIO-RAD). The membranes were then blocked at room temperature for 1 h in 5% BSA blocking solution prepared in TSW buffer (10mM Tris-Cl pH-7.4 (v/v), 0.9% NaCl (w/v), 0.1% Triton X-100 (v/v) and 0.02 % SDS (v/v). Then membrane was incubated with alkaline phosphatase Anti mouse IgG-goat polyclonal antibody (abm) at1:4000 dilutions in TSW buffer for 1 h at room temperature. After three times of washing with TSW the membrane was treated with Anti-His tag antibody mouse clonal (abm) at 1:4000 dilutions in TSW buffer for 1 h at room temperature. Again after washing with TSW, the membrane was soaked in solution of SIGMAFAST™ BCIP® /NBT tablet (Sigma Aldrich) for color development for 5-10 min or until the distinct bands appeared.

Osmotic stress tolerance assay in transformed *E. coli* cells

Functional validation of ZnNAC protein was carried out by liquid culture assay. *E. coli* BL21 (DE3) cells transformed with recombinant plasmid (*pET28a*:: *ZnNAC)* and *E. coli* BL21 (DE3) non recombinant (*pET28a*) were grown in LB basal medium at 37 ÚC at 200 rpm until OD_{600} reach at 0.6, followed by induction of protein expression by addition of 1 mM IPTG for 12 h. Equal numbers of cells from each culture were transferred to fresh 20 ml LB media supplemented with 50 µg/ml kanamycin and 10% PEG 6000 to induce water stress condition and the suspension was allowed to grow at 37 ^{o}C with continuous shaking at 200 rpm in kuhner's incubator shaker. Aliquots of the bacterial suspension were harvested at different time intervals (2, 4, 8, 12 and 16 h) and optical density (OD) was calculated at 600 nm spectrophotometrically. Three replicates were taken for each sample and standard deviation and standard error was calculated. Cell number was calculated *E. coli* OD_{600} calculator (*http:// www.genomics.agilent.com/biocalculators/ calcODBacterial.jsp*).

RESULTS

Full length *ZnNAC* gene isolation

Rapid Amplification of cDNA Ends (RACE) technique was used to obtain full length coding cDNA sequence of NAC gene in *Z. nummularia* (*ZnNAC*). Four set of primers (*ZnNAC* R1, *ZnNAC* R2, *ZnNAC* R3 and *ZnNAC* R4) were designed to amplify 5' cDNA of *Z. nummularia*. Amplicon size of 1 kb was obtained with primer *ZnNAC* R1, 900 bp was obtained with primer *ZnNAC* R2, 850 bp was obtained with *ZnNAC* R3 and 750 bp of amplicon was obtained with *ZnNAC* R4 primer (Figure1). For 3'RACE, another four set of primers (*ZnNAC* F1, *ZnNAC* F2, *ZnNAC* F3 and *ZnNAC* F4) were designed and amplified with 3' cDNA of *Z. nummularia*. Amplicon of size 1.2 kb was obtained with primer *ZnNAC* F1, 1 kb was obtained with primer *ZnNAC* F2, 900 bp was obtained with *ZnNAC* F3 and 800 bp of amplicon was obtained with *ZnNAC* F4 primer (Figure 1). Assembly results showed that the complete sequence of cDNA of *ZnNAC* gene was 1726 bps long including 123 bps up-stream and 538 bps downstream untranslated regions (UTR). The open reading frame (ORF) region of *ZnNAC* gene was found to be 1059 bp long encoding a protein of 352 amino acids. Coding DNA sequence (CDS) sequence of *ZnNAC* gene showed 82% identity with *Prunus mume* NAC domain-containing protein in BLASTn analysis.

Amplification of NAC gene from genomic DNA

Primers were designed for full length amplification of *ZnNAC* gene, which was successfully amplified from cDNA template and amplicon of 1059 bp was obtained. On contrary when same primers i.e. *ZnNACgDNA-F* and *ZnNACgDNA-R* (Table 1) *tried with* genomic DNA template 1333 bp amplicon size was obtained, including start codon and stop codon. The cDNA sequence of *ZnNAC* was submitted to NCBI GenBank and accession No KT225022 was obtained. Comparisons of the cDNA sequence of *ZnNAC* and the corresponding genomic DNA sequence revealed that gene contain 3 exons with 2 introns (Figure 2). The genomic analysis found that genomic fragment encompassing three exons of size 185, 265, 609 bp with two introns of size 118 and 156

bp respectively.

