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Research paper

A temperature-responsive gene in sorghum encodes a glycine-rich protein that interacts with calmodulin

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ABSTRACT

Imposition of different biotic and abiotic stress conditions results in an increase in intracellular levels of Ca^{2+} which is sensed by various sensor proteins. Calmodulin (CaM) is one of the best studied transducers of Ca^{2+} signals. CaM undergoes conformational changes upon binding to Ca^{2+} and interacts with different types of proteins, thereby, regulating their activities. The present study reports the cloning and characterization of a sorghum cDNA encoding a protein (SbGRBP) that shows homology to glycine-rich RNA-binding proteins. The expression of *SbGRBP* in the sorghum seedlings is modulated by heat stress. The SbGRBP protein is localized in the nucleus as well as in cytosol, and shows interaction with CaM that requires the presence of Ca^{2+}. SbGRBP depicts binding to single- and also double-stranded DNA. Fluorescence spectroscopic analyses suggest that interaction of SbGRBP with nucleic acids may be modulated after binding with CaM. To our knowledge, this is the first study to provide evidence for interaction of a stress regulated glycine-rich RNA-binding protein with CaM.

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1. Introduction

Plant cells encounter different developmental, hormonal and environmental stimuli, which are sensed by various secondary messengers (Ca^{2+}, cAMP, IP3, etc) [1]. Calcium is one of the most important secondary signal transducers whose intracellular levels are modulated in response to different biotic and abiotic stress conditions. Under normal conditions, the plant cells maintain low level of cytosolic Ca^{2+} (nearly 100 nM) [2]. Imposition of different stresses can lead to transient increase in intracellular Ca^{2+}, which is detected by different Ca^{2+} sensors such as calmodulin (CaM), CaM-like (CML) proteins [3], Ca^{2+}-dependent protein kinases (CDPKs) [4], and calcineurin B-like proteins [5]. CaM, a conserved, small acidic protein comprising of 148 amino acid residues, is one of the best characterized Ca^{2+}-sensors. CaM consists of four EF-hand motifs, each of which is involved in binding to Ca^{2+}. Conformational changes in CaM, after binding to Ca^{2+}, lead to interaction with different target CaM-binding proteins (CaMBPs) which comprise of disparate members that perform a diverse range of roles in the cell [6].

Exposure of plants to different abiotic stress conditions results in differential induction and repression of different proteins that enable the plant cells to tolerate adverse environmental conditions. Earlier studies have shown that several of the proteins modulated by stress also show interaction with CaM [7], implying that these proteins play an important role in stress adaptation response of plants. Identification of stress-regulated CaMBPs in plants is,

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therefore, imperative for understanding the molecular mechanisms of plant stress tolerance, a highly complex and multigenic trait. Protein microarray studies in *Arabidopsis thaliana* demonstrated that there may be several hundreds of different proteins that may bind to CaM in plants [8]. We are focusing our attention on identification of different CaMBPs that are expressed in sorghum, which is considered as a model crop for studying mechanisms responsible for tolerance to heat and drought stress [9]. Due to lack of conservation in the amino acid sequence of the CaM-binding domains (CBDs) in different CaMBPs [10], these proteins are identified only through their functional interactions with CaM-affinity matrix. Earlier studies in our lab on sorghum resulted in identification of several CaM-interacting proteins [11]. These studies demonstrated that HSP90 interacts with CaM, and steady state levels of this protein are regulated through Ca^{2+}-CaM pathway(s) [11,12]. Except for these reports, information on the proteins that bind to CaM in sorghum, to our knowledge, is lacking.

Glycine-rich proteins are implicated in several cellular functions such as plant defence response [13], pollination [14], osmotic- [15] and cold stress [16], regulation of flowering and development [17], etc. In the present study we report the cloning of cDNA encoding a stress-modulated glycine-rich RNA-binding protein in sorghum (designated as SbGRBP) that interacts with CaM in a Ca^{2+}-dependent manner. To our knowledge, interaction of CaM with a glycine-rich protein has not been reported earlier. Further, we also provide *in vitro* evidence that SbGRBP binds to both single- (ssDNA) and double-stranded DNA (dsDNA) and that the interaction is modulated by CaM.

2. Materials and methods

2.1. Purification and identification of CaM-binding proteins

Extraction and purification of CaM-binding proteins (CaMBPs) was carried out as described earlier [11]. The proteins were concentrated by dialysis against 50% glycerol, quantified [18] and resolved by 10% SDS-PAGE [19] followed by staining with Coomassie Brilliant Blue R-250 under aseptic conditions. The individual protein bands were excised [11] and subjected to MALDI-ToF/ToF MS analysis using ABI Sciex 5800 ToF/ToF system coupled with LC-MALDI at Advanced Instrumentation Research Facility, JNU, New Delhi, India. Analysis was done in a positive-ion detecting reflectron mode and the instrument was calibrated with a mixture of calibration proteins standards (CAL-1 from SIGMA, USA). Homology of the peptide fragments with known proteins in the database was established by analysis using the software MASCOT.

