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The CarERF genes in chickpea (*Cicer arietinum* L.) and the identification of CarERF116 as abiotic stress responsive transcription factor

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Abstract The AP2/ERF family is one of the largest transcription factor gene families that are involved in various plant processes, especially in response to biotic and abiotic stresses. Complete genome sequences of one of the world's most important pulse crops chickpea (*Cicer arietinum* L.), has provided an important opportunity to identify and characterize genome-wide ERF genes. In this study, we identified 120 putative ERF genes from chickpea. The genomic organization of the chickpea ERF genes suggested that the gene family might have been expanded through the segmental duplications. The 120 member ERF family was classified into eleven distinct groups (I-X and VI-L). Transcriptional factor *CarERF116*, which is differentially expressed between drought tolerant and susceptible chickpea cultivar under terminal drought stress has been identified and functionally characterized. The *CarERF116* encodes a putative protein of 241 amino acids and classified into group IX of ERF family. An in vitro *CarERF116* protein-DNA binding assay demonstrated that *CarERF116* protein specifically interacts with GCC box. We demonstrate that *CarERF116* is capable of transactivation activity of and show that the functional transcriptional domain lies at the C-terminal region of the *CarERF116*. In transgenic *Arabidopsis* plants overexpressing *CarERF116*, significant up-regulation of several stress related genes were observed. These plants also exhibit resistance to osmotic stress and reduced sensitivity to ABA during seed germination. Based on these findings, we conclude that *CarERF116* is an abiotic stress responsive gene, which plays an important role in stress tolerance. In addition, the present study leads to genome-wide identification and evolutionary analyses of chickpea ERF gene family, which will facilitate further research on this important group of genes and provides valuable resources for comparative genomics among the grain legumes.

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Introduction

Abiotic stress primarily drought, temperature extremes, and salinity is the major limiting factor that prevents crops from realizing their full yield potential. Tolerance to these stresses is a complex process, involving various changes in signal transduction, gene expression, and ultimately metabolic and physiological processes. Abiotic stress induces the expression of various regulatory genes (such as transcriptional factors AP2/ERF, bZIP, DREB, MYB etc.) that are involved in stress tolerance. Alterations in expression of several transcription factors and their downstream genes in response to different biotic and abiotic stress have been reported in plants [see review by (Mizoi et al. 2012; Saibo et al. 2009)]. Among these APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily is one of the largest group of transcription factors (TFs) in plants. The AP2/ERF genes contain a conserved DNA binding AP2 domain of ~58–60 amino acids (Okamuro et al. 1997). In *Arabidopsis*, based on the number and composition of AP2 domain, the superfamily is divided into AP2 (contains two AP2/ERF domains), ERF (contains single AP2/ERF domain) and Related to ABI3/VP1 (RAV) (contains single AP2/ERF and B3 domain) families. ERF family further subdivided in two subfamilies ERF and DREB subfamily (Sakuma et al. 2002), or 12 groups (I to X, VI-L and Xb) (Nakano et al. 2006). The ERF proteins have been mainly implicated in biotic stress responses, however recent studies have uncovered additional role of some ERF proteins in abiotic stress responses. For example the pepper ERF gene, *CaPF1*, was induced by ethylene (ET), jasmonic acid (JA), and cold stress response and gave tolerance to freezing temperatures, pathogens, heavy metals (cadmium, copper and zinc) and heat stress, in transgenic *Arabidopsis* (Yi et al. 2004), Virginia pine (Tang et al. 2005) and potato (Youm et al. 2008).

Recently sequenced chickpea genome comprises of 28,269 genes and organized into 15,441 gene families (orthologous groups using orthoMCL) (Varshney et al. 2013). Genome-wide analysis of gene families provides valuable insight into evolutionary conservation or functional diversification of the gene family. To our knowledge, no information is available on genome-wide identification and characterizations of ERF genes in the chickpea. Given the critical role of ERF transcription factors in biotic and abiotic stress, we identified 120 members of ERF family and systematically analysed genomic organization, gene duplication and phylogenetic relationships. Additionally, we performed full length cloning and functional characterization of an ERF gene *CarERF116* using detailed expression profiling under various biotic and abiotic stresses, in vitro protein interactions, transcription activation activity test in yeast cells, overexpression in *Arabidopsis* plants and expression profiling of downstream target genes using *Arabidopsis* ATH1 genome arrays in transgenic *Arabidopsis* plants overexpressing *CarERF116*.

Methods and methods

Genome-wide identification and analysis of ERF genes

Whole genome nucleotide and protein sequences encoding AP2/ERF transcription factors from *Arabidopsis* and soybean were retrieved from transcription factor database 3.0 (http://plntfdb.bio.uni-potsdam.de/v3.0/). Chickpea CDC Frontier genome and annotation data were downloaded from the ICRISAT chickpea data resource (http://www.icrisat.org/gt-bt/ICGGC/GenomeManuscript.htm). *Arabidopsis* and soybean ERF sequences were used to identify homologous peptides from chickpea by performing a BLASTP search. Chickpea EST sequences were also searched to eliminate any additional ERF members. The identified candidate protein sequences were subjected to the National Center of Biotechnology Information (NCBI) CD search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) databases to ensure the presence of the AP2 domain. After removing the repeat sequences and incomplete sequences, remaining protein sequences were used for further analyses. Phylogenetic trees were constructed using MEGA 5.2 (Tamura et al. 2011) with the maximum likelihood method using a bootstrap value of 1,000 and pairwise gap deletion mode. The potential duplicated genes in chickpea genome were analysed by MCScanX (Wang et al. 2012) using default criteria.

