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Materials and Methods

3.1 Seed Material

Young seedlings of sorghum were used for the purification of calmodulin-binding proteins. The seeds of *Sorghum bicolor* (L.) Moench were procured from National Seed Corporation and Indian Agriculture Research Institute, New Delhi.

3.2 Chemicals

All the necessary chemicals were of analytical and molecular biology grade and were procured from Sigma-Aldrich, USA; GE Healthcare Bio-Sciences AB, Uppsala, Sweden; Enzo Life Sciences, Plymouth Meeting, USA; Alexis Grünberg, Germany and local suppliers.

3.3 Seed Germination

The seeds of sorghum (*S. bicolor* L. Moench) cultivar GK908 were surface sterilized with 0.1% (m/v) mercuric chloride and 70% ethanol, followed by washing with autoclaved double distilled water (ADDW). Seeds were imbibed overnight (12 h) in ADDW at 37 °C and germinated in petri plates containing autoclaved non-absorbent wet cotton, which were incubated in the dark at 37 °C for 24 h.

3.4 Purification of Calmodulin-Binding Proteins (CaMBPs)

The CaMBPs were purified from the sorghum seedlings, which were subjected to osmotic- and heat stress. Osmotic stress was imposed by irrigating the 24 h-old seedlings with 0.75 M mannitol solution for 24 at 37 °C under dark and heat stress was imposed by exposing the seedlings to 45 °C for 24 h. Seedlings were harvested after stress imposition and stored in liquid N₂ for further analysis.

3.4.1 Extraction of proteins

5 g of sample was ground in liquid N₂ with mortar and pestle. Fine powder was transferred to autoclaved pre-chilled Oakridge tubes and 25 ml of ice-chilled extraction buffer [50mM Tris buffer (pH 7.5), 0.1% Triton X-100, 2 mM DTT, 30 µl protease inhibitor cocktail] was added. Tubes were vortexed briefly for homogenization of powder with buffer. Tubes containing protein solution were kept on ice for 30 min and centrifuged in fixed angle rotor at 10,000 rpm at 4 °C for 30 min. Supernatant was collected and transferred to fresh, pre-chilled Oakridge tubes and re-centrifuged at
16000 rpm at 4 °C for 40 min. After centrifugation, the supernatant was collected in 250 ml autoclaved centrifugation bottle after filtering through 0.45 µm filters, using vacuum filter assembly, and kept on ice at 4 °C for protein estimation. Total proteins were estimated according to Bradford’s micro method (Bradford, 1976) using different concentrations of bovine serum albumin (BSA) (2-10 µg) as a standard. In brief, different volumes of BSA stock solution were diluted with ADDW to final concentrations of 2, 4, 6, 8 and 10 µg in 100 µl reaction volume. 1ml of Bradford’s reagent was added and tubes were vortexed gently. Absorbance was recorded at 595 nm on a UV/Visible Spectrophotometer (Lambda Bio 20, Perkin Elmer).

3.4.2 Calmodulin-Sepharose 4B affinity chromatography

3.4.2.1 Column packing

Cooling jacket column (XK 16/20 Amersham Bioscience) was fixed upright on a stand with the help of clamps and the outer jacket of column was connected to water circulator at 4 °C. Small volume of buffer was injected from lower adaptor with the help of syringe. Slurry of Calmodulin-Sepharose 4B was poured gradually into column from upper outlet, after removing upper adaptor, with the help of 5 ml pipette tip along the inner surface of the wall of column and, if necessary, with gentle tapping to expel any air bubbles. The outlet stopper was immediately opened after pouring the matrix. Matrix was allowed to settle down at a flow rate of 680 µl/min (70% of operational flow rate) using peristaltic pump (Pharmacia LKB, Pump P1). The bed volume and void volume of packed column were 23.5 ml and 7.8 ml, respectively.

