Expression and specificity of EcNAC1 transcription factor from *Eleusine coracana* in *Escherichia coli*

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ABSTRACT

Dehydration stress is an important environmental factor which affects the crop yield. *Eleusine coracana* (Finger millet) is one of the relatively drought tolerant crop plant and it can also grow with significant levels of salinity. The present work presents cloning, expression and purification of the moisture stress induced transcription factor, *EcNAC*1. The *EcNAC*1 gene was cloned into pGEX-4T1 expression vector and transferred into *Escherichia coli* BL21 (DE3) pLysS strain. The positive clones were grown and induced with IPTG for 4h at 30ºC. The SDS-PAGE analysis of the lysate proteins showed the 66 kDa fusion protein (i.e., GST tag 26 kDa, *EcNAC1* approximately 41 kDa). The fusion protein was insoluble and localized as inclusion body. The protein was separated by preparative SDS-PAGE, electroeluted and antiserum was raised in adult healthy rabbits. The antiserum specifically detected *EcNAC*1 transcription factor from finger millet with no cross reactivity in western blot analysis.

Keywords: *EcNAC1* transcription factor, *Eleusine coracana*, anti-*EcNAC1* antiserum, recombinant protein, prokaryotic expression

INTRODUCTION

Dehydration stress is an important environmental factor which affects the crop yield. Adaptation of the plants to drought stress is mainly by maintaining cellular water balance necessary for functioning of enzymes and expression of genes. The major events of plant response to dehydration stresses are perception and transduction of the stress signals. The protection of plant cells from dehydration stress damage includes molecular chaperons, osmotic adjustment proteins, ion channels, transporter and antioxidation or detoxification proteins. The expression of these functional proteins is largely regulated by specific transcription factors. Many transcription factors have been identified which are involved in the plant response to drought stress [1]. Transcription factors are the DNA binding proteins that regulate gene expression. They regulate cell development, differentiation and cell growth by binding to a specific DNA site (or set of sites) and regulate gene expression. NAC transcription factor is a plant specific gene family found to be involved in response to various environmental stresses [2].

Isolation of stress specific genes from *Eleusine coracana* and its overexpression in drought susceptible crop species can be useful in improvement of drought tolerance [3]. In this direction *EcNAC*1 was cloned and overexpressed in tobacco for validation of the gene. For efficient analysis of protein expression in transgenic plants, protein expression analysis using antibody against *EcNAC*1 would be a mandate. In this study, we presented the cloning, expression and purification of

*EcNAC*1 transcription factor from *Eleusine coracana* (finger millet) and study the specificity of antiserum raised against it.

MATERIALS AND METHODS

Bacterial strain and vector plasmid

Escherichia coli DH 5α and BL21 (DE3) pLysS and the protein expression vector pRSET A (Invitrogen, USA), pGEX-4T1 (GE, USA) was made available from Department of Animal Science, Central University, Hyderabad, India. The pTZ57R/T vector harboring *EcNAC*1 transcription factor gene was available in the Department of Crop Physiology, University of Agricultural Sciences, Bangalore, India.

Cloning and construction of expression vector

The full-length cDNA of *EcNAC*1 gene was amplified by polymerase chain reaction (PCR) using *Bam*HI-FP (5'-CAGGATCCATGACCATGGGAGGAGGG-3') and *Eco*RI-RP (5'-CCTAGAATTCTGTATTTACAGAGGTCGC-3') primers (Genbank Accession No: EU439937.1). Amplification was performed in a total volume of 50 µl containing 5 µl of 10X PCR buffer (Fermentas, USA), 5 µl of 2 mM of each dNTPs, 1 µl of T/A plasmid harbouring *EcNAC*1 gene (100 ng), 5 pm of each forward and reverse primer, and 1µl (2.5 U/µl) unit of Pfu polymerase (Fermentas, USA). PCR was carried out in an Eppendorf thermal cycler using initial denaturation at 94ºC for 5 min followed by 30 cycles consisting of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec and extension at 72ºC for 90 sec, with a final extension step consisting of 72ºC for 10 min. The PCR products were purified by PCR purification kit (Qiagen, Germany) according to the Manufacturer's instructions. The purified gene fragment was cloned in frame into the *Bam*HI/*Eco*RI site of pRSET A vector. Subsequently the *EcNAC*1 gene fragment was released from pRSET A vector and subcloned in frame into the *Bam*HI/*Eco*RI site of pGEX 4T1 vector. The recombinant plasmid was transferred into *E. coli DH5α* cells.