Following online and stand-alone tools, databases and software's were used in the ERF analysis. Sequence homology search tools (BLASTN and BLASTX: http://blast.ncbi.nlm.nih.gov/Blast.cgi); Protein sequence analysis (ExPASy proteomics Tools: http://expasy.org/tools/, TASSER: http://minnou.cchmc.org/; Secondary structure analysis tool: http://www.ibi.vu.nl/programs/yaspinwww/, http://imtech.res.in/raghava/apssp/; nuclear localization signals prediction: http://www.predictprotein.org/, http://wolfpsort.org/); Plant transcription factor databases (PTFDB: http://planttfdb.cbi.edu.cn/); Plant promoter analysis (PLACE: http://www.dna.affrc.go.jp/PLACE/, plantCARE: http://bioinformatics.psb.ugent.be/webtools/plantcare/html/); Microarray data analysis: GeneSpring® (Agilent Technologies); QPCR analysis: MxPro™ QPCR Software and Microsoft office 2007 (Microsoft Corporation); DNA and amino acid analysis: BioEdit 7.1.3.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), Lasergene DNASTAR (DNASTAR, Inc).

Plant material and stress treatment

Chickpea (*Cicer arietinum* L.) accession ICC 4958, a relatively drought tolerant cultivar was used in this study. For imposing terminal drought stress, plants were subjected to water stress in a pot experiment at flowering stage using the dry down procedure as described in (Deokar et al. 2011). Three week old chickpea seedlings were treated with jasmonic acid (20 μM), salicylic acid (100 μM), gibberellic acid (100 μM), NaCl (150 mM) and sampled at 0, 3, 6, 12, and 24 h after treatment. For heat and cold stress, seedlings were exposed to 37 °C and 4 °C, respectively and sampled at 0, 3, 6, 12, and 24 h after treatment. Dehydration stress was imposed by placing the seedlings over a plain tissue paper at room temperature (25±1 °C), and sampled at 0, 3, 6, 12, and 24 h after treatment. All stress treatments were conducted in triplicates and five seedlings from each experimental treatment were pooled for RNA isolation.

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown under a 16-h-light/8-h-dark photoperiod at 23± 1 °C. *Arabidopsis* seedlings were subjected to osmotic stress induced by mannitol solution of water potential −0.7 MPa (54.7 g/l mannitol) and −1.07 MPa (120.4 g/l mannitol). For freezing stress, *Arabidopsis* seeds were kept at −20 °C for 24 h and then kept for germination at room temperature. For evaluating seed germination ability, more than 50 seeds each of wild type (WT) and 35S::CarERF116 *Arabidopsis* in triplicate was placed on Whatman filter paper saturated with distilled water or different concentrations of mannitol or ABA and incubated at 4 °C for 48 h and then kept at 23±1 °C for germination. Seeds were considered as germinated once radicals completely penetrated the seed coat. Germination was scored daily up to 7 days and expressed as a percentage to the total number of seeds plated.

RNA isolation and quantitative real time PCR

Total RNA from plants was isolated using TRIzol reagent (Invitrogen) and treated with DNase for complete elimination of DNA contamination. The DNase treated RNA was converted into cDNA using the oligodT (18 mer) primer and Super-Script III reverse transcriptase according to the manufacturer's protocol (Invitrogen, USA). The obtained cDNA was diluted to 5 ng/μl concentration. SYBR green qPCR was performed in 96 well plates using the Stratagene Mx3000P system and SYBR FAST qPCR Master Mix (2x) Universal (KAPA Biosystems). All qPCR reactions were run in triplicates with a no-template control to check for contamination. Reaction mixture of 10 μl contained 1X SYBR FAST qPCR master mix, 200 nM gene specific forward and reverse primers and 5 ng cDNA. Q-PCR was conducted with the following parameters: 3 min at 95 °C (enzyme activation), 40 cycles with each cycle consisting of 3 s at 95 °C (for denaturation) and

30 s at 60 °C (for annealing/extension). Finally melting curve analysis was performed from 65° to 95 °C in 0.5 °C steps each lasting 5 s to confirm presence of a single product and absence of primer-dimers. Two internal controls GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, GenBank accession no. AJ010224) and HSP90 (Heat shock protein 90, GenBank accession no. GR406804) were used to normalize the variations in cDNA samples. The fold changes were calculated using the $2^{-\delta\delta Ct}$ method.

Identification of 5′- and 3′- ends of *CarERF116* cDNA and isolation of its 5′ upstream sequence

To isolate full-length *CarERF116* cDNA and to map the transcription start site (TSS), the 5′- and 3′-RACE (Rapid Amplification of cDNA Ends) were performed according to the SMARTer RACE cDNA Amplification Kit (Clontech, USA) and GeneRacer RACE kit (Invitrogen, USA) respectively. For 5′- RACE, one μg of total RNA, isolated from chickpea plants subjected to water deficit stress, was used for reverse transcription with SMART ScribeTM reverse transcriptase (100U) with the 5′-CDS primer-A. A set of nested PCR primers was designed from the available truncated EST sequence. CarERF1-5′-NES1: TTTGCCTTTTCTCACCAACTTTGATGC, CarERF1-5′-NES2: CAGCTTTATCATAAGCCCTTGC AGCTT. The Primers 5′-NES1 and 5′-NES2 were used sequentially in combination with the UPM primer to generate 5′ end of *CarERF116* gene. For 3′ RACE, cDNA was prepared by reverse transcription of RNA using 3′-AP adapter primer (Invitrogen, USA) and MuMLV-RT (Promega, USA) and used as template for isolation of 3′ end of the gene. Two 3′ nested primers CarERF1-3′-NES1: GACTCCTTCATGTTGGAAAGGG T, CarERF1-3′-NES2: CCTCCTTTGTCACCATTATCTC CAC along with a GeneRacer 3′-primer were used sequentially to obtain the 3′ end of *CarERF116* gene. The obtained RACE PCR products were eluted from the gel, cloned in pGEM-T easy cloning vector and sequenced.