3.4.2.2 Column regeneration

Calmodulin-Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) matrix was regenerated according to manufacturer’s instruction. The regeneration procedure was performed at 4 °C, using circulating water bath (Amersham Bio Sciences, MultiTemp III) as following:

- Column was washed with 3 bed volumes of 100 mM ammonium carbonate buffer (pH 8.6) containing 2 mM ethylene glycol-bis(2-aminoethyl ether)-N, N, N’, N’-tetraacetic acid (EGTA) followed by washing with 3 bed volumes of 1M NaCl containing 2 mM CaCl₂.
Column was then washed with 3 bed volumes of 100 mM sodium acetate buffer containing 2 mM CaCl$_2$. This was followed by washing with 3 bed volumes of binding/equilibration buffer [50 mM Tris buffer (pH 7.5), 200 mM NaCl, 2 mM CaCl$_2$, 2 mM DTT, 0.2 mM phenylmethanesulfonyl fluoride (PMSF)].

For short term storage, 0.1 mM sodium azide was used while for long term storage, the equilibration buffer was replaced with ice cold 20% ethanol.

### 3.4.2.3 Purification of CaMBPs

**Analytical method**

- Purification of CaMBPs on regenerated Calmodulin-Sepharose 4B matrix was carried out at 4 °C by passing 50 mg of total proteins through the column.
- The flow rate of protein solution was 200 µl/min during binding and final concentration of protein was 200 µg/ml. Column was extensively washed by equilibration buffer (3-4 bed volumes) at flow rate of 400 µl/min, until the absorbance of flow through reached zero at 280 nm.

**Preparative method**

- For large scale purification of CaMBPs, 400 mg of total proteins solution (285 ml) and 50 ml of regenerated CaM-Sepharose 4B affinity matrix were poured in hybridization bottles (240x68 mm) and placed on low profile roller (Innova 4230, New Brunswick Scientific, Edison, NJ, USA) for 16 h at 4 °C. Final concentration of protein was 200 µg/ml.
- After binding, the matrix was allowed to settle down. The supernatant was discarded and the matrix was loaded on to the column gently using wide bore 5 ml pipette tip.
- Column was packed at a flow rate of 400 µl/min and extensively washed by equilibration buffer (3-4 bed volumes) at flow rate of 200 µl/min, until the absorbance of flow through reached zero at 280 nm.
- The proteins were eluted with elution buffer [50 mM Tris buffer (pH 7.5), 200 mM NaCl, 10 mM EGTA, 2 mM dithiothreitol (DTT), 0.2 mM PMSF] in which CaCl$_2$ was replaced with EGTA.
- Eluted protein solution was dialyzed against 10 mM tris (pH 7.5) and 0.2 mM PMSF for 24 h at 4 °C with continuous stirring.
Proteins were concentrated by dialysis against 50% glycerol and estimated according to Bradford (1976).

For further purification of the affinity-purified CaMBPs, various chromatographic techniques were used.

### 3.4.3 Fractionation of CaMBPs by gel exclusion chromatography

40 ml of Sepahcryl S-200 (GE Health Sciences) was loaded on the XK42/10 cooling jacket column. Column was packed at a flow rate of 200 µl/min using peristaltic pump followed by washing with 5-6 bed volumes of tris buffer saline (TBS) [50 mM Tris (pH 7.5), 150 mM NaCl, 0.2 mM PMSF]. The bed volume and void volume were calculated using blue dextran.

2.25 mg of affinity-purified CaMBPs (2.25 ml) containing 0.2 mM PMSF were loaded on to the matrix carefully without disturbing the bed.

The proteins were fractionated with TBS (pH 7.5) at a flow rate of 100 µl/min in 1 ml fractions at 4 °C and stored at –20 °C till further analysis.

The column was regenerated with 4 bed volumes of 0.2 M NaOH followed by 5 bed volumes of TBS (pH 7.5) containing sodium azide (0.2 mM) and stored at 4 °C.

### 3.4.4 Cation-exchange chromatography

Activation of pre-swollen carboxymethyl (CM)-cellulose (4g) (Sigma-Aldrich, USA), cation exchange matrix, was carried out by suspending in 50 ml of buffer [50 mM sodium acetate (pH 5.5), 0.2 mM PMSF]. The matrix was incubated at 37 °C for 15-18 h for activation with intermittent stirring.