Expression of recombinant protein in *E. coli*

Expression of recombinant protein in *E. coli* BL21(DE3) pLysS cells was studied in the positive clones carrying recombinant plasmid pGEX 4T1-*EcNAC*1. The positive clones were grown in LB medium containing ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) at 37ºC, and when A_{600nm} reached to 0.4-0.5 the culture was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 30ºC. To identify and localize the *EcNAC*1 protein produced by *E. coli*, the cytosolic and pellet (insoluble fraction containing inclusion bodies) fractions of the bacterial culture were electrophoresed through 12.5% SDS polyacrylamide gel along side a standard protein molecular weight markers (Fermentas, USA). The SDS-PAGE gel was stained with Coomassie brilliant blue in Methanol:Acetic acid:Water (45:10:45) and destained in the same solution. The stained bands were visualized under an illuminator.

Electrophoretic elution of fusion proteins from gel

In order to extract the protein from the polyacrylamide gel, the method of electro-elution was applied using dialysis membrane [4,5]. Protein band with 66 kDa size was excised and cut into small fragments. The stain CBB-R250 from the gel fragments was removed as per the method described [6]. Briefly, destaining solution containing 50% isopropanol and 1.5% SDS in gel running

buffer was added to gel pieces in glass tube and the tubes were capped with parafilm. Tubes were placed in 37ºC water bath set for overnight without agitation. After cooling at room temperature, the liquid was removed and the gel fragments containing the appropriated protein were used for electrophoretic elution.

Preparative SDS-PAGE electrophoretic elution was done as per the method described earlier [7]. Briefly the gel fragments were equilibrated twice in 0.1 M Tris-HCl buffer (pH 6.8) and 1.0% solution of 2-mercaptoethanol for 20 min. A final equilibration of the gel fragments in 0.1 M Tris-HCl buffer (pH 6.8) containing 1.0% (w/v) SDS was performed. The gel fragments were then placed in a dialysis tube with tris-glycine buffer containing SDS (25mM Tris, 192 mM glycine, and 0.1% SDS) and electroelution was performed at 50 V for 10 h at 4ºC in Tris glycine buffer containing 0.1% SDS (pH 8.3). At the end of electrophoretic elution, the polarity of the electrodes was changed for 1 min in order to release the absorbed protein on the dialysis tubes. The electroeluted protein sample was again dialyzed twice in PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4).

Polyclonal antibody production

Antibodies against electroeluted fusion protein (GST-*EcNAC*1*)* were raised in a New Zealand white rabbit. The purified protein (100 μg) in 1.0 ml of PBS was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at 6 to 8 sites at the back of the neck, of an adult healthy rabbits of body weight 2.5-3.0 Kg. Three-booster injections with 50 μg each with Freund's incomplete adjuvant were given at every three weeks intervals. The animals were bled two weeks after each injection and the serum was separated, aliquoted and stored at -20ºC.

Western blot analysis

SDS-PAGE separated proteins were electroblotted onto a nitrocellulose membrane at a constant current of 90 mA at room temperature for 1 h. After blocking the leftover free sits with blocking buffer (PBS containing 5% (w/v) skimmed milk powder, the membrane was incubated with the antiserum antibodies raised against GST-*EcNAC*1 (1:1000 diluted in PBS containing 0.05% Tween-20 and defatted milk powder) at 37ºC for 2 h. After washing three times, goat-anti-rabbit IgG conjugated with HRP (diluted 1:5000 in blocking buffer) was added and incubated for 1 h at room temperature and the blot was developed with DAB (3,3'Diaminobenzidine) substrate.