The 5′ -upstream sequence (CarERF116 promoter region) was isolated by genome walking with the help of the Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA) according to manufacturer's instructions. In brief, five libraries were prepared by digesting chickpea genomic DNA by *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, and *Sma*I, and further ligated with genome walker adapters. The adapter ligated DNA libraries were used as template for genome walking PCR. Two nested PCR primers (CarERF116-GW1: GTTGCAAA AACTGAAGGTGGATTCAAAGG, and ERF116-GW2: TGGAAGAAGGGA TTGTGATGTTCAAAGAGG along with AP1 and AP2, were sequentially used to obtain the 5′-upstream sequences.

Southern blotting analysis

Genomic DNA was isolated from chickpea leaves (cv. ICC 4958) by a modified CTAB method and purified by phenol extractions (Dellaporta et al. 1983). 15 μg genomic DNA was completely digested with *Eco*RI, *Hin*dIII, *Xba*I (New England Biolabs, UK). The digested DNA was run on 1 % agarose gels and transferred onto Hybond N+nylon membranes (Amersham Pharmacia Biotech, USA) by capillary blotting as described in (Sambrook 2001). PCR amplified full-length *CarERF116* was gel-purified and labeled with α-^{32}P-dCTP, using the DecaLabel™ DNA labeling kit (Fermentas Life Sciences, UK). The hybridization signals were detected using a PharosFX plus PhosphorImager (Bio Rad, USA).

Generation of transgenic *Arabidopsis* plants

Full-length cDNA of *CarERF116* was amplified from cDNA by PCR using a set of PCR primers (CarERF1-ORF-F: CGAGGATCCAATATGCAACAAACCTTTGAATCCACC and CarERF1-ORF- R: ACTGAGCTCATATCAATAACC CATCATGAGTGG) containing the *Bam*HI and the *Sac*I site, respectively. The resulting amplicon was digested with *Bam*HI and *Sac*I, and ligated between the CaMV 35S promoter and the NOS terminator in the pBI121 binary vector containing the kanamycin as a selectable marker.

This construct was mobilized into *Agrobacterium tumefaciens* strain GV3101 using freeze-thaw method (Chen et al. 1994). *Arabidopsis* Col-0 was transformed with these constructs by the floral-dip method (Clough and Bent 1998). T_1 seeds were selected on MS medium containing kanamycin (50 mg/l). To assess *CarERF116* expression, RT-PCR analysis was performed on T_2 transformants. Three independent transgenic lines for each construct were used for further analysis.

Yeast one hybrid assay

For yeast one hybridization based transactivation assay, complete and partial sequences of *CarERF116* open reading frame (without stop codon) was amplified from the chickpea cDNA with the specific PCR primers containing *Bam*HI site in forward primer and *Sal*I in the reverse primer. The purified PCR product was digested with *Bam*HI and *Sal*I and cloned in frame with GAL4 DNA binding domain in the digested pGBKT7 vector (Clontech, USA). The positive clones containing appropriate inserts were transformed into yeast Y2HGold strain (Clontech, USA) containing four reporter genes (AUR1-C, ADE2, HIS3, and MEL1) under the control of GAL4 promoter.

Protein expression and in vitro binding assay (EMSA)

For production of recombinant protein, *CarERF116* protein with C-terminal His-Tag was expressed in *Escherichia coli*. The *CarERF116* open reading frame (without stop codon) was amplified with primers flanked by restriction sites for *Nde*I and *Xho*I to clone in pET-29a(+) (Novagen, USA). The CarERF116-HIS protein was produced by inducing *Escherichia coli* strain BL21 (DE3) containing a pET-29a-CarERF116 by 0.5 mM IPTG concentration at 26 °C. The recombinant protein was purified with Ni-NTA chromatography spin columns (Qiagen, USA) according to the manufacturer's instructions. For the EMSA analysis, following wild type (W) and mutant (M) double stranded oligonucleotides were designed as wild type GCC box sequence, W-GCC-F1: CATAAGAGCCGCCACT, mutant type GCC box sequence M-GCC-F1: CATAAG ATCCTCCACT, wild type DRE box sequence W-DRE-F1: ATACTACCGACAT, M-DRE-F1: ATACTGCCGACAT. 0.5 μg DNA elements and 100 and 200 ng of CarERF116-HIS fusion proteins were mixed in EMSA buffer (100 mM HEPES, pH 7.5, 0.5 M KCl, 25 % glycerol, 5 mM DTT, 5 mM EDTA) at room temperature for 30 min, and then were loaded onto an 8 % non-denaturing polyacrylamide gel. After electrophoresis in 1.0X TBE buffer, the gel was stained with SYBR green (Molecular probes) for visualization of DNA bands.

Microarray analysis

Total RNA was isolated from three-week old *Arabidopsis* plants grown under controlled conditions in a glasshouse. Microarray expression analysis was performed using Affymetrix ATH1 array GeneChip (Affymetrix, USA). Total RNA was isolated using Trizol reagent (Invitrogen) and processed as recommended by Affymetrix technical manual. For further analysis, the GeneChip data files were imported into GeneSpringGX (Agilent Technologies, USA).

