Promoter of CaZF, a Chickpea Gene That Positively Regulates Growth and Stress Tolerance, Is Activated by an AP2-Family Transcription Factor CAP2

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Abstract

Plants respond to different forms of stresses by inducing transcription of a common and distinct set of genes by concerted actions of a cascade of transcription regulators. We previously reported that a gene, CaZF encoding a C2H2-zinc finger family protein from chickpea (Cicer arietinum) imparted high salinity tolerance when expressed in tobacco plants. We report here that in addition to promoting tolerance against dehydration, salinity and high temperature, the CaZF overexpressing plants exhibited similar phenotype of growth and development like the plants overexpressing CAP2, encoding an AP2-family transcription factor from chickpea. To investigate any relationship between these two genes, we performed gene expression analysis in the overexpressing plants, promoter-reporter analysis and chromatin immunoprecipitation. Northern analysis of transcripts that exhibited enhanced accumulation upon expression of CAP2 or CaZF in tobacco plants were found common. Transient expression of CAP2 in chickpea leaves resulted in increased accumulation of CaZF transcript. Gel mobility shift and transient promoter-reporter assays suggested that CAP2 activates CaZF promoter by interacting with C-repeat elements (CRTs) in CaZF promoter. Chromatin immunoprecipitation (ChIP) assay demonstrated an in vivo interaction of CAP2 protein with CaZF promoter.

Introduction

Environmental stresses such as changes in temperature, water and salt content in the soil are the major obstacles affecting plant growth and crop productivity. To cope with these stresses, plants undergo physiological and developmental adaptations that are manifested partly by altered gene expression. Changes in gene expression during stress responses have been extensively studied [1–3]. Transcriptional regulators are one of the important groups of proteins that contribute to the stress-adaptation process by regulating expression of genes that are important for changes in cellular metabolism in response to stress. Regulatory functions of a number of transcription factors, such as dehydration-responsive element binding proteins (DREBs), basic helix-loop-helix (bHLH) proteins, MYB, MYC, WRKY and zinc-finger proteins have been described important for stress-adaptation [4–6].

Among several kinds of transcription factors involved in stress responses, the protein family with the Cysteine-2/Hisidine-2-type (C2H2) zinc finger domains also called as the classical or TFIIIA-type finger is one of the best-characterized DNA-binding proteins found in eukaryotes [7,8]. They were first identified in Xenopus oocytes [9]. These proteins have single or multiple canonical CX_2

_3CX_2

_3FHX_2

_3H zinc finger motifs with two conserved cysteine and histidine residues. Plant C2H2 zinc finger proteins contain a non-variant QALGHH motif in each of the fingers which serves as a DNA binding motif. In plants the zinc finger proteins can have one to four fingers and the adjacent fingers are separated by a stretch of amino acids of variable lengths [10,11]. The first plant C2H2 zinc finger protein ZPT2-1 (earlier named as EPF1) was identified from petunia for its ability to bind EPSPS core element (TGACAGTGTCA) present in the promoter of its target gene EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) [12]. Subsequently many other ZPT2-related zinc finger proteins having two zinc finger domains from different plant species were reported such as, WZF1 from wheat [13], SUPERMAN (SUP) [14] and AZF1 and AZF2 from Arabidopsis [15], Mzpt2-1 from M. sativa [16], SCOF-1 from soybean [17]. A number of them have been shown to play regulatory roles in plant growth and development, and also in stress response and defense activation pathways. STZ (ZAT10), an Arabidopsis’ TFIIIA-type zinc-finger protein reportedly provided drought-tolerance [18] and conferred tolerance to heat, salinity and osmotic stress when overexpressed in Arabidopsis [19]. STZ was also able to complement salt-sensitive phenotype of a yeast mutant [20]. Rice C2H2 zinc finger proteins ZFP179, ZFP182, ZFP245 and ZFP252 improved salt and drought tolerance in the overexpressing plants [21–24]. Arabidopsis proteins AZF1, AZF2, AZF3 and STZ were shown to repress transactivation abilities of DREB1A and AREB2 in an Arabidopsis protoplast transient analysis with chimeric promoter-reporter constructs [18]. These proteins possess a C-terminal DLNL/FAR (DLN box) sequence. This motif is also present in the proteins belonging to class II ethylene responsive factor (ERFII) family [25] and was shown to be essential for their function as transcription
repressors in plants [26]. Previously, we have shown that CaZF overexpression improved salt tolerance in transgenic tobacco [27]. Overexpression of different ZPT2-related zinc finger proteins have resulted in different phenotypes. While STZ, AZF1 and AZF2 were shown to function as transcription repressors; ectopic expression of STZ imparted drought-tolerance in contrast to the salt-sensitive phenotype of the plants overexpressing AZF1 and AZF2. Plants overexpressing any of these three genes displayed growth retardation [15]. However, ectopic expression of rice genes \(ZFP252\) or \(ZFP179\) did not exhibit any growth defect in rice [23,24]. All these observations suggest that these proteins play diverse roles in different developmental and defense pathways.