RESULTS AND DISCUSSION

Drought stress is an important environmental factor which affects the crop yield. *Eleusine coracana* (Finger millet) is one of the relatively drought tolerant crop plant and it can also grow with significant levels of salinity. Recent studies reported that drought tolerance can be achieved by developing transgenic plants, over-expressing stress specific transcription factors [8,9]. In this direction *EcNAC*1 was cloned and overexpressed in tobacco for validation of the gene. The transcription factors regulate the expression of downstream genes involved in stress response and differential expression of these transcription factors brings about variation in stress adaptation. The quantitative difference besides functional efficiency may play a role, therefore precise quantification is essential. For efficient analysis of protein expression in transgenic plants needs antibody against *EcNAC*1 protein.

In this direction, *EcNAC*1 gene was initially cloned in frame into the *Bam*HI/*Eco*RI site of pRSET A vector and sequenced. The positive clones were induced with different IPTG concentration (0.1-1.0 mM) and temperature. Very low levels of protein expression was noticed

with pRSET A as expression vector, therefore *EcNAC*1 gene was subcloned inframe into the *Bam*HI/*Eco*RI site of pGEX 4T1. This vector carried an N-terminal Glutathione-S-transferase (GST) protein tag, thereby facilitating the purification of recombinant protein by affinity chromatography in which glutathione immobilized on agarose acts as a ligand. The recombinant plasmid was transferred into *E. coli DH5α* cells and the positive clones were identified by colony PCR using gene specific primers. The restriction analysis of the recombinant plasmid with *Bam*HI and *Eco*RI showed the release of 1.1 kb insert along with 4.9 kb vector backbone fragment on 1.0% agarose gel (Figure 1). The recombinant plasmid was transferred into *E. coli* BL21 (DE3) pLysS cells and the positive clone was selected for protein expression. The expression of GST-*EcNAC*1 [26+40 kDa] protein was clearly seen in induced culture but not in uninduced culture (Figure 2). The optimal conditions for the GST-*EcNAC*1 protein expression was at 30ºC temperature, 0.4 mM IPTG concentration and 4 h induction time.

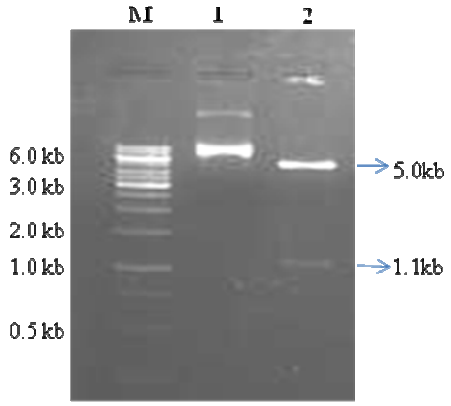

Figure 1. Recombinant plasmid pGEX-4T1-*EcNAC*1 digestion. Lane M: 1kb DNA ladder (Fermentas, USA); 1: uncut pGEX-4T1-*EcNAC*1 plasmid; 2: product from pGEX-4T1-*EcNAC*1 digested with *Bam*HI and *Eco*RI.

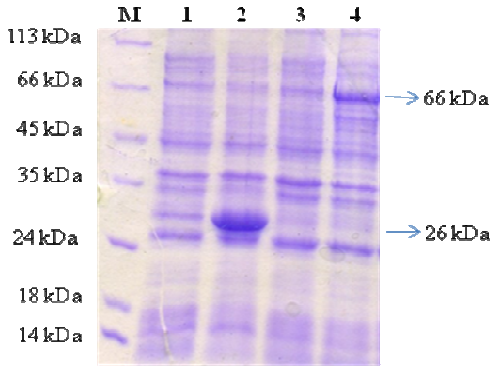

Figure 2. Expression and identification of recombinant fusion protein. Lane M: Page Ruler Unst.Protein moleculat weight marker (Fermentas, USA); 1: Total cell protein of pGEX-4T1plasmid transformed *E. coli* cell without induction; 2: pGEX-4T1 plasmid transformed *E. coli* cell with 0.4mM IPTG induction; 3: Total cell protein of pGEX-4T1-*EcNAC*1 plasmid transformed *E. coli* cell without induction; 4: pGEX-4T1-*EcNAC*1 plasmid transformed *E. coli* cell with 0.4mM IPTG induction.

