African Journal of Biotechnology Vol. 7 (18), pp. 3231-3234, 17 September, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Overexpression of an abiotic-stress inducible plant protein in the bacteria *Escherichia coli*

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Accepted 15 August, 2008

The aim of our work was the overexpression of the abiotic stress-inducible dehydrin protein, namely RAB16A, from rice in the BL21 strain of *Escherichia coli*. The *Rab16A* transcript of 0.5 Kbp was amplified from the total RNA of the salt-tolerant indica rice cultivar Nonabokra by RT-PCR and cloned into the expression vector pGEX-3X. The 47 kDa protein, expressed as GST: RAB16A fusion protein, after 2 mM IPTG-mediated induction, was collected as S10 fraction and purified through glutathione-sepharose affinity resin. Immunoblot analysis with the maize dehydrin antiserum showed cross-reaction with the above band, but not with GST protein alone, showing functional expression of the heterologous RAB16A protein in the bacterial system.

Key words: Fusion protein, Glutathione-sepharose, GST: RAB16A, pGEX-3X.

INTRODUCTION

Earlier studies on the regulation of gene expression have shown that a group of stage-specific mRNAs and proteins, popularly called late embryogenesis abundant (LEA), which accumulate in late embryogenesis as long-lived mRNA in mature dry grains, are induced precociously in immature embryos when abscisic acid (ABA), the universal stress hormone, is supplied in the culture (Sanchez-Martinez et al., 1986), or even in vegetative tissues like roots, shoot and leaves in response to various abiotic stress like water deficit, high salinity and low temperature or exogenous ABA treatment. Several groups of LEA proteins have been identified from different cereal crops, of which the group 2 LEA, also called dehydrins, are the most conspicuous of soluble proteins, induced by osmotic stress (Close, 1996). The first published sequence of a plant dehydrin was the RAB21 (or RAB16A) protein from rice (Mundy and Chua, 1988). The major ORF of the transcribed region of this gene is 489 bp encoding the 162 amino acids of RAB16A protein. The protein accumulates in rice embryo, leaves, roots and callus-derived suspension cells upon treatment with exogenous NaCl and ABA or during drought and cold acclimation. The potentiality of RAB16A protein in conferring salt tolerance in transgenic plants has been established earlier (RoyChoudhury et al., 2007) and the protein was found to express at a much higher level in salt-tolerant rice cultivars than salt-sensitive ones (Roychoudhury et al., 2008). Despite considerable research in this field, definitive proof of the intracellular localization of RAB16A protein or its structure-function correlation remains elusive. Several previous propositions have indicated RAB21 to be a nuclear protein, marked by the presence of nuclear localization signals (NLS), and suggested protein targeting from the cytoplasm to the nucleus, being mediated by heavy phosphorylation with kinases during stress (Goday et al., 1994; Jensen et al., 1998). However, this remains yet to be well established through clear experimental evidences and a distinct knowledge of the RAB16A protein structure along with its transport and localization will aid our understanding of the role of this protein in cell signaling pathway during tolerance mechanism. Thus, for the further detailed characterization of the RAB16A protein,

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Abbreviations: cDNA, complementary DNA; **FL**, full length; **GST**, glutathione S-transferase; **IPTG**, isopropyl thiogalactoside; **ORF**, open reading frame; **PCR**, polymerase chain reaction; and **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