The excess of buffer was decanted along with fine particles to obtain slurry of reasonable thickness and it was degassed under vacuum for 10 min.

Activated CM-cellulose slurry was poured gradually into XK16/10 cooling jacket column from upper outlet, with the help of wide bore 1 ml pipette tip along the inner wall of column. Outlet stopper was immediately removed to drain excess buffer from the column and the matrix was allowed to settle down evenly under gravitational force.
The column was packed at a flow rate of 400 µl/min. The bed volume of the column was 23 ml. Column was equilibrated with 5 bed volumes of equilibration buffer [50 mM sodium acetate (pH 5.5), 0.2 mM PMSF].

Total of 2.0 mg of CaM-affinity purified protein sample (2.0 ml) was loaded onto the matrix carefully without disturbing the bed. The peristaltic pump was connected to the column through upper adaptor (inlet) and the lower adaptor (outlet) was connected with UV detector and recorder.

Proteins were allowed to bind by passing it through the column at a flow rate of 50 µl/min. The flow through was collected and reloaded on the column.

The final flow through was collected and stored at -20 °C for SDS-PAGE analysis. The column was washed with equilibration buffer [50 mM sodium acetate (pH 5.5), 0.2 mM PMSF] till the absorbance of buffer reached zero at 280 nm.

The proteins were eluted with the buffer [50 mM sodium acetate (pH 5.5), 0.2 mM PMSF] containing a linear gradient of NaCl (0 - 500 mM). Eluted proteins were collected in 1ml fractions and stored at –20 °C.

The column was regenerated with 4 bed volumes of 1M NaCl followed by 5 bed volumes of regeneration buffer [50 mM sodium acetate (pH 5.5), 0.2 mM PMSF, 0.2 mM sodium azide] and stored at 4 °C.

3.4.5 Anion-exchange chromatography

For matrix activation, 4.0 g of pre-swollen diethylaminoethyl (DEAE)-cellulose, an anion exchange matrix (Sigma-Aldrich Chemicals Pvt. Ltd., USA), was suspended in 50 ml of Tris buffer [50 mM Tris (pH 8.0), 0.2 mM PMSF] and kept for activation at 37 °C for 15-18 h with intermittent stirring. The excess of buffer was decanted along with fine particles to obtain slurry of reasonable thickness.

XK16/10 cooling jacket column was packed with activated DEAE-cellulose as described in the previous section.

DEAE-cellulose column was equilibrated with 5 bed volumes of equilibration buffer [50 mM Tris (pH 8.0), 0.2 mM PMSF].
2.0 mg of protein sample (2.0 ml) was loaded on to the matrix without disturbing the bed.

Proteins were allowed to bind by passing through the column at a flow rate of 50 µl/min. The flow through was collected and the column was washed with 3 bed volumes of 50 mM tris buffer (pH 8.0).

The proteins were eluted with elution buffer [50 mM Tris buffer (pH 8.0), 0.2 mM PMSF] containing linear gradient of NaCl (0 - 500 mM). Eluted proteins were collected in 1 ml fractions and stored at –20 °C.

The column was regenerated with 4 bed volumes of 1M NaCl followed by 5 bed volumes of buffer [50 mM Tris buffer (pH 8.0), 0.2 mM sodium azide] and stored at 4 °C.

3.4.6 Hydrophobic interaction chromatography

Phenyl Sepharose 6B CL (Pharmacia LKB) was used for hydrophobic interaction chromatography. 10 ml of matrix was mixed with 20 ml of 50 mM sodium phosphate buffer (pH 7.5) and packed into XK16/10 column.

10 mg affinity-purified CaMBPs (10 ml) were mixed with 10 ml binding buffer [50 mM sodium phosphate buffer (pH 7.0), 1.7 M ammonium sulphate, 0.2 mM PMSF] and kept on ice for 30 min.

Protein solution was loaded on to the column and allowed for binding at a flow rate of 100 µl/min. Column was washed with binding buffer till the absorbance reached zero at 280 nm.

The bound proteins were eluted with 50 mM sodium phosphate buffer (pH 7.0) containing linear gradient of ammonium sulphate (1.7 - 0 M) and analyzed by SDS-PAGE.