Overexpression of the GST-*EcNAC*1 at appreciable level was observed in *E. coli* BL21 (DE3) pLysS cells and the fusion protein was predominantly expressed as inclusion body even with 0.1 to 1mM IPTG concentration and 20 to 37ºC incubation temperatures. The inclusion bodies were easily separated from majority of the contaminating *E. coli* proteins by electroelution method [10]. The rapid protein purification by preparative SDS-PAGE electro-elution method was reported by Mohammadian and his co-workers [7]. We followed this method to purify our GST-*EcNAC*1 fusion protein from inclusion body. This one-dimensional preparative gel electrophoresis allows a single and short purification step. The high resolution capacity of this technique leads to a high level of purity of the protein. Moreover, it avoids contamination by other non-specific proteins which often appear during protein purification by column chromatography. The purity of the electroeluted GST-*EcNAC*1 protein was evident from the SDS-PAGE (Figure 3A) with a single band of approximate molecular weight of 66 kDa and the purity of the fusion protein was nearly 98%. The healthy adult rabbits were immunized with 100 µg electroeluted fusion protein for polyclonal antibody production. The animals were bled after the three booster injections and the serum was collected. The serum detected 66 kDa GST-*EcNAC*1fusion protein (Figure 3B) and also 41 kDa *EcNAC*1 transcription factor protein from finger millet with no cross reactivity in western blot analysis (Figure 4). In conclusion, the present study clearly demonstrates that *EcNAC*1 protein could be successfully expressed purified as recombinant protein by preparative SDS-PAGE electro-elution method with 98% purity. The polyclonal antibody detected *EcNAC*1 transcription factor protein from finger millet with no cross reactivity in western blot analysis.

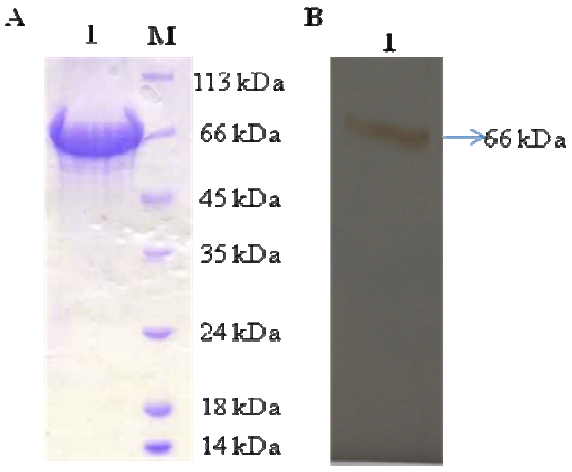

Figure 3. 12.5% SDS-PAGE and western blot analysis of purified recombinant (GST-*EcNAC*1*)* fusion protein. (A) Lane 1: purified fusion protein by preparative SDS-PAGE Electroelution method; M: Page Ruler Unst.Protein molecular weight marker (Fermentas, USA); (B) Lane 1: Western blot analysis of purified fusion protein with polyclonal antibody raised against GST-*EcNAC*1.

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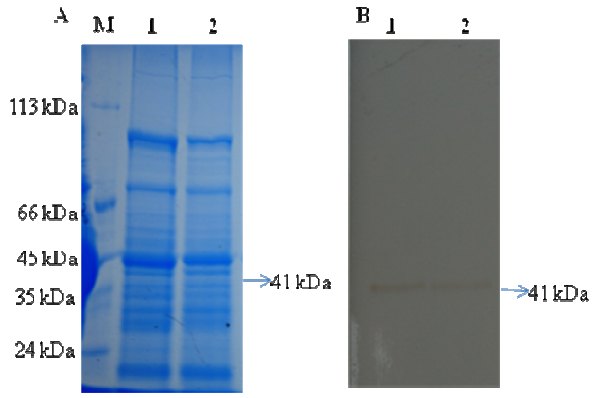

Figure 4. 10% SDS-PAGE and western blot analysis of total cell protein from finger millet. (A) Lane M: Page Ruler Unst.Protein molecular weight marker; 1 and 2: Finger millet total cell protein; (B) Lane 1: western blot analysis of finger millet total cell protein with polyclonal antibody raised against GST-*EcNAC*1.

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