Results

Identification and phylogenetic analysis of ERF genes in the chickpea genome

We identified 147 putative AP2/ERF domain containing transcription factors in chickpea. According to the number and structure of AP2/ERF domain, the 120 genes were classified into ERF family, 16 into AP2, two into RAV family and one as soloist members. In the present study, we focused our attention on the analyses of ERF family members. An unrooted phylogenetic tree of 120 ERF members was constructed (Fig. 1). Further, based on the Nakano classification system of
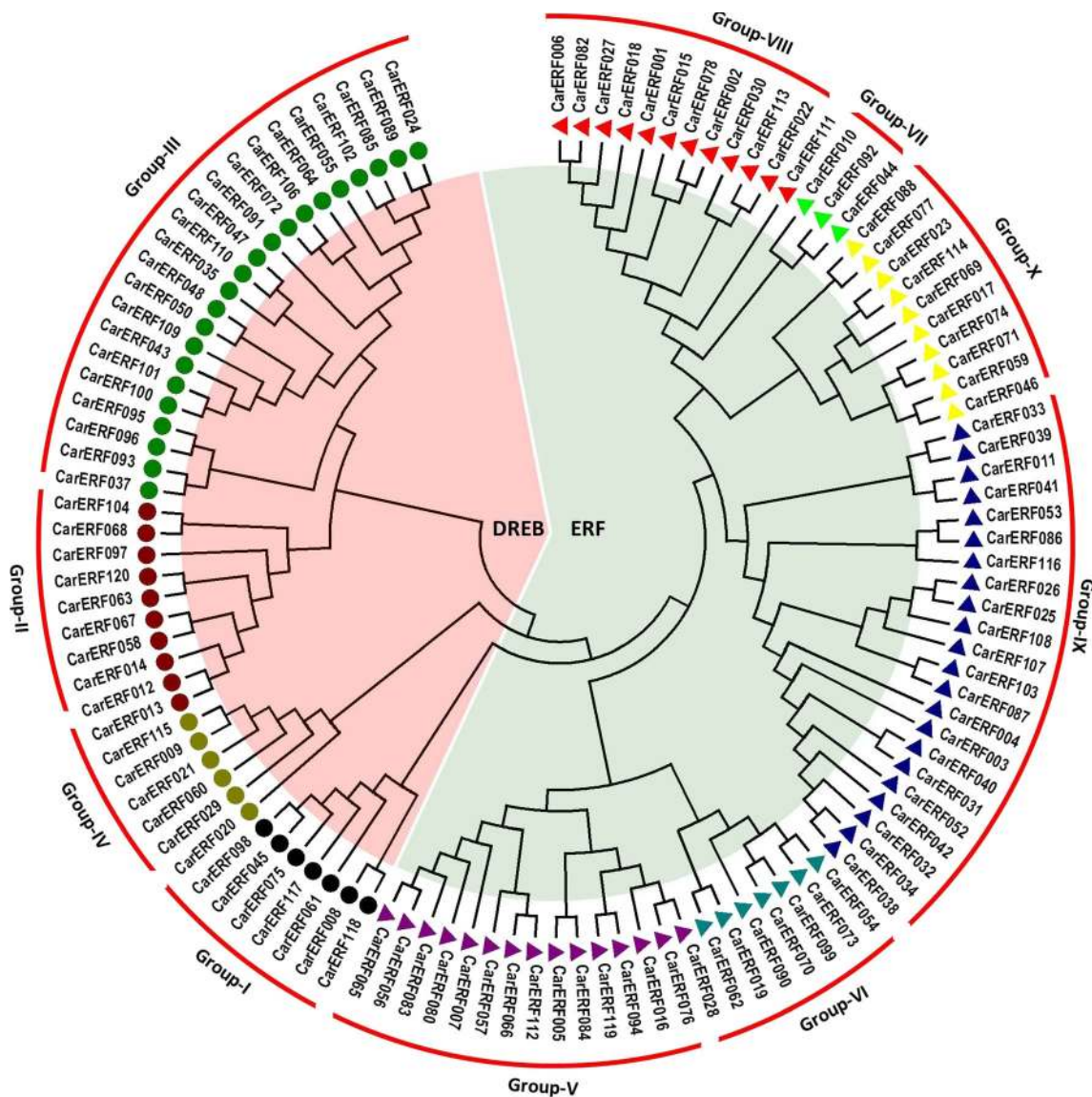
Fig. 1 An unrooted phylogenetic tree of chickpea ERF proteins. The amino acid sequences of the AP2/ERF domain of CarERFs (excluding Group VI-L) were aligned by Clustal W and the phylogenetic tree was constructed using MEGA 5.2 and the maximum likelihood method. Ten groups of CarERF are markers as I-X, as described by Nakano et al., 2006

Arabidopsis ERF family (Nakano et al. 2006), the chickpea ERF family were organized in eleven groups (I-X and VI-L). The group I-IV belongs to DREB subfamily which contains 46 members, whereas group V-X belongs to ERF subfamily and contains 74 genes Group III is the largest group with 23 ERFs, whereas group VII is the smallest group with only three ERFs (Table S1 and S2). Generic names (*CarERF001–CarERF120*) were assigned to all identified ERF genes based on their order on chickpea chromosomes. The identified ERF genes encode peptides ranging from 128 to 429 amino-acids (aa) in length with an average of 238 aa. Whereas, in *Arabidopsis*, the size of ERF genes ranges from 131 to 391 aa with an average of 240 aa. Comparative analysis of ERF gene family size across different plant species including *Arabidopsis* (122), Rice (131), Poplar (198), Grapevine (109), *Medicago truncatula* (106) and caster bean (90) indicates relatively large size variations. Although the size of ERF family in chickpea and *Arabidopsis* is almost the same, but the size of ERF and DREB subfamily varies significantly. The biotic stress responsive ERF subfamily size in chickpea (74 members) is larger than *Arabidopsis* (65 members) whereas, abiotic stress responsive DREB subfamily size of chickpea (46 members) is smaller than *Arabidopsis* (57 members).