2.2. Quantitative real-time polymerase chain reaction

RNA was extracted as described earlier [20] and checked by denaturing gel electrophoresis. cDNA from 1 μg of total RNA was prepared using Superscript III reverse transcriptase (Invitrogen, USA) as per manufacturer's instructions. Quantitative PCR (qRT-PCR) was carried out using specific primers for cDNA encoding the SbGRBP (Supplemental Table 1) with SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, China) on an Mx3000P Real-Time PCR Detection System (Agilent technologies, USA). Each qRT-PCR reaction contained 5 μL SYBR® Green (Applied Biosystems, UK), 0.2 μM forward and reverse primers and 1 μL of cDNA template. The PCR amplification details are as follows: 95 °C for 10 min; 40 cycles of: 95 °C for 30s, 56 °C for 30 s and 72 °C for 30s; followed by a melting-curve program of 95 °C for 1 min, 56 °C for 30s and 95 °C for 30s. The expression was normalized using actin gene as an internal reference.

2.3. Cloning of cDNA encoding SbGRBP

PCR-amplifications for cloning of cDNA encoding SbGRBP and its truncated variants $SbGRBP_{38\text{-}147}$ and $SbGRBP_{61\text{-}147}$, that contained amino acid residues 38–147 and 61–147, respectively, were performed using gene-specific primers with Pfu polymerase. Primer sequences are listed in Supplemental Table 1. PCR conditions used for amplification were 95 °C for 3 min; 35 cycles of 95 °C for 30 s, annealing temperature for 40 s, extension at 72 °C for 1 min followed by 72 °C for 7 min. The amplification was carried out using one unit of Pfu polymerase. The PCR-amplified products were analyzed on 1.2% agarose gel and extracted using gel extraction kit (Merck Specialities Pvt. Ltd., India) as per the manufacturer's protocol. The purified amplicons of cDNA encoding full length SbGRBP and $SbGRBP_{61\text{-}147}$ were cloned in pET-28a(+) cloning vector, whereas the amplicon for $SbGRBP_{38\text{-}147}$ was cloned in pGEX-6P1 vector after digesting with *Eco*RI and *Xho*I. The cloning was confirmed by sequencing.

2.4. Heterologous expression and purification of recombinant proteins

Expression of full length SbGRBP and truncated proteins ($SbGRBP_{38\text{-}147}$ and $SbGRBP_{61\text{-}147}$) was carried out in *E. coli* strain BL21 (DE3) pLysS by inducing with 1 mM isopropyl thiogalactoside when the absorbance at 600 nm reached 0.5–0.6 at 37 °C. After induction, the *E. coli* cells were further incubated for 4 h at 25 °C. Purification of SbGRBP was performed using methods that avoid co-purification of DNA [21]. After centrifugation at 6000 rpm for 10 min at 4 °C, the recombinant cells were pelleted and suspended in lysis buffer [50 mM sodium phosphate (pH 8.0), 8 M urea, 500 mM NaCl, 10 mM imidazole, 0.25% Triton X-100, 10% glycerol, 1 mM phenyl methyl sulfonyl fluoride (PMSF)] followed by sonication for 4 min on ice; each pulse of 9 s with 5 s resting period. The cell lysate was centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was filtered through 0.45 μM filter and passed through Ni-NTA column that was pre-equilibrated with binding buffer [50 mM sodium phosphate (pH 8.0), 10 mM imidazole, 500 mM NaCl] at flow rate of 0.5 ml/min. The column was washed with thirty bed-volumes of linear gradient of urea (8 M - 0 M) at flow rate of 0.5 ml/min for renaturation of the matrix-bound SbGRBP. The column was eluted with three bed-volumes of elution buffer [50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 250 mM imidazole, 0.25% Triton X-100, 10% glycerol, 0.2 mM PMSF]. The eluted proteins were desalted by dialysis against 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl, and stored at −80 °C until further analysis. For purification of GST-tagged protein, the pelleted recombinant cells were suspended in lysis buffer [50 mM Tris-HCl (pH 8.0); 100 mM NaCl] followed by sonication and centrifugation. The supernatant was incubated with GST beads (Novagen) for 2 h according to manufacturer's instructions. The beads were washed with thirty bed-volumes of lysis buffer and the protein was eluted with elution buffer containing glutathione [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 50 mM glutathione]. Homogeneity of the purified proteins was confirmed by SDS-PAGE.

2.5. Calmodulin-pull down assays

100 μg of the purified recombinant SbGRBP protein, suspended in binding buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 0.2 mM PMSF], was mixed in 500 μl CaM-agarose bead (Sigma Aldrich, USA) in sterile tubes [11]. For studying the effect of Mg^{2+}, Mn^{2+}, Zn^{2+} and La^{2+} on interaction of SbGRBP with CaM, the $CaCl_2$ was replaced with $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $LaCl_3$. The tubes were placed on a slow speed roller at 25 °C for 1 h, followed by

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