The plant hormone abscisic acid (ABA) plays an important role in stress-responsive gene expression [28]. Previously, we identified CAP2, an AP2-family transcriptional regulator from chickpea. CAP2 functions as a C-Repeat binding factor (CBF) and binds to DRE/CRT (dehydration responsive element/C-repeat element) (CCGAC) that is often present in the promoter regions of abiotic-stress responsive genes. CAP2 gene expression was induced by dehydration, high salinity and external ABA application. Ectopic expression of CAP2 in tobacco resulted in improved tolerance to drought, salinity and heat and in addition, improved growth of the transgenic plants [29,30]. In this study we reported that overexpression of CaZF in tobacco led to a similar phenotype of improved growth and stress-tolerance as in the case of CAP2 overexpression. A number of tobacco transcripts that exhibited higher accumulation in the CAP2-expressing plants showed higher abundance in the CaZF-expressing plants. Transient expression of CAP2 in chickpea leaves enhanced CaZF gene expression. In a protoplast-mediated transient assay CAP2 induced expression of a reporter gene fused to the CaZF promoter sequence. Nucleotide substitution of C-repeat elements present in CaZF promoter suggested that CRTs are critical for CAP2-mediated activation of CaZF promoter. Chromatin immunoprecipitation followed by PCR-amplification indicated that CAP2 protein interacts with CaZF promoter in vivo. All together, our data demonstrated that CAP2 was able to transactivate CaZF promoter in planta and indicated that CAP2 is a potential transactivator of CaZF gene.

Materials and Methods

Plant materials, Growth conditions and Stress treatments

Chickpea (\(Cicer arietinum\) L. cv. PUSABGD72 provided by Indian Agricultural Research Institute, New Delhi, India) was used in this study. 10-d-old seedlings were subjected to dehydration, salt, cold and hormonal treatments for given time periods (mentioned in the text) as discussed in [31]. Stress-treated seedlings were harvested, immediately flash-frozen in liquid nitrogen and stored at \(-80^\circ C\) for later use. CaZF-overexpressing transgenic tobacco (\(Nicotiana tabacum\) var. \(xanthii\)) lines were constructed as described by [27]. \(T_3\) homozygous transgenic seedlings were used for experiments. Vector transformed \(Vec\) and CaZF-overexpressing CaZFOX tobacco seeds were grown on \(\frac{1}{2}\) MS medium supplemented with 1.5% (w/v) sucrose, pH 5.8, and 0.8% (w/v) agar or in composite soil (agropeat/vermiculite, 1:1) at 25°C, 60% humidity and with a photoperiod of 16 h. Drought, mannitol and high temperature treatments were applied to the transgenic plants as described previously [29,30]. Seedlings were harvested in liquid \(N_2\) and stored at \(-80^\circ C\) for further analyses.

Northern blot, qReal-Time PCR and Southern analysis

Total RNA was isolated from chickpea or tobacco seedlings using the TRI-reagent (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer’s protocol. 20 µg of total RNA was electrophoresed on a 1.2% MOPS-formaldehyde denaturing agarose gel, blotted onto Nylon membrane (HyBond-N; Amersham Biosciences, Buckinghamshire, UK) and then hybridized with a random-prime labeled probe as described in [32]. Washed and dried membranes were then exposed in cassettes to storage phosphor screen for autoradiography. The autoradiographs were scanned on a PhosphorImager (Amersham Biosciences) and the relative intensities of the bands were quantituated using the Image-Quant program (Molecular Dynamics, Sunnyvale, CA). The values obtained were normalized by the background and then expressed as fold change with respect to control.

For semi quantitative RT-PCR and qRT-PCR analysis total RNA was isolated from chickpea or tobacco seedlings using the RNeasy plant mini kit (Qiagen GmbH, Hilden, Germany). 2 µg of total RNA was used to prepare cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR reactions were performed in an optical 48-well plate in Applied Biosystems StepOne™ using PowerSYBR Green to monitor dsDNA synthesis. Chickpea and tobacco \(Actin\) primers were used as internal control. Sequences of all the primers used in this study are listed in Table S1.

The data presented are average of multiple biological replicates as mentioned in the results and figure legends. Error bars represent standard deviation. Statistical differences between the control and experimental samples were computed using Student’s \(t\)-test with paired two-tailed distribution. The \(p\)-value cut-off was considered at \(p<0.005\).

Construction of subtracted cDNA library

Subtracted cDNA library was constructed between antibiotic selected \(Vec\) (driver) and CaZFOX (tester) tobacco by using CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, Palo Alto, CA) as described before [33]. Enriched DNA fragments from the forward subtracted library were directly cloned into \(T/A\) cloning vector (pGEM-T Easy Vector Systems, Promega, USA) and plasmids isolated from white colonies were further sequenced and annotated using Blastx with NCBI non-redundant protein sequence database (blast.ncbi.nlm.nih.gov).

Analysis of transgenic tobacco plants for abiotic stress tolerance

\(T_3\) homozygous seeds of vector control (\(Vec\)) and transgenic CaZFOX tobacco lines were surface sterilized and grown essentially under culture room conditions as described previously [34]. For germination experiments, \(Vec\) and CaZFOX seeds were grown on \(\frac{1}{2}\) MS medium supplemented with 0.3 M mannitol as mentioned in the figures. Plates were photographed after 15 days. For germination under high temperature, the seeds were plated on \(\frac{1}{2}\) MS medium and kept at 39°C for 15 days. Seeds grown on \(\frac{1}{2}\) MS medium for the same period at control condition were taken as controls. For water-deficit stress of the soil grown plants, fresh weights of the stress-treated and recovered plants relative to those grown in control condition for the same period was calculated. All experiments were performed in triplicates and data in the form of mean of multiple experiments with standard deviation is presented. Proline content was determined in vector-control and CaZFOX transgenic plants under control and stress conditions by sulphosalicylic acid method [35].