we have amplified the cDNA of *Rab16A* from the salt-tolerant rice cultivar Nonabokra, cloned the fragment in a bacterial expression vector, creating a system so as to overproduce the protein in huge amounts in *Escherichia coli* (*E. coli*), followed by the protein purification. This would serve as a readily and easily available source of the protein for further biochemical assays or molecular analysis and generating structural or bioinformatic data.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Oryza sativa* L. cv. Nonabokra obtained from Central Soil Salinity Research Institute (Canning, West Bengal, India), were surface sterilized with 0.1% (w/v) $HgCl_2$ for 20 min, washed extensively, imbibed in deionised water for 6 to 8 h and allowed to germinate over water-soaked sterile gauge in Petri dishes at 37°C in dark for 3 days. The germinated seedlings were grown in presence of 0.25 X Murashige and Skoog (MS) complete media at 32°C under 16 h light and 8 h dark photoperiodic cycle with 50% relative humidity and 700 µmol photons m^{-2} s^{-1} for the desired period in a plant growth chamber (NIPPON, LHP-100-RDS, Tokyo, Japan). Salt stress was imposed by treating the 10 day old plants with 0.25 X MS medium supplemented with 200 mM NaCl for 16 h.

RT (Reverse transcriptase)-PCR amplification of *Rab16A* cDNA

Total RNA was isolated from the young leaf tissues of salt-treated (200 mM NaCl, 16 h) seedlings of Nonabokra following the method of Longhurst et al. (1994). RNA samples were treated with RNase-free DNase I (Boehringer Mannheim). RT reactions were carried out with 5 µg of total RNA using Thermoscript™ RT-PCR system kit (Life Technologies, USA) and the oligonucleotide primer pairs Rab16-5A (5'-CGACAAGCTTCACGCGCACAGTAC ACACACAT-3') and Rab21-3B (5'-ATAGAGCTCTGGATCCTCAGTGCTGGCCG GGCAGCTT-3') designed according to the published nucleotide sequence of this gene in the database (Accession No.Y00842) by Mundy and Chua (1988). PCR reaction was done in a thermocycler (Perkin Elmer, 2400, USA) by running 30 cycles of 1 min at 94°C, 65°C for 1 min and 72°C for 2 min. The PCR product was purified following QIAquick PCR purification kit protocol (QIAGEN).

Bacterial overexpression of GST: RAB16A (FL) fusion protein

The purified PCR product of *Rab16A* cDNA was sub-cloned at the BamHI site of the dephosphorylated pGEX-3X (Amersham Biosciences, USA), a glutathione S-transferase (GST) gene fusion vector, and transformed into BL21 strain of *E. coli* cells following the standard protocols (Sambrook and Russell, 2001). The appropriate clone was induced with 2 mM IPTG (freshly prepared) at 44°C for 4 h. The cells were then harvested at 5,000 X g for 5 min at 4°C and washed once with 1X Tris buffered saline (TBS). About 4 ml of buffer A (100 mM Tris Cl, pH 8.0; 10 mM EDTA pH 8.0; 20 mM β-mercaptoethanol; 10 µg ml^{-1} leupeptin and 1 mM PMSF) was added to the pellet and it was sonicated by 5 - 6 pulses of 10 s each, with 30 s interval, keeping the tubes on ice. It was then centrifuged at 10,000 X g for 10 min at 4°C to collect the supernatant (S10), from which the fusion protein was purified using glutathione-sepharose affinity resin.

Purification of the fusion protein

About 300 µL of glutathione-sepharose was mixed with 700 µL of 1X phosphate buffered saline (PBS) in a sterile microfuge tube, mixed carefully by inverting the tube and centrifuged at 2,000 X g for 5 min at 4°C. The supernatant was discarded and this step was repeated at least 3 - 4 times to equilibrate the column material with PBS. It was finally equilibrated in buffer A. The S10 fraction was added to the gel bed, incubated at 4°C with gentle rocking motion for 30 min, centrifuged at 5,000 X g for 10 min at 4°C, and the supernatant discarded. The gel bed was washed in this manner several times and the fusion protein was ultimately eluted from the affinity resin with the elution buffer (50 mM Tris Cl, 10 mM reduced glutathione pH 8.0) at 4°C for 30 min. The band of the fusion protein was cut from the unstained gel with the help of a sterile surgical blade by aligning it with the marker lane, which was only stained. It was then macerated in Downs' homogenizer with 500 µL of 1X PBS and incubated overnight in the same buffer at 4°C. The homogenized sample was ultimately passed through a 24 gauge needle several times and the fraction preserved for the monitoring of the purified fusion protein band through SDS-PAGE analysis.