3.4.7 Concanavalin A-Agarose affinity chromatography

Pre-packed Concanavalin A (Con A)-Agarose affinity matrix (Bangalore Genei Pvt. Ltd., Bengaluru, India) was used to purify the glycoproteins and the entire procedure was carried out at 4 °C.

Column was washed with 3 bed volumes of binding buffer [10 mM Tris buffer (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂] at a flow rate of 1 ml/min.
➢ Total of 1 mg affinity-purified CaMBPs (6.8 ml) were loaded on to the column under gravity.

➢ Flow through was collected in 10 ml sterile vials and column was extensively washed with binding buffer till the absorbance of proteins became zero at 280 nm.

➢ For the elution of Con A-binding proteins, the column was washed with elution buffer [10 mM Tris buffer (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 0.5 M methyl-α-D-mannopyanoside].

➢ Eluted proteins were dialyzed against 10 mM Tris buffer (pH 7.5) for 16 h followed by 50% glycerol for overnight at 4 °C. The proteins were analyzed by SDS-PAGE.

3.5 Polyacrylamide Gel Electrophoresis (PAGE) of Calmodulin-Binding Proteins

3.5.1 Native-PAGE analysis of CaMBPs

CaM-Sepharose 4B affinity-purified proteins were analyzed by native-PAGE under non denaturing conditions as following:

➢ Glass plates were soaked in chromic acid for overnight followed by washing with ADDW. Plates were air dried in hot air oven for 2 h at 100 °C.

➢ 1.5 mm thick spacers were placed between the notched and back glass plates and the glass plates were sealed with cello tape.

➢ 25 ml of 8% continuous polyacrylamide gel was prepared by mixing all components [8% acrylamide/bisacrylamide, 375 mM Tris buffer (pH 8.8), 0.1% ammonium persulfate (APS)] in 200 ml conical flask. Solution was degassed under vacuum and poured between sealed glass plates after adding 0.1% N, N, N’, N’-tetramethyl ethylene diamine (TEMED).

➢ Polyacrylamide gel was allowed for polymerization at RT for overnight. Cello tape was removed from the lower end of the gel plates. The wells were washed with running buffer [25 mM Tris base, 250 mM glycine] using disposable syringe. Upper and lower tanks were filled with 2.5 l tris-glycine running buffer.

➢ 100 µg of protein sample was mixed with sample buffer [Tris buffer (pH 8.8), 20% glycerol, 0.04% bromophenol blue] in 1:1 ratio, and loaded in the wells.
followed by electrophoresis at 4 °C. The gel was stained by silver staining method.

3.5.2 Silver staining

- All silver staining solutions were prepared fresh in ADDW.
- Gel was fixed in fixing solution (50% methanol, 10% acetic acid) for 6-8 h on rocker. Gel was washed thrice with ADDW and dipped in 50% ethanol for 30 min followed by three washings with ADDW.
- Gel was sensitized with sodium thiosulfate (0.05 %) for 1 min and briefly washed twice with ADDW. The gel was dipped in silver nitrate solution (0.2%), containing formaldehyde (0.15%) as reducing agent, for 30 min followed by brief washing with ADDW.
- For development of protein bands, the gel was dipped in cold developing solution [sodium carbonate anhydrous(6%), formaldehyde (0.12%)]. Developing reaction was quenched with acetic acid (4%) after desired colour intensity of protein bands was achieved.
- Gel was documented on ALPHADOC GEL IMAGER [Model Alpha Ease (standard)] and stored in preservative solution [glycerol (10%), acetic acid (1%)] at room temperature (RT).

3.5.3 Sodium dodecyle sulphate (SDS)-PAGE analysis of CaMBPs

- SDS-PAGE analysis was performed according to Laemmli (1970). Glass plates were prepared as described earlier. SDS-PAGE gels of different pore size were prepared according to Sambrook and Russell (2001). Gels were allowed to polymerize for 4 h to overnight according to the size of gels.
- Tris-glycine-SDS running buffer [25 mM Tris base, 250 mM glycine, 0.1% SDS] was filled in both upper and lower tanks. 2X Laemmli buffer [125 mM Tris buffer (pH 6.8), 4% SDS, 20% glycerol, 2% β-mercaptoethanol (β-ME), 0.04% bromophenol blue dye] was used for sample denaturation. Sample and Laemmli buffer were mixed in equal volume followed by heat denaturation at 95 °C for 5 min before loading.
Stacking of the proteins was carried out at 120 V constant and resolving at constant 30 mA. Following electrophoresis, the gels were stained with silver staining method.