Phylogenetic and *in silico* analysis of *ZnNAC*

Multiple sequence alignment of NAC gene from different plant species showed that the conserved residue lies at N-terminal of the NAC domain containing protein between 14th to 156th amino residues residues. These conserved residues may bind with DNA and play crucial role in the transcription (Figure 3). Analysis for phylogenetic relationship reveals that *ZnNAC* shows a close phylogenetic relationship with *Fragaria vesca* NAC (*FvNAC*) and *Rosa chinensis* NAC (*RcNAC*) (Figure 4). Further analysis for physicochemical property showed that *ZnNAC* is slightly basic in nature with theoretical pI 8.51, which leads to efficient binding of the negatively charged DNA to the NAC protein. Study for sub-cellular localisation showed that ZnNAC is a nuclear protein and maybe involved in transcription of DNA.

ZnNAC structure prediction and docking study

Predicted Model of the ZnNAC has 93% residues in most favoured and additionally allowed region of Ramachandran plot so model is stereo chemically stable. The ProQ result with LGscore and MaxSub value 2.241 and 0.180 respectively further establishes the correctness of the predicted model. The root mean square deviation of the predicted model of ZnNAC with respect to templates 4GBZ is 0.207Å, with similar NAC domain architecture. The overall structure of ZnNAC contains seven α-helices and seven anti-parallel β-sheets (Figure 5A). The NAC domain of ZnNAC which binds the DNA is composed of five α-helices and seven anti-parallel β-sheets. Further, docking result of ZnNAC with nonspecific DNA shows that this NAC domain provides a space for binding of DNA with its major and minor groove. The amino acid residues of ZnNAC may be involved in interaction with DNA: are Arg85, Thr100, Lys102, Arg109, Lys114, Lys126, Lys129, Arg138, Lys 145, Arg157, Lys161, Lys162 and Ser163 (Figure 5B).These amino acid residues are either part of β-sheets of NAC domain or it lies in the vicinity of the β-sheets and are likely to contribute to the overall affinity of DNA binding.

Expression analysis of *ZnNAC* transcript by real time qPCR

Expression analysis of *ZnNAC* transcripts was performed in one month old seedlings. Drought stress was induced by 30% PEG treatments for different time intervals (0, 6, 12, 24, 48 and 72 h). After RNA isolation from these samples cDNA synthesis was performed followed by real time qPCR analysis. Differential gene expression of NAC transcription factor suggested that 4.83 fold changes in expressions was observed at 6 h of treatment which increased up to 74.02 fold at 48 h of treatment and was then down regulated 18.83 fold at 72 h of treatment in *Z. nummularia* (Figure 6).

Expression analysis of *ZnNAC* gene in *E. coli*

ZnNAC gene was cloned into bacterial expression vector pET28a at *Nde*I and *Bam*HI site. Double digestion with *Nde*I and *Bam*HI restriction enzyme to released the CDS of *ZnNAC* and linear pET28a vector (Figure 7). ZnNAC protein was successfully expressed with an N-terminal histidine-tag in recombinant *E. coli* BL-21 DE3 bearing pET28a::*ZnNAC* plasmid at 37 °C. SDS-PAGE and western blot was performed to study the over expression of recombinant protein (*pET28a::ZnNAC*) in *E. coli* strain BL21 cells after 1mM IPTG induction at different time intervals (0, 1, 2,3,4, 5, 6 and 16 h). SDS-PAGE revealed that the molecular weight of expressed protein was observed approximately 44.1 kDa. SDS-PAGE analysis showed that protein accumulated proportionately with increase in incubation time up to 16 h (Figure 8). Western blot results demonstrated that the Anti-His tag antibody mouse clonal antibody binds with N-terminal histidine-tag present in recombinant *pET28a::ZnNAC* protein and expressed protein was observed approximately 44.3 kDa in size, validate the results of SDS-PAGE (Figure 9).

Osmotic stress tolerance assay in transformed *E coli* cells

Liquid assay results with *E. coli* BL-21 DE3 transformed with recombinant plasmid *pET28a:: ZnNAC* plasmid alongwith *E. coli* BL-21 DE3 transformed with only vector pET 28a plasmid showed that there was no difference in growth pattern between the tranformants with recombinant plasmid and the transformants with non-recombinant plasmid (control) in LB basal medium containing kanamycin (50µg/ml). Growth of *E. coli* BL-21 DE3 tranformants with *pET28a*::*ZnNAC* plasmid under induced drought stress (LB basal medium containing 10% PEG 6000 supplemented with kanamycin (50µg/ml) was better as compared to transformant with pET28a plasmid alone (control) (Figure 10) in comparison to LB basal medium without PEG 6000. However much better cell growth was observed in the transformant with *pET28a*::*ZnNAC* plasmid as compared to *E. coli* BL-21 DE3 transformant with pET28a plasmid alone (control) (Figure 10).