We performed a multiple sequence alignment of the conserved AP2 domain as well as complete amino acid sequences of the ERF proteins from each group to investigate the sequence features of the CarERF family proteins. The members

of ERF family contain single AP2/ERF domain. The amino acid residues Gly-4, Arg-6, Arg-8, Glu-16 and Ala-37 are completely conserved among the 120 proteins in the ERF family. In addition, 97 % of the ERF family members contain a conserved WLG motif (Fig. S1). These conserved amino acid residues and motifs are a common feature with almost all studied members of the ERF gene family of *Arabidopsis*, soybean and rice (Nakano et al. 2006; Zhang et al. 2008). In addition to AP2/ERF domain, members of Group VI contain a conserved CRF domain in the N-terminal region, which is a characteristic feature of Cytokinin response factors (CRFs) (Fig. S2). CRFs are unique to land plants lineages and involved in normal leaf vascular patterning, plant growth and development and senescence process (Zwack et al. 2013).

Conserved functional motifs were also identified outside the AP2/ERF domain (Fig. S3). For instance, Group II and VIII contain a conserved EAR (ERF-associated amphiphilic repression) motif, which is the most predominant form of the transcriptional repression motif so far identified in plants (Kagale and Rozwadowski 2011). The CarERF group II and VIII consist of 10 and 15 ERF genes respectively. The ERF genes from group VIII of tobacco, *Arabidopsis* and rice have been shown to be involved in GCC box-mediated transcription repressor activity (Nakano et al. 2006). The conserved EAR motif in the chickpea ERF groups II and VIII suggest that these genes may function in GCC box-mediated transcriptional regulation process.

Putative transcription activators, such as Ser/proline/asn/gln-rich domain regions were predominantly present in the majority of chickpea ERFs. Another type of transactivation domain found in group IX as CMIX-1 motif (conserved motif IX-1), which is highly conserved across plant species. The CMIX-1 motif of *Arabidopsis* AtERF#98 gene has been characterised as a EDLL motif and found as strong transcription activation characteristics, as well as ability to partly overcome the repressive activity of EAR repressor motif (Tiwari et al. 2012). We also found conserved putative MAP kinase and/or casein kinase sites in several ERF proteins, such as group VI and VI-L has conserved casein kinase sites, whereas group VII and IX has conserved MAP kinase phosphorylation sites. The phosphorylation of group IX member of *Arabidopsis* ERF gene Pti4 and rice OsEREBP enhances the transactivation activity via GCC box-mediated transcription (Cheong et al. 2003; Gu et al. 2000). The presence of putative kinase and/or casein kinase sites in the chickpea ERF group IX genes, suggesting that these genes might undergo post-translational phosphorylation to regulate downstream genes. Several group specific motifs with unknown function were also identified, such as group VII contain a conserved MCGGAI[I/L] motif. This motif was also found conserved in *Arabidopsis* and soybean group VII (Zhang et al. 2009). Similarly, group V contain a conserved motif sequence QMIEELL[ND].

Genome-wide distribution and duplication events among CarERF genes

To define the relationship between genetic divergence and gene duplication within the CarERF gene family in chickpea, we determined the physical locations of CarERF genes in the chickpea genome. The results showed that the 120 CarERFs were distributed throughout all eight chickpea pseudochromosomes and also in ten unplaced scaffolds (Fig. 2). Maximum of 23 (19.2 %) ERFs were located on Ca4 and a minimum of seven (4.0 %) ERFs were located on Ca2. Genome-wide distribution of CarERFs appeared to be uneven, with relatively highest gene density of one ERF gene per 1.2 Mb on Ca8 and lowest gene density of one ERF gene per 12.2 Mb on Ca6.

We observed seven clusters of ERFs on Ca1, Ca3, Ca4, Ca7 and Ca8. Genes within the clusters belong to the same group of ERFs, such as cluster located on Ca1, containing CarERF012, CarERF013 and CarERF014 (belongs group II), cluster located on Ca4 containing CarERF040, CarERF041 and CarERF042 (belongs to group IX) and cluster located on Ca8 containing CarERF095 and CarERF096 (belongs to group III). Similar patterns of ERF gene clusters were also found in the *Arabidopsis* (Sakuma et al. 2002), grape (Licausi et al. 2010), and poplar genomes (Zhuang et al. 2008). In plants, gene families are mainly expanded by segmental and tandem duplication events. In the chickpea genome, we identified 1,954 (6.9 %) genes as tandem and 5,230 (18.5 %) genes as segmental duplicated. In total, 51 (42.5 %) ERFs were involved in segmental duplication whereas, 14 (11.7 %) genes were involved tandem duplication (Fig. 3).