For stress tolerance experiments of the soil-grown seedlings, two-week-old seedlings grown in composite soil at control condition were subjected to water deficit treatment. Stress tolerance was represented as fresh weight of the treated plants relative to those grown in control condition.
Full length cloning and site directed mutagenesis of CaZF promoter

For CaZF-promoter (proCaZF) cloning, 1.97 kb fragment upstream to the transcriptional start site of CaZF was amplified from a chickpea genomic DNA library constructed with Universal Genome WalkerTM Kit (Clontech, USA) by following the manufacturer’s protocol. In silico analysis of proCaZF was performed by using PLACE signal scan search (http://www.dna.affrc.go.jp/PLACE/signalscan.html) [36] and PLANTCARE search (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) [37] programs. In vitro site directed mutagenesis of the C-Repeat elements (CRTs) present at three different positions in proCaZF was done by mutating “G” of the core motif (ACCGAC) to “T”.

For mutagenesis PCR various (CUM/F and CUM/R, M = M1, M2, M3) primer pair combinations (Table S1) were used. Mutagenesis PCR were done on double-stranded plasmid DNA using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) by following parameters: 95°C for 30 sec; 16 cycles of 95°C for 30 sec, 58°C for 1 min, 72°C for 7 min. PCR reaction was digested with methylation-specific restriction enzyme DpnI at 37°C for 6 h for bacteria-derivated template DNA degradation and the digested PCR product was directly transformed into E. coli DH5α cells. The mutation and the fidelity of the rest of the construct were confirmed by DNA sequencing.

Gel mobility shift and promoter activation assay

For determining the in vitro binding activity of CAP2 with CRT present in proCaZF, gel mobility shift assay (GMSA) was carried out as described by [27] with a radiolabeled probe containing tetramer of two CRTs (GACCGACCCA) and its flanking sequences. The oligonucleotides used to amplify the CRT sequence are pCRT1 and pCRT2. Mutations in the probe sequences were done using the primers (pCRT3 and pCRT4) as mentioned in the figures. The recombinant GST-fused CAP2 protein was expressed in E.coli DH5α and purified from bacterial lysates with glutathione-Sepharose beads (Amersham Biosciences) as described in [29].

For promoter activation assay in yeast the proCaZF was cloned in the BamHI restriction site of pYES2.1/V5-His/LacZ vector by using promoter specific primers, pyProF and pyProR to create the pYProCaZF/LacZ construct. Site directed mutagenesis was done in the CRT3 site of the construct as described before and named as pYProM3CaZF/LacZ. CAP2 ORF was amplified with primers pgCAP2/F and pgCAP2/R and cloned in NdeI-EcoRI restriction sites of pGADT7 (BD Biosciences Clontech) to create the pGADCAP2 construct. Both pGADCAP2 and pYProCaZF/LacZ (or pYProM3CaZF/LacZ) were co-transformed in S. cerevisiae strain AH109 by LiOAc method [27] and the transformants were selected on SD (-His/-Leu) medium. Transactivation mants were selected on SD (-His/-Leu) medium. Transactivation activity of transformed DNA. Leaf tissue bombarded with control plasmid DNA (control plasmids with or without proCaZF:GUS/ CaMV35S-Myb-CAP2 plasmids) was mixed with 100 µl protoplasts (1×10⁶), followed by addition of 110 µl PEG (50%) solution and incubated at room temperature (RT) for 30 min. After incubation, 1 ml of W5 solution was added, centrifuged, resuspended in 1 ml of incubation solution and incubated in dark at 28°C for 48 h. 5 µg of p35S:EFPI plasmid (pEFPI-1; BD Biosciences Clontech, Palo Alto, CA, USA) [39] was included in each transfection experiment as a control for normalization of transfection efficiency.

Protoplast isolation and transfection

Protoplasts were isolated from 3-d-old tobacco Bright Yellow-2 (BY-2) suspension cells, cultured and maintained in flasks at 28°C in dark, as described by [38] with few changes. Details of the protocol and solution recipes are mentioned in Methods S1. 10 µg DNA (control plasmids with or without proCaZF:GUS/ CaMV35S-Myb-CAP2 plasmids) was mixed with 100 µl protoplasts (∼1x10⁶), following by addition of 110 µl PEG (50%) solution and incubated at room temperature (RT) for 30 min. After incubation, 1 ml of W5 solution was added, centrifuged, resuspended in 1 ml of incubation solution and incubated in dark at 28°C for 48 h. 5 µg of p35S:EFPI plasmid (pEFPI-1; BD Biosciences Clontech, Palo Alto, CA, USA) [39] was included in each transfection experiment as a control for normalization of transfection efficiency.

Protoplast extraction and quantitative GUS assays

Transfected protoplasts were collected by centrifugation at 1000 rpm, RT for 5 min, re-suspended in 500 µl of extraction buffer (100 mM sodium phosphate, pH 7.0, 0.5 mM PMSF) and disintegrated by vortexing. Total protein in the supernatants was measured by the Bradford method using bovine serum albumin (BSA) as the protein standard and GUS activity was measured using the β-glucuronidase substrate 4-methylumbelliferyl β-D-glucuronide (MUG) [40]. An average of GUS activity of three replicates was reported here. Two-step Student’s t-tests were conducted between the samples containing reporter plasmid and reporter plasmid/effecter plasmid, and between the samples containing reporter plasmid/effecter plasmid and mutated reporter plasmid/ effecter plasmid.

Transformation by particle bombardment

CaMV35Ss-Myb-CAP2 construct was introduced with control plasmid into leaf epidermal cells of potted chickpea (PUSABGD72) plant by particle bombardment using Helios gene gun (BioRad, www.bio-rad.com), according to the manufacturer’s instructions. The leaf cells were bombarded with 1.0 µm gold particles at a helium pressure of 120 psi and a distance of 2 cm from the tissue. Plants were incubated at 25°C for 48 h to allow expression of transformed DNA. Leaf tissue bombarded with control plasmids only was taken as control. Control and two different experimental tissues were harvested after incubation period, flash frozen in liquid nitrogen and used for RNA isolation.