3.6 2-Dimensional Gel Electrophoresis

3.6.1 Protein precipitation and solubilisation

- 10 mg CaM-affinity purified proteins were precipitated using pre-chilled 20% trichloroacetic acid (TCA)/acetone containing 0.07% β-ME and 15 µl/ml protease inhibitor cocktail and incubated at -20 °C for 2 h.
- The precipitated proteins were centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet was washed thrice with pre-chilled 100% acetone (containing 0.07% β-ME) for 5 min each.
- The sample was solubilised in solubilisation buffer [5 mM Tris buffer (pH 7.5), 2% CHAPS, 8 M urea, 2% NP-40, 15 µl/ml protease inhibitor cocktail, 2% Ampholine (pH 3.5-10)] and kept for 30 min at 10 °C followed by protein estimation according to Bradford (1976).

3.6.2 Isoelectric focusing

- Isoelectric focusing (IEF) was performed in tube gels. Tubes were washed with chromic acid followed by extensive washing with running water. The inner surface of glass tubes was sialinized using Repel-Silane ES (GE Health Sciences). Tubes were washed with ADDW and used for IEF.
- IEF was carried out according to O’Farrell (1975). 5% polyacrylamide gel [containing 5% acrylamide/bisacrylamide, 8 M urea, 2% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), 2% NP-40, 2% Ampholine (3.5-10.0), 50 mM DTT, 0.1% APS, 0.1% TEMED] was prepared in tubes (120x5 mm) for IEF.
- Tubes were placed in tube gel electrophoresis apparatus (GT1, Hoefer Pharmacia Biotech Inc., San Francisco CA, USA) and lower and upper tanks were filled with 10 mM orthophosphoric acid and 20 mM NaOH buffer, respectively.
- Gels were overlaid with 50 µL of gel overlaying buffer (8 M urea). IEF gels were pre-run at 200 V for 15 min, 300 V for 30 min, 400 V for 30 min and then
power supply was switched off. Upper tank buffer was decanted and the upper surfaces of gels were briefly washed with ADDW.

- Sample proteins (150 µg) were mixed in sample buffer [8 M urea, 2% CHAPS, 2% NP-40, 2% Ampholine (3.5-10.0), 50 mM DTT, 10% glycerol] in 1:1 ratio and loaded on top of the gel after a brief spin at 10,000 rpm at 10 °C. Sample was overlaid with 50 µL of gel overlaying buffer (8 M urea).

- Upper tank was refilled with 1 l upper tank buffer (20 mM NaOH) and IEF was performed at 400 V for 14 h followed by 800 V for 1 h.

- Gels were removed from tubes after IEF and dipped in 20 ml equilibration buffer [50 mM Tris buffer (pH 6.8), 2% SDS, 10% glycerol, 5% β-ME, 8 M urea, 0.04 % bromophenol blue] for 20 min. Tube gels were transferred to SDS-PAGE slab gel and overlaid with low melting point agarose. The separation in the second dimension was performed in polyacrylamide gel (11% T and 2.6% C) at constant current of 30 mA for 3 h and then transferred to fixing solution [50% methanol and 10% acetic acid] for overnight.

- The gels were stained with ProteoSilver Plus silver staining kit (Sigma-Aldrich, USA).

### 3.7 Calmodulin-Binding Gel-Overlay Assay

- CaM-affinity purified proteins were separated by 2-D gel electrophoresis and transferred on to Hybond C membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at 0.8 mA/cm² using TE 70 semi-dry western blotting apparatus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

- The membrane was incubated for 16 h at 4 °C in renaturation buffer [50 mM Tris buffer (pH 7.5), 150 mM NaCl, 1% BSA (v/v), 1 mM CaCl₂, 0.01% Tween 20] for renaturation, followed by washing twice for 5 min each in binding buffer [50 mM Tris buffer (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, 1% BSA].