Isolation and sequence analysis of a drought responsive *CarERF116*

One of the differentially expressed ESTs in response to terminal drought stress, coding for AP2/ERF protein was identified in our earlier studies (Deokar et al. 2011). We delineated the 5′ and the 3′ end and isolated the full-length cDNA from ICC 4958 (GenBank accession no. JX034738). The full-length cDNA sequences showed 100 % homology with *CarERF116* (Ca_23626) of CDC frontier genome and classified as a member of group IX ERF gene family. The *CarERF116* contains an open reading frame (ORF) of 726 bp that encodes for a protein of 241 amino acids. This ORF is flanked by 3′ and 5′ untranslated region (UTRs) of 247 bp and 93 bp, respectively. *CarERF116* protein has a molecular mass of 27.24 kDa and an isoelectric point (pI) of 9.39. The predicted 241 amino acid long polypeptide is composed of 23 negatively charged (Asp+Glu) and 32 positively charged (Arg+Lys) residues. The relatively high percentage of positively charged amino acids suggests that the protein product of *CarERF116* is probably a nuclear protein and could be a putative transcription factor protein. *CarERF116* showed high homology with some of the known ERF proteins of *Glycine max* (70 %), *Arabidopsis*
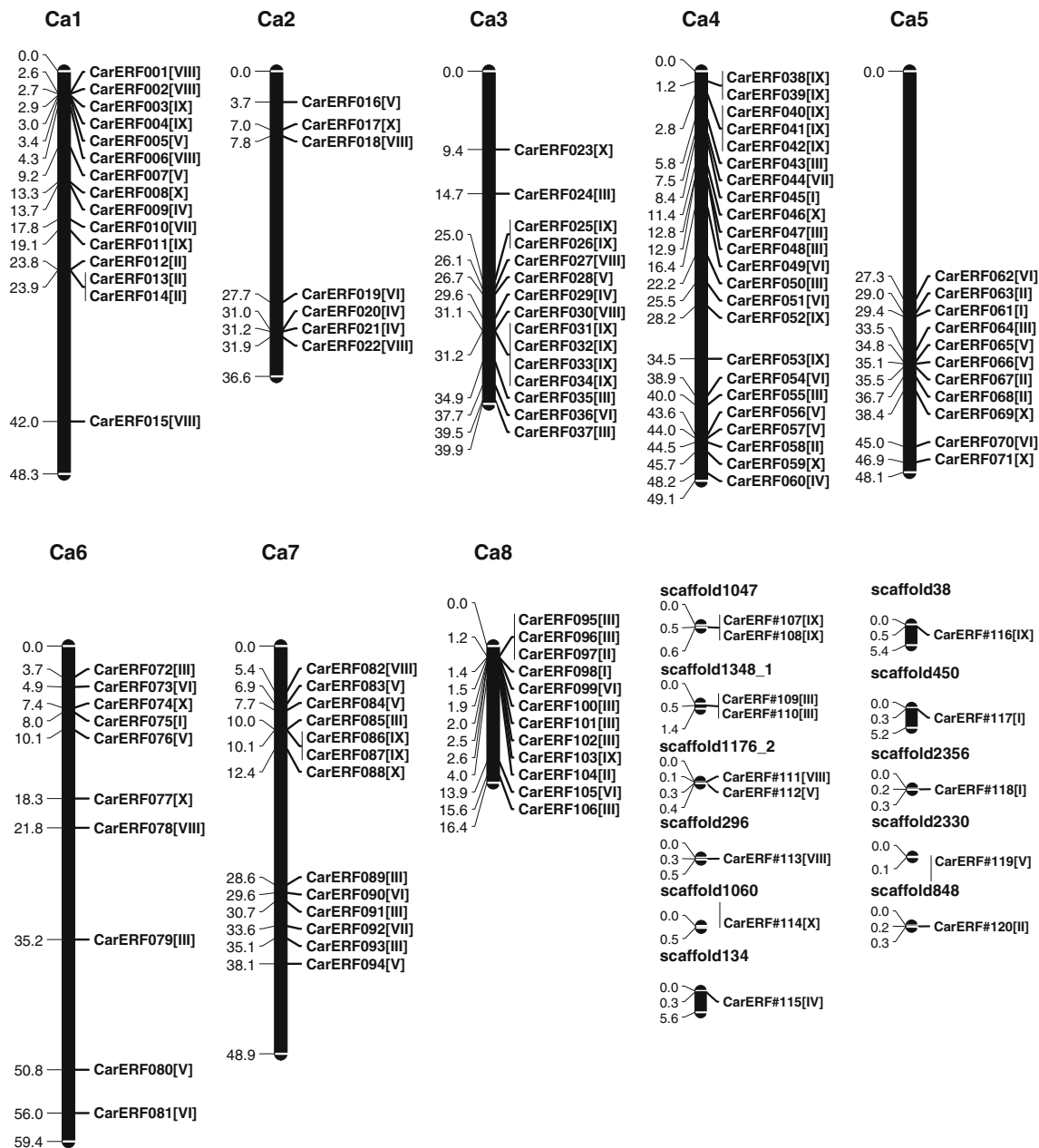
Fig. 2 Chromosomal locations of chickpea ERF (CarERF) genes. Out of 120 CarERFs, 108 were mapped to the eight chickpea pseudochromosomes, while 12 ERFs resides on 11 unplaced scaffolds of chickpea genome. The scale is in megabases (Mb)

thaliana (53 %), *Arabidopsis lyrata* (52 %) and *Populus trichocarpa* (46 %). Although sequence homology was more in the AP2 DNA binding region, two conserved motifs outside the AP2 domain, towards the C-terminus were also found. These two conserved motifs were also found in group IX of *Arabidopsis* ERF family (Nakano et al. 2006) (Fig. S4).

Structural characterization of *CarERF116* protein

The molecular and biochemical functions of newly identified genes can be predicted using three-dimensional (3D) structural analysis of protein sequences. For 3D homology modelling