Chromatin immunoprecipitation (ChIP)

Chromatin extraction and immunoprecipitation was performed essentially as described by (31) with slight modifications. Elaborate procedure with buffer compositions is described in Methods S1. Briefly, 1.5 g of tobacco shootlets harboring both proCaZF:GUS and CaMV35Ss-Myb-CAP2 constructs, grown under sterile conditions were harvested and fixed with 1% formaldehyde. Formaldehyde was quenched by adding glycine (final concentration, 0.3 M). Tissue was washed repeatedly with ice-cold Tris buffered saline (TBS). Fixed tissue was ground to a fine powder and resuspended in 1 ml lysis buffer I with protease inhibitors. The
suspension was then sonicated and chromatin was pelleted at 15000 rpm, 4°C for 15 min. The supernatant was incubated overnight with preimmune serum or anti-Myc antibody (C-9956, Sigma-Aldrich, USA) and 40 μl Protein A-sepharose beads (Amersham Biosciences) at 4°C with gentle shaking. Beads were washed twice each with wash buffer, LNDET buffer and TE. The chromatin-immune complex was eluted and the DNA cross-linking was reversed by with NaCl. Protein was degraded by adding protease solution at 45°C for 2 h and DNA was precipitated with ethanol. Immunoprecipitated DNA was amplified with CaZF-promoter specific primers (Uchip/F and Uchip/R). In each PCR reaction, corresponding input was taken in parallel for PCR validation.

**Results**

**CaZF expression analysis under different stress treatments**

We have reported earlier the cloning of CaZF from chickpea [27]. Briefly, CaZF is a Cys2-His2 zinc finger protein of 280 amino acids and is nuclear localized. The expression of CaZF in 10-d-old chickpea seedling was monitored by northern blot analysis under different stress conditions and hormonal treatments. Northern blot analysis with a CaZF specific probe showed a basal level of expression under control condition. Increased steady-state accumulation of CaZF transcript was observed after 5 hour (h) of treatment with low temperature, dehydration (DH), salt, abscisic acid (ABA), methyl-jasmonate (MeJA) and salicylic acid (SA), except wounding (Figure 1A). The time-dependent expression kinetics of CaZF under dehydration, high salinity and low temperature showed that the transcript quickly accumulated at a ten-fold higher level within 0.5 h under dehydration and then reduced at 3.5–4 h higher up to 3 h of treatment. Similarly, two-fold high transcript accumulation was observed within 0.5 h of cold treatment, and then further reached to five-fold high after 1 h followed by a reduction to three-fold after 3 h. While under salt stress, two-fold increase in transcript accumulation was observed within 0.5 h and further accumulated up to 12-fold after 1 h and decreased to 8-fold at 3 h (Figure 1B). Organ-specific expression of CaZF was observed in root, stem and leaves in normal and stressed conditions. Expression was strongly induced in all the tissues under dehydration stress, specifically in root and stem under salt stress and in stem under cold treatment (Figure 1C).

**Phenotypic features of transgenic tobacco plants constitutively expressing CaZF**

In our previous study, we reported construction of transgenic tobacco plants overexpressing CaZF (CaZF FOX lines) under CaM135S promoter [27]. Two single copy insertion homozygous lines (from T3 homozygous seeds) each with relatively high (CaZF FOX lines L212 and L316) and low expressing (CaZF FOX lines L100 and L211) CaZF were selected for further experiments. No differences in germination period and frequency were observed between the transgenic and the control (Vec) seeds on control media [Figure 3A (i), B (ii)]. Germination of seeds was delayed under stress as compared to that in the control condition. The germination efficiency of CaZF FOX lines was above 85% in comparison to about 8% for the vector-control seeds after 15 days of sowing on mannitol-supplemented medium [Figure 3A (iii)]. Under higher temperature, only 4% vector-control seeds showed germination after 15 days, while CaZF FOX seeds showed germination efficiency above 55% [Figure 3B (iii)]. Seeds of vector-control and CaZF FOX plants did not show any difference in germination period and frequency in control media [Figure 3A (i), B (ii)].

**Stress tolerance of soil-grown CaZF FOX transgenic plants**

We monitored the stress-tolerance of the soil-grown transgenic lines under water-deficit (WD) condition. Percent fresh weight under stress conditions relative to that grown in control condition was presented to compare the stress-tolerance of the control and transgenic plants. Fifteen-day-old vector control and CaZF FOX plants grown in control condition were not irrigated for 2 weeks for water deficit stress. For recovery, pots were watered for a week. CaZF FOX transgenic seedlings recovered more than the vector control plants, which appeared to be mostly withered (Figure 4A). As shown in Figure 4B, the CaZF FOX seedlings retained 32–40% of relative fresh weight, whereas vector-control seedlings retained only 10–15% fresh weight compared with the unstressed seedlings.

To investigate the physiological basis for the improved stress tolerance of transgenic tobacco, proline content was measured in 15-day-old CaZF FOX and vector-control plants under control and stressed conditions. After water-deficit stress, CaZF FOX plants accumulated approximately 1.5 to 3-fold higher amount of proline compared to the control seedlings (Figure 2C). CaZF FOX lines also showed longer and robust root hair (Figure 2D). Longer root phenotype in CaZF FOX overexpressing lines was investigated for altered cellular organization at the root tip by confocal imaging (Figure 2E). CaZF FOX root tips showed no gross deformation in the cellular arrangement. However, roots of CaZF FOX lines showed a relative increase of approximately two-fold in the number of dividing cells of the meristematic region (Figure 2F). Increased cell number was evident in the epidermal, cortical and stellar layers and that caused increase in cell layers in CaZF FOX roots indicating enhanced cell division. Subsequently to the enhanced cell division, the zone of cell division is also longer in these seedlings.