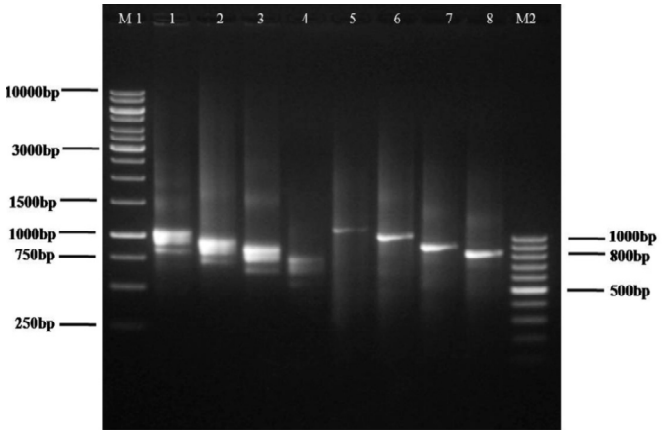

Figure 1: 5' and 3' RACE of *ZnNAC* gene in *Z. nummularia*. Lane M: *GeneRuler 1kb DNA Ladder, Lane 1-4 (5' RACE): Amplification of ZnNAC R1, ZnNAC R2, ZnNAC R3 and ZnNAC R4', Lane 5-8 (3' RACE): Amplification of ZnNAC F1, ZnNAC F2, ZnNAC F3 and ZnNAC F4, Lane M2: GeneRuler 100 bp DNA Ladder*

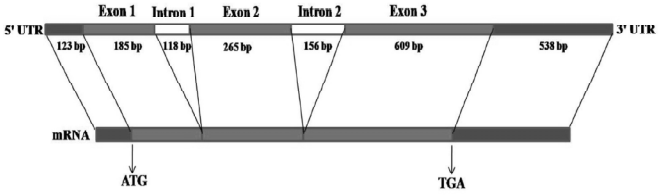

Figure 2: Schematic representation of genomic DNA and mRNA structure of *ZnNAC*. Genomic DNA contains three exons that were separated by two introns

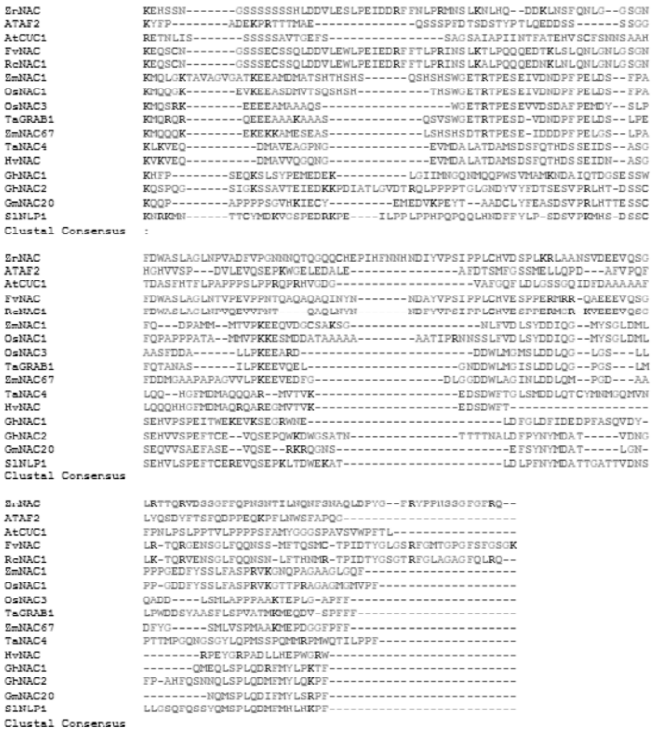

Figure 3: Protein containing NAC Domain from different plant species are analysed for conserved amino acid residues. The conserved residues lie near the N-terminal of the protein