- The membrane was then incubated with 1.50 µg of biotinylated CaM (Alexis Grünberg, Germany) at 4 °C for overnight in binding buffer. After washing thrice for 10 min each with renaturation buffer, the membrane was treated with 1µg/ml of streptavidin-alkaline phosphatase conjugate (Sigma-Aldrich, USA) in
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10 ml of binding buffer for 1 h. In control experiments, CaCl$_2$ was substituted with by 5 mM EGTA.

- Bound CaM was visualized by using nitro-blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyphosphate (BCIP) (Sigma-Aldrich, USA) as substrates.

3.8 Mass Spectrometric Analysis of CaMBPs

- 2D-PAGE gel was stained with MALDI-ToF/MS compatible silver staining method. The excision of bands was performed under aseptic conditions using laminar air flow hood. Gel pieces, containing proteins, were transferred to 1.5 ml sterile eppendorf tubes and destained with 500 µl acetonitrile (ACN) for 10 min at RT.

- Destained proteins were reduced in situ at 56 °C for 30 min using 10 mM DTT. Gel pieces were chilled and thoroughly washed with ACN followed by alkylation for 10 min in dark at RT using 50 µl of iodoacetamide (55 mM). DTT and iodoacetamide were prepared in 100 mM ammonium carbonate. For removal of iodoacetamide, gel pieces were washed with ACN.

- In gel digestion with trypsin (Promega, Medison, WI, USA) was carried out for 16 h at 37 °C. Peptides were extracted from the gel by diffusion in 40 µl trifluoroacetic acid (TFA) and ACN in 2:3 ratio by incubation in an ultrasonic bath for 20 min at RT, followed by incubation for 20 min in 50 µl ACN.

- The samples were desalted and concentrated using strong cation exchange, 12 µm, 300 Å pore size Zip Tip pipette tips (ZiptipsSCX, Millipore, USA). Briefly, peptide solution was lyophilized and dissolved in TFA at final concentration of 0.1%. Zip Tip pipette tips were equilibrated thrice with 10 µl TFA (100%). The peptide solution was loaded on Zip Tip pipette tips by performing 10-15 aspirate-dispense cycles in 200 µl eppendorf tubes. Zip Tip pipette tips were washed five times with 10 µl of 1% TFA, followed by elution of bound peptides with 3-5 µl of elution buffer [80% ACN, 20% TFA]. The samples were spotted onto the target plate as per the manufacturer’s instruction along with α-cyano-4-hydroxycinnamic acid matrix (10 mg/ml in 70% ACN solution and 30% of 0.1% TFA) and analysed using MALDI-ToF, AXIMA-CFR Plus spectrometer (Shimadzu/Kratos, Manchester, UK) equipped with a 337-nm nitrogen laser.
Analysis was carried out in a positive-ion detecting reflectron mode and the external calibration was carried out using a mixture of calibration protein standards CAL-1 from (Sigma-Aldrich, USA). Proteins in the tryptic fragments were analyzed using the Mascot search engine (www.matrixscience.com) based on the Mass Spectrometry protein sequence DataBase (MSDB).

3.9 Purification and Identification of Heat Shock Protein SbHsp73

Of the several proteins, which showed interaction with CaM after 2D-CaM gel overlay assay, two of the proteins, corresponding to 73 kDa and 85 kDa, were identified as members of Hsp70 and Hsp90 family, respectively. The two proteins were designated as SbHsp73 and SbHsp85, respectively. Both SbHsp73 and SbHsp85 showed development of colour within 5-10 min after incubation of the blot in the NBT/BCIP substrate, and were selected for further studies.

SbHsp73 was purified and used for raising polyclonal antibodies, which were employed for immunobloting studies. Western blot analysis of SbHsp85 was carried out with anti-\textit{N. crassa} Hsp80 antibodies, which are reported to cross-react with plant Hsp90 (Roychowdhury et al., 1992; Pareek et al., 1995), and were gifted by Dr. Manju Kapoor (Emeritus Professor, University of Calgary, Alberta, Canada).