Protein immunoblot analysis

About 10 µg of the purified fusion protein was transferred electrophoretically to a PVDF (Hybond-P from Amersham Pharmacia) membrane, blocked with 1% (w/v) non fat dried milk in 1X TBS containing 0.05% (v/v) Tween 20 for 2 h at room temperature (25°C), washed repeatedly with the same buffer and incubated with the maize anti-dehydrin (RAB16 like) antibody (a generous gift from Prof. Timothy J. Close) at 1:1000 dilution overnight at 4°C. The membrane was next incubated with goat anti-rabbit IgG alkaline phosphatase conjugate at 1: 1000 dilutions. The cross-reacted band of GST: RAB16A (FL) fusion protein was detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates.

RESULTS AND DISCUSSION

Because of the vast fund of knowledge about its genetics, biochemistry and molecular biology, *E. coli* is the system of first choice for expression of many heterologous proteins. Genetic manipulations are straightforward and many foreign proteins are well-tolerated and may be expressed at high levels. Expression of fused reading frames generates hybrid proteins, in which the protein of interest is attached to a carrier protein (Uhlen and Moks, 1990; La Vallie and McCoy, 1995). Fusion proteins expressed from pGEX vector contains a GST moiety and can therefore be purified to near homogeneity by affinity chromatography on glutathione-agarose matrix. Bound GST fusion proteins are readily displaced from the column by elution with buffers containing free glutathione. Rice RAB proteins are essentially believed to act as free radical scavengers and structural stabilizers with chaperone-like properties having an array of nuclear and cytoplasmic targets (Rorat, 2006). In order to further elucidate the mechanism of RAB16A protein action, our objective was the overproduction of the protein in *E. coli* as a fusion protein followed by its purification. RT-PCR using gene specific primers with total RNA from salt-treated Nonabokra leaf showed *Rab16A* transcript of approximately 0.5 Kbp (Figure 1a). The PCR product was subcloned at the BamHI site of *E. coli* GST-fusion expres-

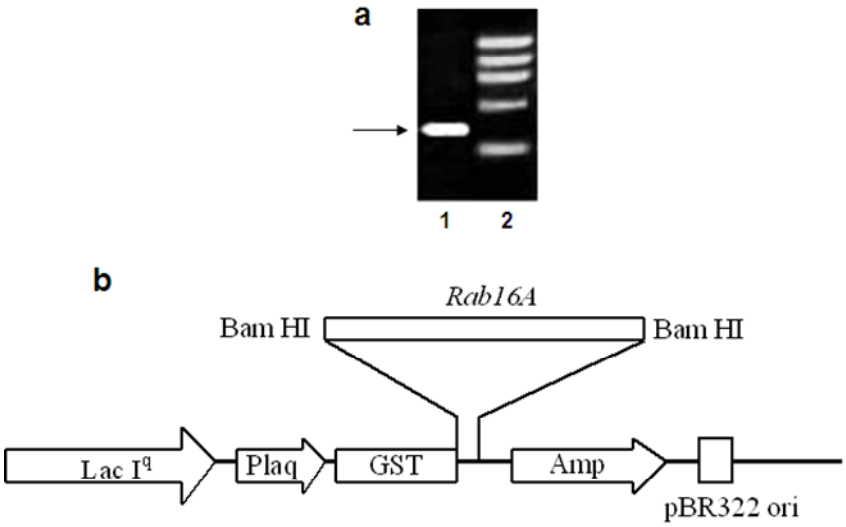

Figure 1. RT-PCR amplification of *Rab16A* transcript and its cloning in pGEX-3X vector at BamHI site: (**a**) 0.5 Kbp RT-PCR product (lane 1) along with marker (lane 2), and (**b**) physical map of the recombinant plasmid pGEX-3X: *Rab16A*.