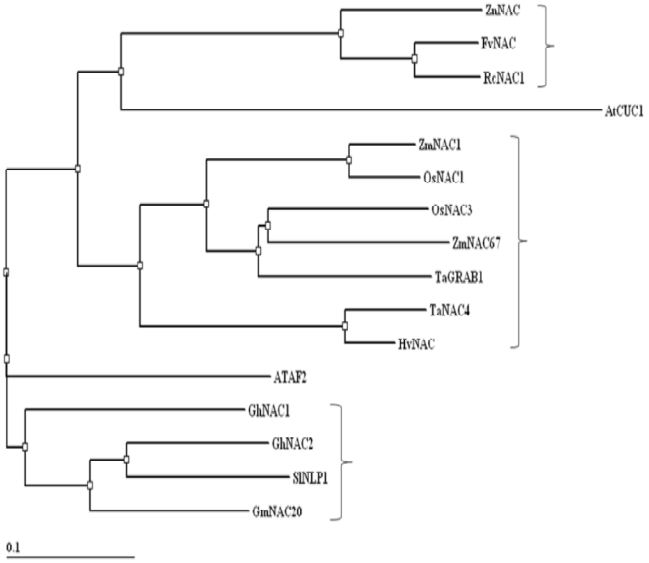

Figure 4: Phylogenetic analysis of ZnNAC with other NAC protein of other plant species

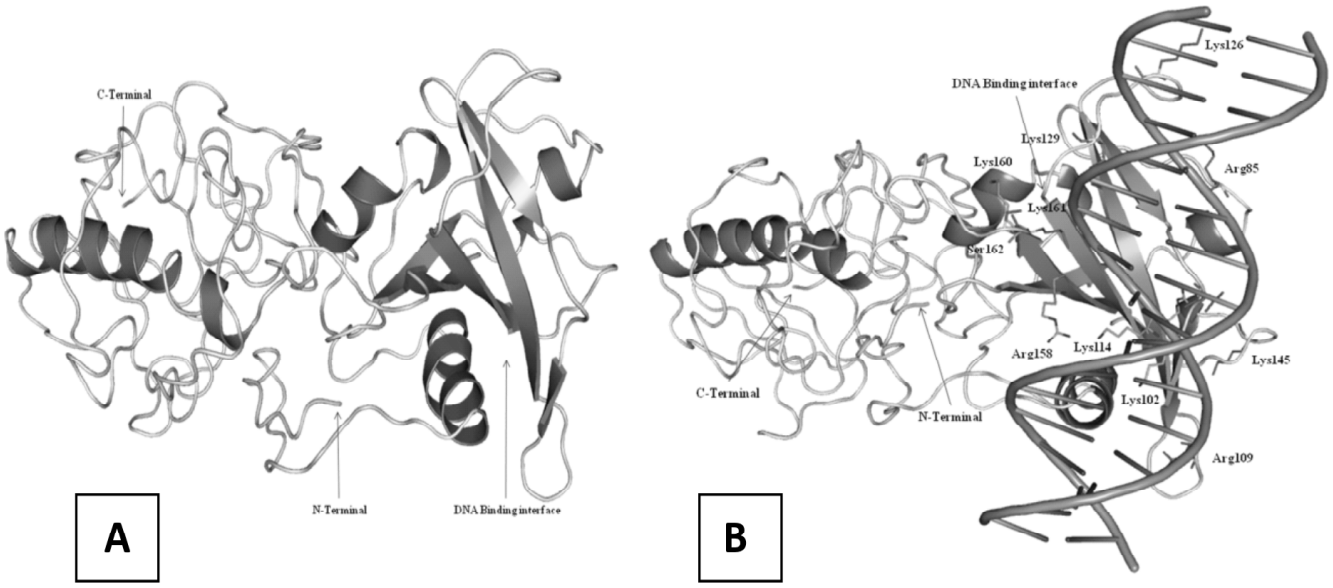

Figure 5: Figure shows the (A) overall structure of the ZnNAC having conserved DNA binding domain at N-terminal. (B) Positively charged amino acid like Arg and Lys of the the β- sheet and loop near β-sheets of the NAC domain play a crucial role in binding of DNA with of ZnNAC