Enhanced growth phenotype of 15-day-old transgenic seedlings was clearly depicted by larger leaf lamina. Average surface area of the third (from the bottom) leaves of 10 plants from each transgenic line was measured and found to be approximately 2.5–4.5 fold higher in CaZF FOX plants in comparison to the control samples (Figure 2G). To explore the reason behind the increase in the leaf lamina in the transgenic plants, the ventral epidermal peel from both sides of mid-rib in the leaf base, middle lamina and leaf tip of the vector control and CaZF FOX lines were compared for cell size. Six samples from each leaf and leaves from two plants of each line were investigated. Leaf cell size of the CaZF FOX plants was found to be larger than that of the control leaves (Figure 2H).
than the vector-control plants. Under control condition, vector-control and CaZF FOX plants accumulated similar amount of proline, indicating that other stress regulated factors are required along with CaZF to regulate the accumulation of proline under stress (Figure 4C).

Analysis of altered gene expression due to CaZF overexpression in transgenic plants

Physiological and biochemical experiments suggested that CaZF overexpression might have altered expression of some genes related to abiotic stress signaling and helped in the cellular survival and recovery. To gain an insight into alteration of gene expression, specifically to identify the up regulated genes in the CaZF transgenic plants, a subtractive cDNA library was constructed with the mRNAs isolated from the 2-week-old seedlings of vector control (driver) and CaZF FOX (tester) lines grown in control condition. After screening out the redundant sequences, 222 high-quality unique EST sequences were generated, annotated and classified according to their putative functions (Table S2). Many of the well-known abiotic stress-responsive genes such as HSFs, CBF/DREB, catalase, CIPK16, Ca2+-binding proteins and MAPK1 were identified, indicating their likely contribution towards the stress tolerance of transgenics. For validation of enhanced expression of the ESTs, expression of 6 ESTs (MRP-like ABC transporter,

Figure 1. Expression analysis of CaZF in response to various stresses in chickpea. (A) Expression patterns of CaZF gene induced under different treatments. Total RNA was isolated from 10-d-old chickpea seedling treated with distilled water (control, Con), 100 μM abscisic acid (ABA), 4°C (Cold), dehydration (DH), 50 μM methyl-jasmonate (MeJA), 5 mM salicylic acid (SA), 250 mM NaCl (Salt) and wound for 5 h. (B) Time-course of accumulation of CaZF mRNA under dehydration, cold and salt treatments for 0.5 h, 1 h and 3 h. (C) Organ-specific expression of CaZF in root (R), shoot (S) and leaf (L) after cold, salt and dehydration treatments for 3 h. 20 μg of total RNA was electrophoresed on formaldehyde denaturing gel, blotted onto nylon membrane and hybridized with 32P-radiolabeled CaZF cDNA. The lower panel in each figure shows ethidium bromide-stained ribosomal RNAs as loading controls. The relative intensities of the bands were quantitated by densitometry in PhosphorImager scanner and are presented in the form of fold changes below each blot.

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Auxin efflux carrier family protein-like protein, Heat-shock factor, Lipid transfer protein, CIPK16 and Elongation-factor 1α) was verified by qRT-PCR (Figure S1A–F).

**CAP2 activates CaZF promoter**

CAP2 overexpression in tobacco plants caused drastic increase in leaf cell size, leaf surface area and in the number of lateral roots. Further, the CAP2-expressing plants demonstrated more tolerance to water-deficit and salt stress [29]. CaZF overexpression resulted in similar developmental and stress tolerance phenotypes as that of CAP2-overexpressing plants. In addition, a number of ESTs identified in CaZF OX subtracted cDNA library (Dehydrin, Heat shock factor, Heat-shock protein 70, Heat-shock protein 90, Secretory peroxidase, Glyceraldehyde-3-phosphate dehydrogenase, Myo-inositol-1-phosphate synthase and H⁺-transporting ATPase) were found to be similar to those in CAP2 OX subtracted cDNA library [30]. Expression of abiotic stress marker genes like NtERD10B and NtERD10C was similarly enhanced in the CaZF and CAP2 transgenic seedlings under unstressed conditions (Figure S1G–H) [this report and [29]. Similarity of the phenotypes of CAP2 OX and CaZF OX transgenic plants and their gene expression prompted us to investigate a relationship between these two genes.

To investigate whether CAP2 expression enhances CaZF expression in chickpea, we transiently expressed CAP2 in chickpea leaves by introducing CAP2 expression construct (CaMV35S:Ca:Myc-CAP2) in chickpea leaves by particle bombardment resulting in increase in CAP2 expression (Figure 5A). Subsequently, expression of CaZF was increased by about five folds as determined by semiquantitative RT-PCR and qRT-PCR (Figure 5B). To further elucidate the effect of CAP2 expression on CaZF expression, 1970 bp of CaZF promoter (proCaZF) region was cloned by genome walking. Bioinformatics analysis of proCaZF showed