For further purification of SbHsp73, the CaMBPs, purified after CaM-Sepharose affinity chromatography, were resolved on preparative SDS-PAGE gel.

Gel was subjected to Zn\(^{2+}\)-reverse staining according to Ortiz et al. (1992). Briefly, the gel was incubated in 0.2 M imidazole and 0.1% SDS for 15 min at RT. After brief washing with ADDW, the gel was soaked in 0.2 M ZnSO\(_4\) till the development of transparent bands against a white background was observed.

The 73 kDa band, which corresponded to the CaM-binding protein on Hybond C membrane after 1D-CaM gel overlay assay, was excised and the protein was eluted and electro-dialyzed against 50 mM tris buffer (pH 7.5) for 1 h using Electro-Eluter (Model 422, Bio-Rad laboratories, U.S.A.), as per the manufacturer’s instructions.

The identity of the extracted protein (Hsp73), as a member of Hsp70 family, was reconfirmed by MALDI-ToF/ToF analysis before immunization. The eluted
protein was digested with trypsin and the digested peptides were used for obtaining mass spectrum using MALDI-ToF/ToF (Autoflex II ToF/ToF, Bruker Daltonics, Germany). The obtained spectrum was further used for MASCOT search to get the homology of the peptide fragments with known proteins in the database. This analysis clearly indicated the protein (SbHsp73) to be a member of Hsp70 family.

3.10 Raising of Polyclonal Antibodies

The polyclonal antibodies against SbHsp73 were raised in female New Zealand White rabbits (2.0 kg) according to Harlow and Lane (1988). 100 µg of purified SbHsp73 protein was mixed with Freund’s complete adjuvant in 1:1 ratio and mixed thoroughly using Luer-Lok syringe and injected subcutaneously on dorsal thorax and lumbar area of female rabbits.

First and second antigen boosters were given to rabbits after 20 and 35 days of primary immunization, respectively. For booster, 100 µg of protein was mixed with Freund’s incomplete adjuvant in 1:1 ratio and injected intramuscularly on both the hind legs.

Rabbits were bled from marginal ear vein and blood was collected in sterile vial. The whole serum was separated from blood by incubating the vials at 37 °C for overnight, followed by centrifugation at 14000 rpm. The serum was transferred to a fresh sterile vial and stored at -20 °C for titre determination and immunobloting studies.

3.11 Studies on the Effect of Ca$^{2+}$-Channel Blockers and CaM-Antagonists, and Heat Stress-Induced Regulation of SbHsp73 and SbHsp85

To study the effect of CaCl$_2$, Ca$^{2+}$ channel blockers [lanthanum (III) chloride (LaCl$_3$) and verapamil] and CaM antagonists [chlorpromazine (CPZ), N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) and W5 (inactive analogue of W7)], the 24 h-old-seedlings were transferred on to sterile, cotton bedded disposable 90 mm petriplates, which were irrigated with 30 ml of these solutions, and incubated at 37 °C for further 24 h. Exposure of seedlings to
inhibitors for 24 h ensured that the intracellular concentration of these compounds was not a limiting factor. Final working concentrations used for different inhibitors were; LaCl$_3$ - 400 µM and 10 mM; verapamil - 50 µM, 400 µM and 1 mM; CPZ - 50 µM and 200 µM; W7 - 50 µM and 500 µM; W5 - 500 µM; CaCl$_2$ - 1 mM and 10 mM. Except for W5 (100 mM in DMSO) and W7 (100 mM in methanol), all stock solutions (100 mM) were prepared in water. The control plants were treated with water in an identical manner, except for W5 and W7 controls, which were irrigated with water containing equal volume of respective solvents.

Stock of 1 mg of geldanamycin (GDA) (Sigma-Aldrich, USA) was prepared in 100 µl of DMSO. 48 h-old germinated sorghum seedlings were placed in 2.0 ml amber tube, which were covered with aluminium foil and 1.0 ml of 5 µM GDA solution was added in each vial and kept at 37 °C for various time intervals. For controls, equal volume of DMSO was added in place of GDA stock.