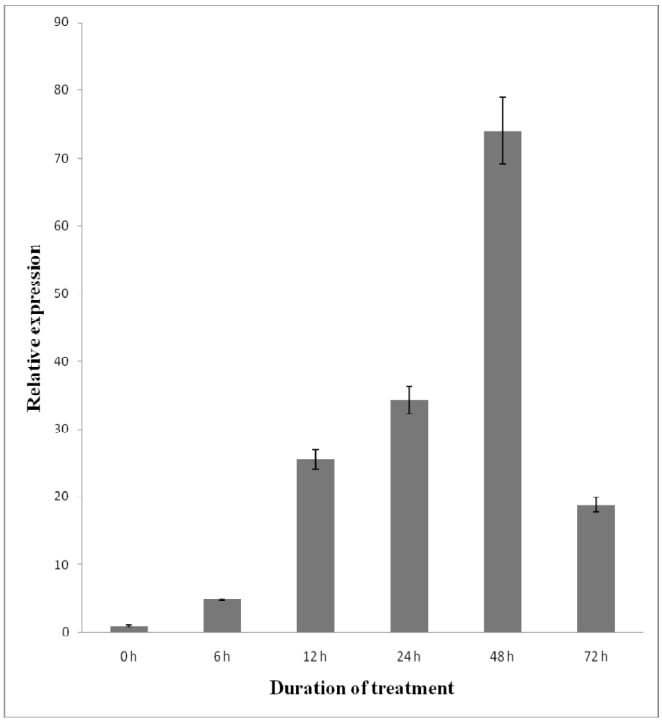

Figure 6: Differential gene expression study of NAC transcription factor gene in *Z. nummularia* subjected to 30% PEG 6000 treatment for different time duration

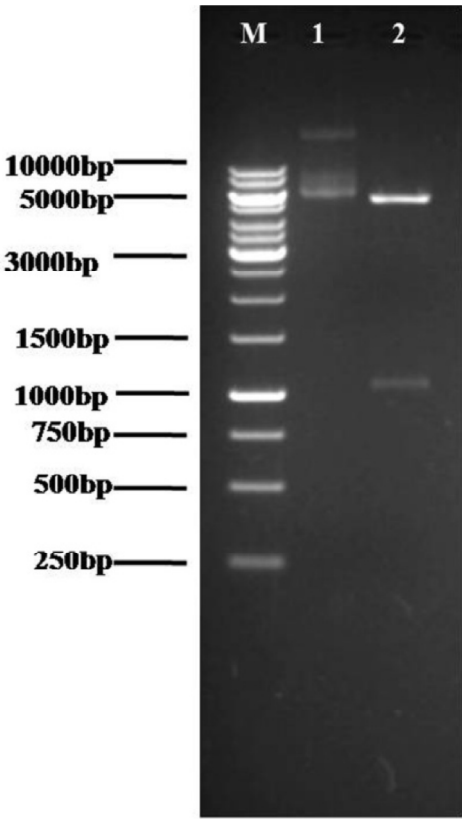

Figure 7: Restriction digestion analysis of recombinant *pET28a::ZnNAC* clone. Lane M: *GeneRuler 1kb DNA Ladder, Lane 1: Undigested plasmid of recombinant clone, Lane 2: Restriction digestion of plasmid of recombinant clone with Nde*I and *BamH*I restriction enzyme