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**Figure 2. Phenotype analyses of CaZF overexpressing (CaZF OX) transgenic tobacco plants.** (A) Tobacco seedlings of four independent T₃ homozygous lines harboring CaZF (CaZF OX, L100, L211, L212, L316) or empty vector (Vec) were grown vertically under control conditions (½ MS, 25°C) for 15 days. Scale bar is 1 cm. (B) Comparison of number of lateral roots, and (C) fold increase in rate of taproot length growth of vector-control and CaZF OX seedlings. The means of three measurements of thirty seedlings of each line are shown. (D) Comparison of root hair of 6-day-old vector-control and CaZF OX (L212 and L316) tobacco seedlings. Scale bar is 1 mm. (E) Confocal imaging of root apices of 6 d-old vector-control and CaZF OX tobacco seedlings stained with fluorescent dye Propidium iodide (PI) at equal magnification. Arrow marks the start of elongation zone. Scale bar is 100 μm. (F) Graphical representation of fold increase in number of dividing cells in the meristematic zone of roots of 6-day-old CaZF OX plants is presented in the right panel. (G) Comparison of surface area of the third (from the bottom) leaves of 15-day-old vector-control and CaZF OX plants. (H) Epidermal peels from the ventral surface of the middle lamina of the vector control and transgenic CaZF OX(L316) leaves showing cell size. Scale bar is 10 μm. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison with the vector-control (Vec) at p<0.005.

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**Figure 3. Germination efficiency of CaZF OX tobacco lines in mannitol and under high temperature.** Seeds of CaZF OX transgenic lines (L100, L212, L211 and L316) and vector-control (Vec) plant were sown on ½ MS-agar [A (i), B (i)] or ½ MS-agar supplemented with 0.3 M mannitol [A (iii)] and incubated for 15 days at 25°C for germination. Seeds from the same plants were plated on ½ MS-agar and incubated at 39°C for 15 days for high temperature treatment [B (ii)]. Graphical representation of percent germination of seeds after 15 days on incubation from three independent experiments is presented below [A (iii), B (iii)]. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison with the vector-control (Vec) at p<0.005.

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presence of three authentic C-repeat elements (CRT: CCGAC) at the positions −1913 (CRT1), −1713 (CRT2) and −1337 (CRT3) along with putative abscisic acid responsive elements, auxin-responsive elements, MYC and MYB binding sites upstream to the transcription start site (Figure S2). 1.97 kb CaZF promoter was fused to β-glucuronidase (GUS) reporter gene to make a reporter construct (proCaZF:GUS) (Figure 5C). This reporter and the effector constructs (CaMV35S:c-Myc-CAP2) were introduced individually or together in tobacco leaf explants by Agrobacterium-mediated transformation. GUS activity in the antibiotic selected shootlets was found to be enhanced about 3-fold in presence of CAP2 (Figure 5D), Ability of CAP2 to activate CaZF promoter in Saccharomyces cerevisiae was also analyzed. Expression of the reporter gene was increased by more than 80-fold in presence of CAP2 (Figure S3), demonstrating that CAP2 was able to activate proCaZF. In order to dissect the roles of three CRTs in CaZF promoter in transcriptional regulation, these CRTs were mutated individually or in combination by replacing 'G' with 'T' (CCGAC/CCTAC). Effect of these substitutions in transcriptional regulation, these CRTs were mutated individually or in combination by replacing ‘G’ with ‘T’ (CCGAC/CCTAC). Effect of these substitutions in transcriptional activation of the reporter construct was tested in the tobacco BY2 protoplast transient assay system by co-introduction of the mutated reporter constructs along with the effector construct (CaMV35S:c-Myc-CAP2). Figure 5E shows that presence of CAP2 led to almost 4-fold enhancement of GUS activity. Mutation (M1) in CRT1, which is the farthest (~1913) from the transcription start site, did not alter the GUS activity significantly, however, mutation in CRT2 (M2, −1713) and CRT3 (M3, −1337) significantly reduced the GUS activity, indicating critical role of these CRTs in CAP2 mediated CaZF promoter activation. Mutations in combination also suggested that CRT2 and CRT3 are the most important for CAP2-mediated activation of CaZF promoter.

CAP2 binds to CaZF promoter

We reported earlier the sequence specific interaction of CAP2 protein with CRT [29]. In order to analyze whether CAP2 protein can directly bind to the CRT present in CaZF promoter, we performed GMSA with different DNA probes containing repeat sequence of CRT3 and it’s flanking bases and purified E. coli-expressed CAP2 protein fused in-frame to glutathione-S-transferase (GST). Figure 6A clearly shows that GST-CAP2 protein could specifically bind to the radiolabeled probe as the binding was competed out by 100× cold probe. The in vitro binding of CAP2 protein to the probe was sequence-specific, as replacement of ‘G’ with ‘T’ within the CRT (CCGAC/CCCTAC) abolished the binding while change in the flanking sequences did not affect the binding.

Finally, to determine whether the CAP2 protein interacts with CaZF promoter within the plant cell we performed chromatin immunoprecipitation (ChIP) assay. The CaMV35S:c-Myc-CAP2 fusion construct was co-introduced along with proCaZF:GUS construct into tobacco explants by Agrobacterium-mediated transformation. Antibiotic-selected transformed shootlets were harvested and fixed with 1% formaldehyde in fixation buffer. The DNA-protein complex was immunoprecipitated by anti-c-Myc antibody. The DNA fragments that co-immunoprecipitated with anti-c-Myc antibody were identified by PCR amplification using the primers containing the flanking bases of CRT3 of the CaZF promoter.
CAP2 Trans-Activates CaZF Promoter

Discussion

We presented this study as a continuation of our previous study [27] where we reported that CaZF can provide salt-tolerance when expressed in tobacco plants. CaZF belongs to zinc finger family of proteins and contains two typical Cys2/His2 zinc finger domains with a DLN-box/EAR-motif at its C-terminus. A number of similar plant Cys2/His2 zinc finger proteins, such as STZ/ZAT10, AZF1, AZF2 were shown to repress transactivation properties of the well-known transcription activators like DREB1A and AREB2 in transient assays [18]. Like CaZF, Cys2/His2 zinc finger proteins ZFP179 from rice [24] and ThZF1 from salt cress [42], showed transactivation property in yeast system. Repressor activity of C2H2 zinc finger proteins having EAR motif is mediated through interaction with TOPOLESS and its related proteins in plants [26]. Therefore, possibility of transactivation of reporter genes by CaZF in yeast system due to lack of EAR motif-TOPOLESS pathway in yeast cannot be ruled out.