of complete *CarERF116* sequence, we performed BLAST analysis to find homologous sequences, but no significant homologous template was found. We used ab initio modeling by I-TASSER method, which implements TASSER (threading/assembly/refinement) on I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The *CarERF116* structure was estimated with a RMAS score of 14.3±3.8 and C Score of −3.56 (Fig. 4b). The structure consists of a DNA binding domain of three β-sheets packed with one α-helix. In addition to this four α-helix structure outside the AP2 domain are also observed, which may be involved in the structural stability of *CarERF116* protein.
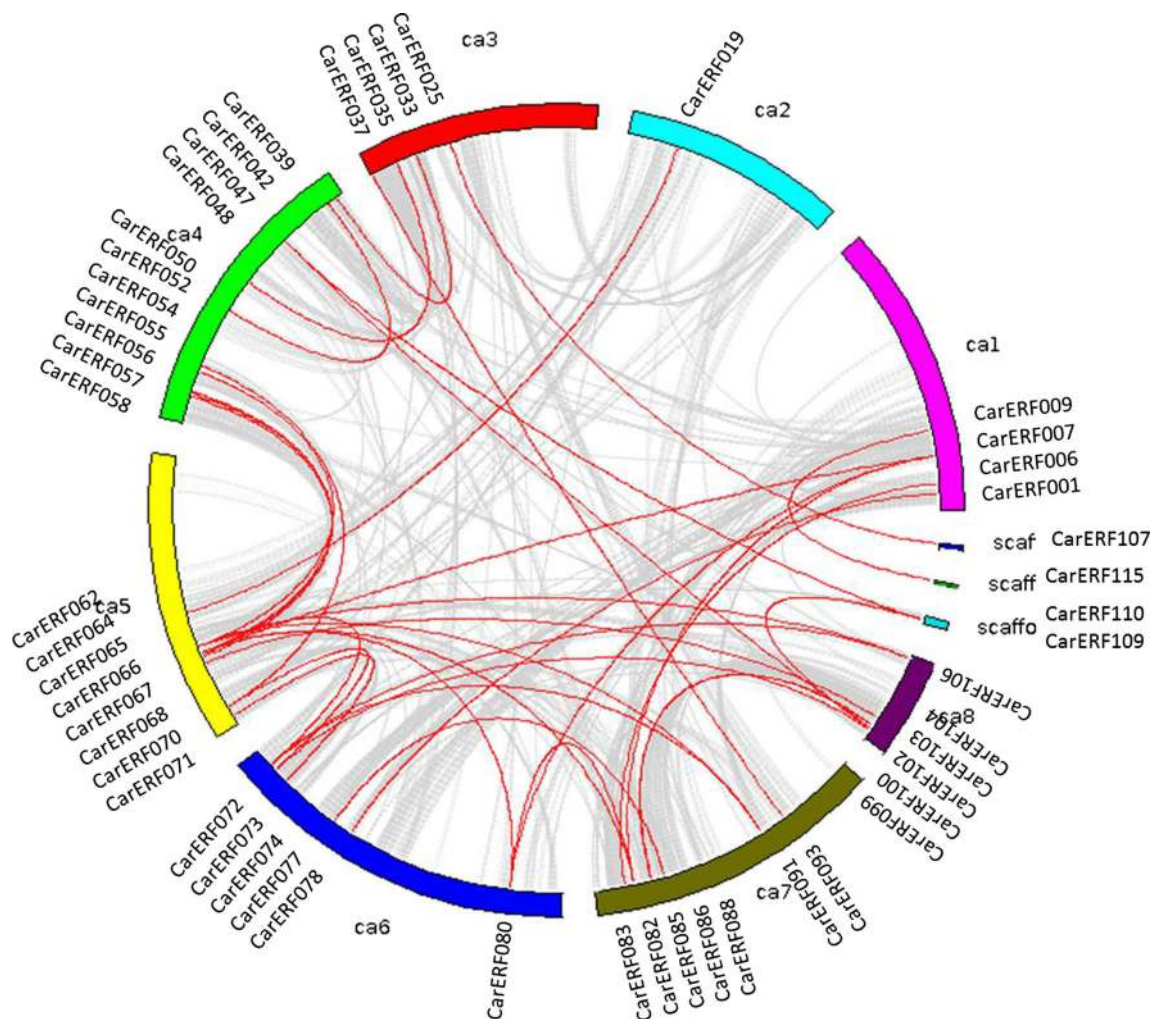
Fig. 3 Genomic distribution of segmentally duplicated ERF genes in chickpea. Red lines indicate duplicated CarERF genes over the gray background of collinear blocks in the chickpea genome

The AP2/ERF domain consists of about 60–70 aa and involved in DNA binding. The predicted secondary structure of the DNA binding domain of *CarERF116* fits completely with the expected AP2/ERF domain, showing three β-strands and an amphipathic α-helix. The AP2 domain of *Arabidopsis AtERF1* (3GCC) was taken as a template for homology modeling of DNA binding domain of *CarERF116* protein. The overall root mean square deviation (RMSD) between the AP2 domain of *CarERF116* and template (3GCC) is 0.5 Å. The C-score of 1.58 indicates the high quality of the predicted protein model (C-score is used for estimating the quality of predicted models and typically in the range of −5 to 2, where a higher value signifies a model with a high confidence and vice-versa) (Fig. 4c). The three β-sheet consisting of strand one (V-5 to R-8), second (G-11 to D-19) and third (S-25 to G-30) packed antiparallel with α-helix (A-35 to R-50). The structure of α-helix relative to the β- sheet is due to the interaction of the alanine residues in the α-helix and the larger hydrophobic residues in the β-sheet as suggested by (Allen et al. 1998). This domain is positively charged and predicted to bind to the negatively charged DNA double helix. Comparison between *Arabidopsis* AtERF1 and *CarERF116* at the DNA binding sites, reveal that the key residues were more than 90 % conserved (10 out of 11 amino acids), including R-150 (AtERF1)/R-105 (*CarERF116*), G-151/106, R-153/108, R-155/110, W-157/112, E-163/118, R-165/120, R-170/125, W-172/127 and T-175/130 (Fig. 4b). These 11 residues of AtERF1 were involved in making contacts with a GCC box of target genes (Balaji et al. 2005). The conserved residues in the DNA binding domain of *CarERF116* suggest that the *CarERF116* protein interacts in a similar fashion with the GCC box elements of target genes in chickpea.