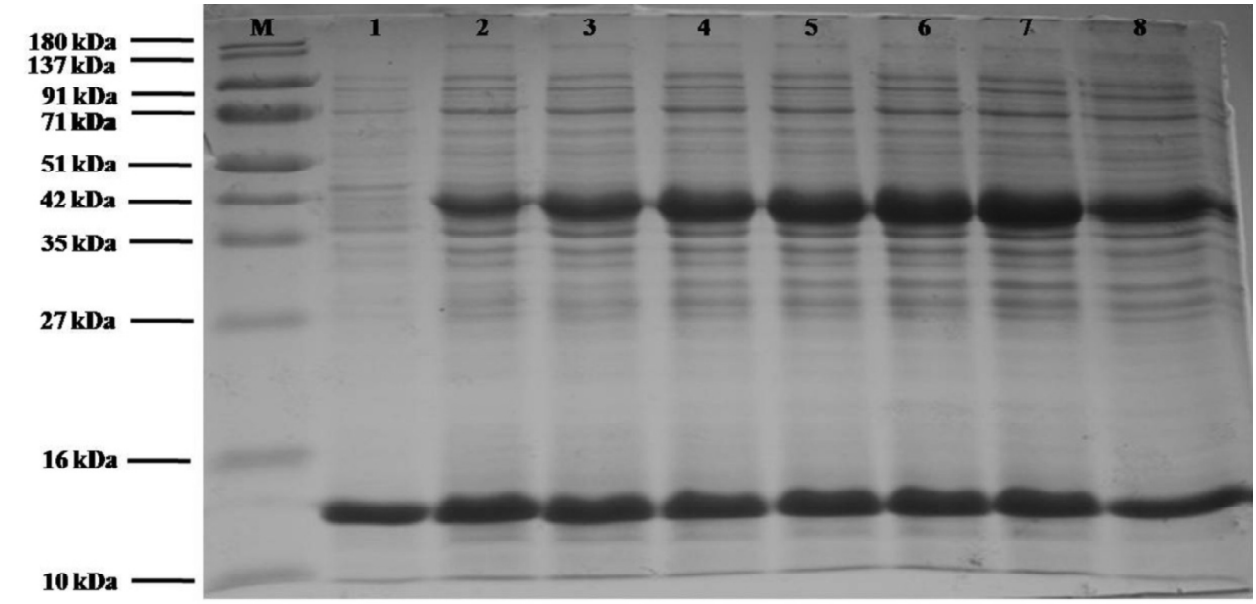

Figure 8: SDS PAGE pattern of recombinant protein (*pET28a::ZnNAC*) in *E. coli* strain BL21 cells. Lane M: 4 color Prestained protein ladder (Pure gene), Lane 1- 8: Protein samples of culture collected after 1mM IPTG induction at 0, 1, 2, 3, 4, 5, 6 and 16 h time intervals

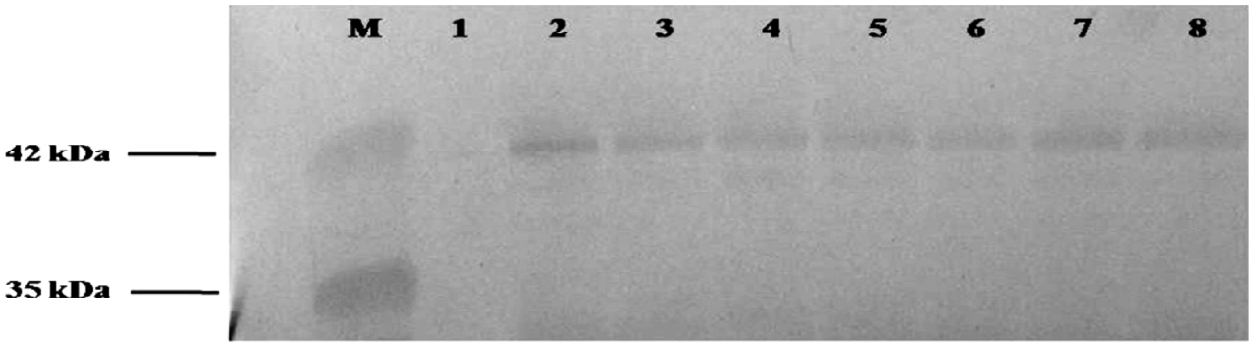

Figure 9: Western blot pattern of recombinant protein (*pET28a-ZnNAC*) in *E. coli* strain BL21 cells. Lane M: 4 color Prestained protein ladder (Pure gene), Lane 1- 8: Protein samples of culture collected after 1mM IPTG induction at 0, 1, 2, 3, 4, 5, 6 and 16 h time intervals

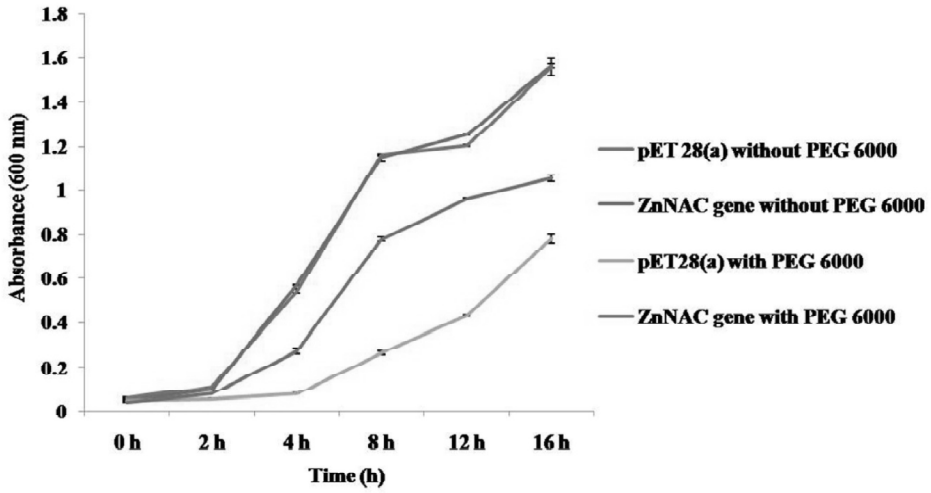

Figure 10: Growth curve assay. Optical density of pET28a and *pET28a::ZnNAC* in LB and LB media with PEG6000 (10%)