Inducible expression of AZF1 and AZF2 resulted in severe salt-sensitivity, however, constitutive expression of STZ/ZAT10 promoted drought-tolerance and; CaZF and ZFP179 promoted salt-tolerance when overexpressed in plants (this report and [24]). Therefore, it appears that in planta functions of this family of proteins cannot be generalized just on the basis of structural homology. Though these proteins were shown to bind the same sequence motif (AG/CT repeat) within an EP2 sequence in in vitro experiments, optimal binding efficiency of these proteins to their target sequences within a plant cell and its effect on expression of the target genes or requirement of other auxiliary proteins seems to play a major role in determining their functions in plant. To elaborate, overexpression of STZ/ZFP179 and CaZF enhanced expression of the oxidative stress response genes in their respective systems e.g. overexpression of STZ/ZFP179 enhanced ascorbate peroxidase 2, and Fe-superoxide dismutase 1 [18]; overexpression of ZFP179 enhanced peroxidase activity in the transgenic rice [24] and overexpression of CaZF enhanced expression of catalase and secretory peroxidase genes in tobacco. However, transcript levels of similar ROS-responsive genes were not altered in AZF1 and AZF2 overexpressing plants. Inducible expression of AZF1 and AZF2 repressed the expression of DREB1A [15], while the overexpression of ZFP179 enhanced expression of DREB2A [24] and overexpression of CaZF enhanced expression of DREB2 and DREB4. Enhancement or reduction in expression of the DREB-family genes in these overexpressing plants does not necessarily indicate that those DREB-family genes are under transcriptional control of these zinc finger proteins. Expression of all the four zinc finger protein-encoding genes mentioned here is highly enhanced by external application of ABA, whereas, DREB1A and DREB2A genes are not ABA-responsive. Alteration in expression of these DREB-family genes in these overexpressing lines might be direct, or, due to any feed-back response.

Many plants accumulate cellular osmolytes like proline for adjusting the intracellular osmotic potential under abiotic stress [43]. Thus, proline accumulation was measured to explain one of the possible reasons for enhanced abiotic stress tolerance of CaZF-overexpressing plants. Although CaZF-FOX plants exhibited higher proline content in stressed conditions, we did not detect any clone encoding proline synthetase gene (P5CS) in the subtracted cDNA library. The reason might be similar to that in the ZFP179-overexpressing plants, where there was no enhancement in OaP5CS gene expression in unstressed condition, however, the

Figure 5. Activation of CaZF-promoter by CAP2 in plant cell. (A, B) Enhancement of CaZF expression by transient overexpression of CAP2 in chickpea. Young leaves of 10-day-old chickpea seedlings were transformed with CaMV35S:Myo-CAP2 by particle bombardment. Chickpea leaves were harvested after 48 h of incubation. Leaves transformed with empty vector (pcambia1302) were taken as control (Con). 2 μg of total RNA was reverse transcribed for cDNA preparation. CAP2 (A) and CaZF (B) expression was analyzed in the control and experimental tissues by semi-quantitative RT-PCR (27 cycles) and qReal-Time PCR. The expression level of Actin gene was taken as an internal control. Results from two biological replicates (Expt 1, Expt 2) are shown. (C) Schematic diagram of the effector and reporter constructs used in the co-transfection experiments. Full-length CAP2 cDNA was fused with 2X-Myo at N-terminus and cloned under CaMV-35S promoter in pcambia1302 to construct effector plasmid. proCAZF with three CRTs was fused with Gus gene in pBI101 to construct reporter plasmid. (D) Both the effector and reporter plasmids as mentioned in the figure were co-introduced in to tobacco leaf explants by Agrobacterium-mediated transformation and antibiotic-selected shootlets were used for the Gus assay. Expression of kanamycin resistance gene (NPT II) as assessed by qRT-PCR was used for normalization of results in the transformed shootlets. (E) The effector and reporter constructs were co-introduced into tobacco BY2 protoplasts as mentioned in the table. CAP2 stands for the effector plasmid and pro-CaZF stands for the reporter plasmid. pro-CaZF (M1–M3) stands for the reporter plasmids with mutations in CRT1-CRT3 in pro-CaZF. Gus activity was measured fluorometrically after 48 h of transformation. The empty vectors without CAP2 (pcambia1302) or proCaZF (pBI101) were used as controls. Transfection efficiency of the CaMV-35S-EYFP1 plasmid included in protoplast experiment was used for normalization. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison to the controls at p < 0.005. doi:10.1371/journal.pone.0056737.g005
gene expression was much higher in the overexpressing plants under stressed condition suggesting that other stress-dependent factors are also required for enhancement in expression of some of the stress-responsive genes. Enhanced growth phenotype of the CaZF-overexpressing plants can be attributed to elevated expression of ESTs encoding auxin-efflux carrier protein, AUX/IAA and ABC-transporters (Figure S1) and, thereby, increased auxin transport (Figure S4 and Methods S1). For instance, the retarded growth phenotype of AZF2-overexpressing plants can be attributed to the down regulation of expression of some of the auxin-responsive genes [15].