48 h-old seedlings grown at 37 °C were used for heat shock treatment. Effect of heat shock was studied after 24 h of inhibitor treatment by incubating the seedlings at 45 °C and 50 °C for 3 h in an incubator.

3.12 Immunoblot Analysis

Total proteins (25 µg) were isolated from whole seedlings and separated on 8% SDS-PAGE gel followed by transfer on to Hybond C membrane.

Membrane was incubated in blocking buffer [200 mM Tris buffer (pH 7.5), 1.4 M NaCl, 0.01% Tween-20, 3% BSA] for 2 h at RT.

After washing twice for 10 min each with TBST [200 mM Tris buffer (pH 7.5), 1.4 M NaCl, 0.01% Tween 20], followed by TBS [200 mM Tris buffer (pH 7.5), 1.4 M NaCl], the membrane was probed with anti-Neurospora crassa Hsp80 polyclonal antibodies (1:1000) and anti-SbHsp73 antibodies (1:2500) in blocking buffer for 2 h at RT.

Membrane was washed twice with TBST followed by TBS for 10 min each at RT and probed with alkaline phosphatase-conjugated goat anti-rabbit IgG in TBS (1:15000) for 2 h.
After washing twice for 10 min each with TBST followed by TBS, the antigen-antibody complex was visualized using NBT/BCIP solution as a substrate.

Equal loading of total proteins was confirmed by staining parallel gels with coomassie brilliant blue R-250.

For densitometer scanning of the blots, the intensity of SbHsp85 and SbHsp73 was normalized with respect to the pre-treatment stage (48 h), whereas the intensity of SbHsp87 and SbHsp75, which were induced only under heat stress, was compared relative to the water grown seedlings that were heat-stressed at 45 °C.

3.13 Statistical Analysis

The data obtained were subjected to one way analysis of variance using the software SPSS version 11 (SPSS for Windows, Rel. 11.0.1. 2001. Chicago: SPSS Inc.).

3.14 Bioinformatic Analysis of CaMBPs

All the sequences of *S. bicolor* Hsp90 and Hsp70 family members were obtained from the gene bank server (http://www.ncbi.nlm.nih.gov/) and saved as flat FASTA format files. Rice (Chen *et al.*, 2006) and *Arabidopsis* Hsp90 protein sequences (Krisha and Gloor, 2001) and *Arabidopsis* and Spinach Hsp70 protein sequences (Guy and Li, 1998; Lin *et al.*, 2001; Sung *et al.*, 2001a) were used as queries for the search of SbHsp90 and SbHsp70 family members, respectively. PSI-BLAST and Smith-Waterman algorithm (Smith and Waterman, 1981) based SSEARCH tools were used for search of Hsp90 proteins from Sorghum genome. BLOSUM50 (Henikoff and Henikoff, 1992) matrix was used for SSEARCH with default values. Gap opening and extension penalties were (-12, -2), and low complex regions were not considered in the search.

Similarity and identity of the sorghum Hsp90 and Hsp70 proteins were calculated by pairwise alignment (Smith and Waterman, 1981). The Hsp90 and Hsp70 sequences were aligned with CLUSTALX, using default alignment option with the PAM series of matrices. NJPlot (Perrière and Gouy, 1996) was used to generate the distance matrix-based unrooted phylogenetic trees.

The functional motif or domains of sorghum Hsp90 and Hsp70 members were determined by Prosite (Sigrist *et al.*, 2010) (http://ca.expasy.org/prosite/), MEME (http://meme.nbcr.net/meme4_6_0/cgi-bin/meme.cgi) and conserved domain (www.ncbi.nlm.nih.gov/Structure/) database. CaM-binding sites were determined by using online CaM target database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html). CaM-binding site analysis (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/analysis.html) for α-helix formation was carried out by calculating average hydrophobicity (Kyte-Doolittle values), the average hydrophobic moment (Kyte-Doolittle values with Eisenberg equation) and the average propensity for α-helix formation (Chou-Fasman values).