Table 1
List of *ZnNAC* primers used in different experiments

S.No	Primer code	Primer sequence	Used in application
1	qNAC-F	CTGGGTGGTTCGGGTAATTT	RT-qCR
2	qNAC-R	CATTGTTGTCCCTGCGTTTG	RT-qCR
3	qZjef 1-F	GCTGACTGTGCTGTTCTCATC	Primers designed from *Ziziphus jujuba* elongation factor gene used as a reference gene
4	*qZjef 2-R*	GACACCAAGAGTGAAAGCGAG	
5	*ZnNAC R1*	GACGAGTCGACCCGCTGAGTGG	5' RACE of *NAC* gene
6	*ZnNAC R2*	GGCAAAGAGGTGGGATGGAAGGGAC	5' RACE of *NAC* gene
7	*ZnNAC R3*	CAGCAAGACTGGCCCAATCGAAATTAC	5' RACE of *NAC* gene
8	*ZnNAC R4*	CTTGAGCGAATTCATACGAGGCAGATTG	5' RACE of *NAC* gene
9	*ZnNAC F1*	CGCTCAAGAATCTACATCAGGGCGAC	3' RACE of *NAC* gene
10	*ZnNAC F2*	CCAGGAAATGATAACCAAACGCAGGG	3' RACE of *NAC* gene
11	*ZnNAC F3*	CTATGTCCCTTCCATCCCACCTCTTTG	3' RACE of *NAC* gene
12	*ZnNAC F4*	CGACTCGTCGGGTTTCTTCCAGCCC	3' RACE of *NAC* gene
13	*ZnNAC_NdeI-F*	**CATATG**ATGGGTGTGCCGGAAAC	Primer for ZnNAC constructs preparation in pET28a. Under line indicates the site for *Nde*I and *BamHI* restriction site.
14	*ZnNAC_BamHI-R*	**GGATCC**TCACTGTCTGAATCCGAACC	
15	*ZnNACgDNA-F*	ATGGGTGTGCCGGAAAC	Amplification of genomic region of *ZnNAC* in genomic DNA of *Z. nummularia*
16	*ZnNACgDNA-R*	TCACTGTCTGAATCCGAACC	

Table 2
List of accession number and details of nucleotide, amino acid sequences of NAC gene in other plant species retrieved from NCBI database

S.No	Plants	Family	Accession no	Length of sequences		Sequence homology (%)	
				CDS (bp)	Deduced amino acid	Nucleotide	Amino acid
1	*ZnNAC*	Rhamnaceae	KT225022*	1059	352		
2	*ATAF2*	Brassicaceae	NM_147856	852	283	36	32
3	*AtCUC1*	Brassicaceae	NM_112380	933	310	35	27
4	*FvNAC domain72*	Rosaceae	XM_004291619	1065	354	71	72
5	*RcNAC1*	Rosaceae	KP784446	1056	351	71	72
6	*ZmNAC1*	Poaceae	NP_001123932	939	312	38	31
7	*OsNAC1*	Poaceae	ABD52007	945	314	38	33
8	*OsNAC3*	Poaceae	NP_001059213	831	276	33	30
9	*TaGRAB1*	Poaceae	CAA09371	864	287	36	30
10	*ZmNAC67*	Poaceae	ACG31804	894	297	34	31
11	*TaNAC4*	Poaceae	ADE34585	945	314	35	31
12	*HvNAC*	Poaceae	CAM57977	837	278	33	30
13	*GhNAC1*	Malvaceae	ACI15341	831	276	35	35
14	*GhNAC2*	Malvaceae	ACI15342	900	299	38	31
15	*GmNAC20*	Fabaceae	ACC66314	807	268	36	32
16	*SlNLP1*	Solanaceae	ACG50001	891	296	36	33

DISCUSSION

NAC family, represents one of the largest plant transcription factor families, is only found in plants [27] and characterized in many plant systems including Arabi-dopsis, wheat, soybean, rice and *Citrus* [18, 28-33]. But to date isolation and characterization of *NAC* family in *Ziziphus* species still remains unknown which need to be explored.

Therefore in the present study, full length coding DNA sequence of *NAC* transcription factor was isolated and characterized at molecular level from hardy and drought tolerant plant *Z. nummularia* and functionally validated in *E. coli* cells. In comparison with the sequences of other *NAC* genes we found genomic sequence of *ZnNAC* shared very common feature of genomic organization, first two exon regions encode to conserved region at N-terminals and last exon encodes the highly divergent C terminal transcriptional activation domain [34, 35].