CaZF promoter, like the promoters of AZF and ZFP179 possesses multiple C-repeat and ABA-responsive elements. Apart from that it also contains five auxin-responsive elements (Figure S2). Regulation of these promoters by AREBs and ARFs is subject to experimentation.

In summary, we have presented a detail study of the effect of CaZF-overexpression in plant. CaZF-expression promoted tolerance of the transgenic plants against dehydration and high temperature along with an overall growth in the aerial and subaerial parts of the plants. We have shown that transient expression of CAP2, a chickpea gene for CBF/DREB-like transcription factor, caused enhanced accumulation of CaZF transcript in chickpea leaves. CAP2 protein was able to bind to C-Repeat/dehydration-responsive element present in the CaZF promoter and activate transcription as determined by GMSA and protoplast transient transactivation assay respectively. Finally, chromatin immunoprecipitation (ChIP) suggested that CAP2 has ability to bind CaZF promoter in plant cell. Our results indicated that CAP2 is a potential transactivator of CaZF.

Figure 6. CaZF promoter is a target of CAP2. (A) The DNA binding ability of CAP2 to CRT in CaZF promoter was analyzed by gel mobility-shift assay (GMSA). Dimers of the sequences shown in the left panel were used as probes. CRT nucleotides are shown in bracket and the mutations (M1 and M2) created within the probe by replacing bases are shown in lower case. GMSA was performed with purified GST-CAP2 fusion protein expressed in bacteria using cold or 32P-labeled probe. (B) ChIP assay indicating CAP2 interacts with CaZF promoter in vivo. Tobacco leaf explants were transformed with proCaZF:GUS and CaMV-35S:c-Myc-CAP2 constructs along with control plasmids. Antibiotic-selected shootlets were harvested and fixed with 1% formaldehyde. The DNA-protein complex was immunoprecipitated against anti-c-Myc antibodies. Quantitative PCR was performed using proCaZF specific primers flanking the CRT3 region. Lanes 1–3, Input (total DNA-protein complex); lanes 4–6, DNA-protein complex immunoprecipitated with anti-c-Myc antibody. Empty plasmids without proCaZF (pBI101) or c-Myc-CAP2 (pCAMBIA1302) were used as controls.

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Supporting Information

Table S1 Oligonucleotide sequences used in this study. (DOC)

Table S2 Functional categorization of ESTs generated by subtracted cDNA library between CaZF/ox and vector control. (DOC)

Figure S1 Quantitative RT-PCR of selected genes. (A–F) mRNA abundance of some of the library genes was determined by qRT-PCR. GO308103, MRP-like ABC transporter; GO308100, Auxin efflux carrier family protein-like protein; GO308183, Heat-shock factor; GO308200, Lipid transfer protein; GO308212, CIPK16 and GO308225, Elongation-factor 1a. (G–H) Expression of abiotic stress marker genes NtERD10B (AB049336) and NtERD10C (AB049337) in vector control and CaZF-overexpressing transgenic tobacco lines was analyzed by quantitative Real-time PCR in normal growth condition. Tobacco Actin is used as an internal control for normalization. Data shown here is a mean of three independent experiments. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison with the vector-control (Vec) at p<0.005. (TIF)

Figure S2 In-silico sequence analysis of CaZF-promoter (proCaZF). CaZF promoter region was analyzed by PLACE and PlantCare softwares. Some of the elements worth to mention are highlighted. Abscisic acid responsive element as bold italics, C-repeat elements (CRTs) as bold underline, auxin-responsive elements [ARFAT site (TGTCTC), CATATGGMASUR site (CATATG), NTBBFIARROLB site (ACTTTA), DOFCOREZM site (AAAG) and TGA Element (AAGCAG)] as lowercase...
underlying, MYC consensus as underlined and MYB consensus as bold lowercase. (TIF)

**Figure S3** CAP2 transactivate proCaZF in yeast. Activation of reporter gene LacZ under CaZF promoter by CAP2 in Saccharomyces cerevisiae. Activation of reporter gene LacZ under CaZF promoter by CAP2 in Saccharomyces cerevisiae, A, Yeast strain PJ69-4A co-transformed with the constructs indicated in the left plate were grown on SD (-His, -Ura) or in SD complete medium. B, Schematic representation of effector and reporter constructs. C, Interaction of proCaZF with CAP2 in yeast. Yeast strain BGY213 was co-transformed with the constructs as indicated in model plate. Trans-activation of proCaZF by CAP2 is shown by β-galactosidase assay of the transformants. Activity is presented as fold increase in activity. Assay was done by taking three independent transformed colonies in triplets. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison with the pYproCaZF::LacZ (Lane 2) at p<0.005. (TIF)

**Figure S4** Basipetal-auxin transport assay in the roots of the vector-control and CaZF-FOX plants. Comparison of basipetal root auxin transport in 8-d-old vector control (Vec) and CaZF-FOX (L100, L212, L211 and L316) seedlings in root segments above the site of auxin application at the root tip. An average of auxin transport in root segments was calculated and data presented was fold increase in average auxin transport, relative to vector control tobacco. Results from three independent experiments are presented. The error bars indicate the standard deviation (SD). * indicates significant differences in comparison with the vector-control (Vec) at p<0.05. (TIF)

**Methods S1** Protoplast isolation and co-transfection analysis, Chromatin immunoprecipitation (ChIP) and Auxin transport assay. (DOC)

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**Author Contributions**

Conceived and designed the experiments: DC. Performed the experiments: DJJ. Analyzed the data: DJJ. Wrote the paper: DC DJJ.

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