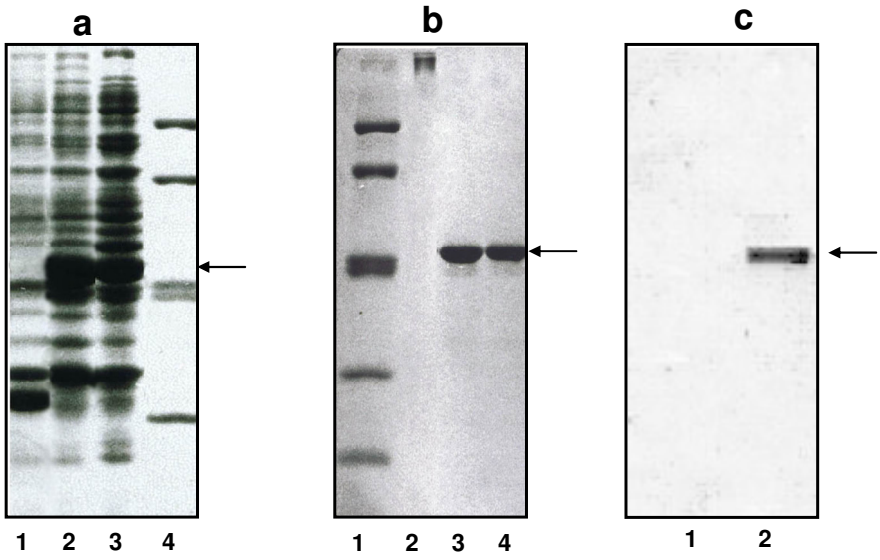

Figure 2. Analysis of bacterially expressed fusion protein GST: RAB16A in 12% SDS-polyacrylamide gel and immunoblot analysis: (**a**) fusion protein expressed after induction with IPTG (lanes 2 and 3), no expression of protein in lysate loaded in lane 1, marker protein in lane 4, (**b**) purified recombinant fusion protein of around 47 kDa (lanes 3 and 4) along with marker protein (lane 1), purified band absent in lysate containing only the pGEX-3X: GST (lane 2), and (**c**) hybridization of the maize dehydrin antiserum with the fusion protein (lane 2) but not with GST protein alone (lane 1).

sion vector pGEX-3X (Figure 1b) and the fusion protein GST: RAB16A, induced after IPTG induction, was observed in 12% SDS-PAGE (Figure 2a). Following purification through glutathione-sepharose, a single band of the fusion protein of 47 kDa (21 kDa of RAB16A FL + 26 kDa of GST) was visualized again in 12% SDS-PAGE (Figure 2b). Immunoblot analysis with the maize dehydrin antiserum (a generous gift from Prof. Timothy J. Close) showed cross-reaction with the 47 kDa GST: RAB16A protein band, but not with the GST protein alone, indicating functional expression of the rice RAB16A (FL) protein in *E. coli* (Figure 2c); the pre-immune antiserum also did not cross-react with the 47 kDa polypeptide band (data not shown) pointing to the specificity of the binding.

Proteolytic cleavage of RAB16A protein from the remainder of the fusion protein is underway so as to derive the protein in a native and biologically active form. In future, the cellular localization of the protein will be established either by immuno-electron microscopy and its targeting to the nucleus will be studied through transgenic approaches either by using purified RAB16A protein tagged to fluorescent markers like green fluorescent protein (GFP) or creating translational fusion of the protein to β-glucuronidase (GUS) reporter protein followed by histochemical and biochemical analysis for GUS activity. *In vitro* phosphorylation assay of the purified protein with different kinases will also assess whether phosphorylation mediates protein accumulation and function in different cellular compartments.

ACKNOWLEDGMENTS

Financial assistance from Department of Biotechnology, Government of India and Bose Institute research fellowship are gratefully acknowledged.

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