Multi alignment of deduced amino acid sequence of *ZnNAC* protein along with NAC proteins from other plant species retrieved from NCBI databse represents that N- terminals of *ZnNAC* protein was highly conserved whereas C-terminal region contain transcriptional activation domain was smaller and divergent. The DNA-binding (DB) ability of NAC transcription factor is confined in NAC domain present in N- terminals. Six motifs identified in *ZnNAC* protein share common modification sites, such as different phosphorylation, glycosylation, amidation and N-myristoylation site as previously reported in putative *GhNAC* proteins of *Gossypium hirsutum* [34]**.** The negative value of grand average of hydropathicity (GRAVY) of *ZnNAC* indicates the *ZnNAC* as hydrophilic protein and it can be assumed hydrophilic nature of protein increases the accessibility to water molecules during stress conditions. All such physiochemical parameters support the characterization of *ZnNAC* as putative transcription factor. Phylogenetic analysis showed that *ZnNAC* clustered in same clade as *FvNAC* domain72 and *RcNAC1,* which indicates that the deduced amino acid sequence of *ZnNAC* has relatively high homology to dicot species *NAC* family members in comparison to monocot species (Figure 4).

Transcript expression analysis of *ZnNAC* gene in whole seedling of *Z. nummularia* showed up-regulation from 6 h to 48 h in response to 30 % PEG induced drought stress with 4.83 fold change to 74.02 fold changes respectively in the tested seedlings by RT-qPCR. Similar findings were reported for *TaNAC*67, where 28 fold up regulation was found in transcript level at 24 h of 20% PEG 6000 treatment in common wheat [36]. Various reports suggested that over expression of *NAC* genes conferred improved tolerance against abiotic stresses in plants like over expression of three *NAC* proteins (*ANAC019*, *ANAC055* and *ANAC072/RD26*) significantly improved drought tolerance of transgenic *Arabidopsis* plants. Therefore up regulation in transcript level of *ZnNAC* under induced drought stress revealed that *ZnNAC* may be involved in early drought stress response in *Z. nummularia.*

Our results showed recombinant bacterial cells imparted better growth under 10% PEG induced drought stress by over expression of *ZnNAC* protein in *E. coli* and conferring protective function in host cells against damage caused by stress on proteins and cellular membrane. Generally functional validation and characterization of genes are carried out in model plants such as tobacco, Arabidopsis and yeast mutants but recently few reports studied the functional expression screening of isolated plant genes responsive to abiotic stress in *E. coli* [37-39]. Some reports suggested that both prokaryotes and eukaryotes follow some common protective mechanism under stress conditions due to which expression of foreign plant genes carried by plasmid DNA of host *E. coli* cells acquire stress tolerance [40-42]. Dehydration-responsive element binding (*DREB*) transcription factor isolated from *Salicornia brachiata* (*SbDREB2A*) showed better growth of *E. coli* cells transformed with *SbDREB2A* under drought and excess salt stress conditions [38]. In the same way better growth of *E. coli* cells by overexpression of PM2, a group 3 LEA protein from soybean was reported [43]. Many reports suggested that abiotic stress conditions would cause the intra-cellular dehydration and damage of both proteins and cellular membrane in bacterial cells [41, 44, 45]. Therefore improved tolerance of recombinant bacteria cells over-expressing *ZnNAC*, under osmotic stress conditions indicate the involvement *ZnNAC* gene in protective function in host cells against damage caused by stress on proteins and cellular membrane and imparting tolerance to bacteria.

Many transgenic have been developed by using *NAC*TFs for conferring different stress tolerance to the plants (Table 2), but the success rate in conferring stress tolerance to plants is limited Constitutive over expression of NAC genes sometime leads to negative effects in transgenic plants such as dwarfing, late flowering and lower yields [18, 46-48]. NAC over expression may occasionally have an antagonistic effect to other kinds of stresses. Overexpressing *ANAC019* and *ANAC055* not only increased drought tolerance but also decreased resistance to *B. cinerea* [46, 49]. Transgenic with *NAC* over expression have been generally evaluated under green house conditions with major emphasis on plant vegetative stages only [50]. The effect of *NAC* overexpression on

crop yield is yet to be fully explored. Furthermore, most of the studies on NAC TFs only investigated the molecular mechanisms of individual occurring stress situations. Although recent studies have conducted multi-parallel stress experiments and identified different NAC TFs responding to single stress situations [51], the knowledge concerning responses to combinatorial stress factors is scarce, especially interactions among stress factors. In such circumstances understanding the molecular mechanisms of *ZnNAC* TFs networks integrating multiple stress responses will be essential for the development of broad-spectrum stress tolerant crop plants which can cope up with future environmental challenges.

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