Expression profiles of *CarERF116* gene

To identify the potential function of *CarERF116* gene, expression profile was investigated in different tissue of chickpea plants growing under control condition and also in response to different abiotic stress conditions and hormonal responses (Fig. 5). *CarERF116* transcripts were ubiquitously present in

Fig. 4 Three-dimensional structures of the AP2 DNA Binding Protein domain and full *CarERF116* gene. (**a**) Alignment of 3GCC (template used for 3D modeling) with the *CarERF116* AP2 DNA-BD. (**b**) 3D ribbon structure of the AP2 DNA binding domain of *CarERF116* and (**c**) full length *CarERF116*. Yellow color represents the three-β sheets, red color represents α-helix and green color represents chain structure. On the right side, the electrostatic surface potential is shown in color gradient from positive (*blue*) to negative (*red*) color
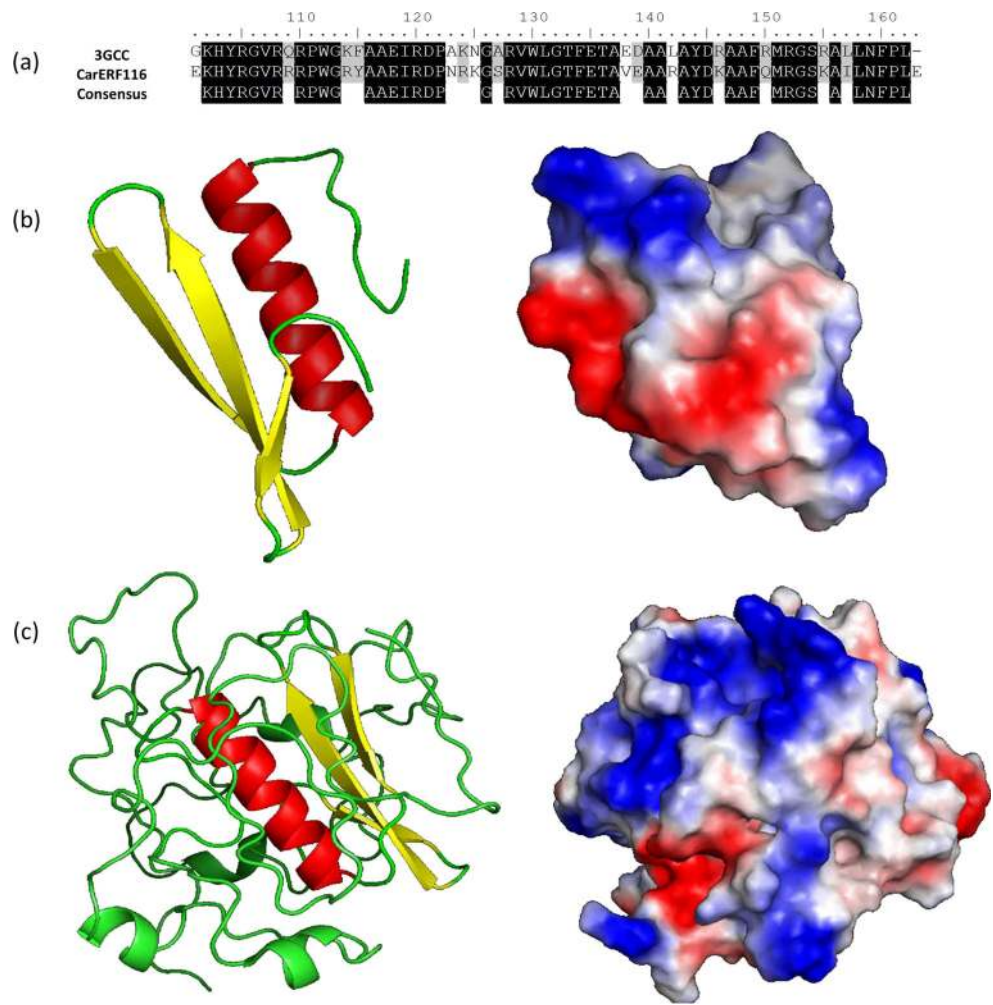
all tissues of chickpea, including leaf, shoot, root, flowers and pods under normal growth conditions. The *CarERF116* transcripts showed relatively high expression in roots compared to other tissues. The expression of *CarERF116* was significantly induced in response to dehydration, salinity and cold stresses. In case of dehydration stress, nine fold induction of the *CarERF116* transcript was detected within one h of the stress treatment. Under cold stress, 15 fold induction of the gene was observed at 3 h. Similarly, in the case of salinity stress, the transcript level of *CarERF116* increased by 11 fold at 6 h. The level of the *CarERF116* transcript gradually declined within 24 h in all these cases. In contrast, we observed very low induction of *CarERF116* under H2O2 treatment. Considering that *CarERF116* might respond to a variety of abiotic stresses, we examined the expression pattern of *CarERF116* in response to exogenous application of ABA, GA3, SA and ethephon. Following the ABA treatment, expression of *CarERF116* increased up to eight fold at 3 h, but rapidly declined at 6 h of treatment. The expression of *CarERF116* increased gradually up to four fold at 12 h of exogenous application of ethephon. Similarly, it increased to up to seven fold at 6 h of GA3 treatment. Under SA treatment, expression of *CarERF116*

increased up to four fold in initial 1 h, and remain relatively constant over 24 h of treatment. This expression profile of *CarERF116* is very similar to some of the members of the ERF group XI of *Arabidopsis* (Fujimoto et al. 2000), rice (Hu et al. 2008), soybean (Zhang et al. 2008) and tomato (Gu et al. 2000). Induction of *CarERF116* by dehydration, salinity and cold as well as ABA, GA3, SA and ethephon suggests that *CarERF116* may have important roles in multiple signaling transduction pathways controlling biotic and abiotic stresses.

Genomic organization of *CarERF116* gene

To analyze the exon/intron organization of *CarERF116* gene, PCR amplification and sequencing of full-length *CarERF116* gene with genomic DNA and cDNA was conducted. A sequence comparison of the genomic clone with the cDNA clone showed that *CarERF116* is an intron-less gene. This finding also supports the previous report in *Arabidopsis*, where the majority of the ERF genes are without an intron (Nakano et al. 2006). The copy number of *CarERF116* gene was estimated by Southern blotting. The genomic DNA of chickpea cultivar ICC 4958 was digested with *Eco*RI, *Hin*dIII
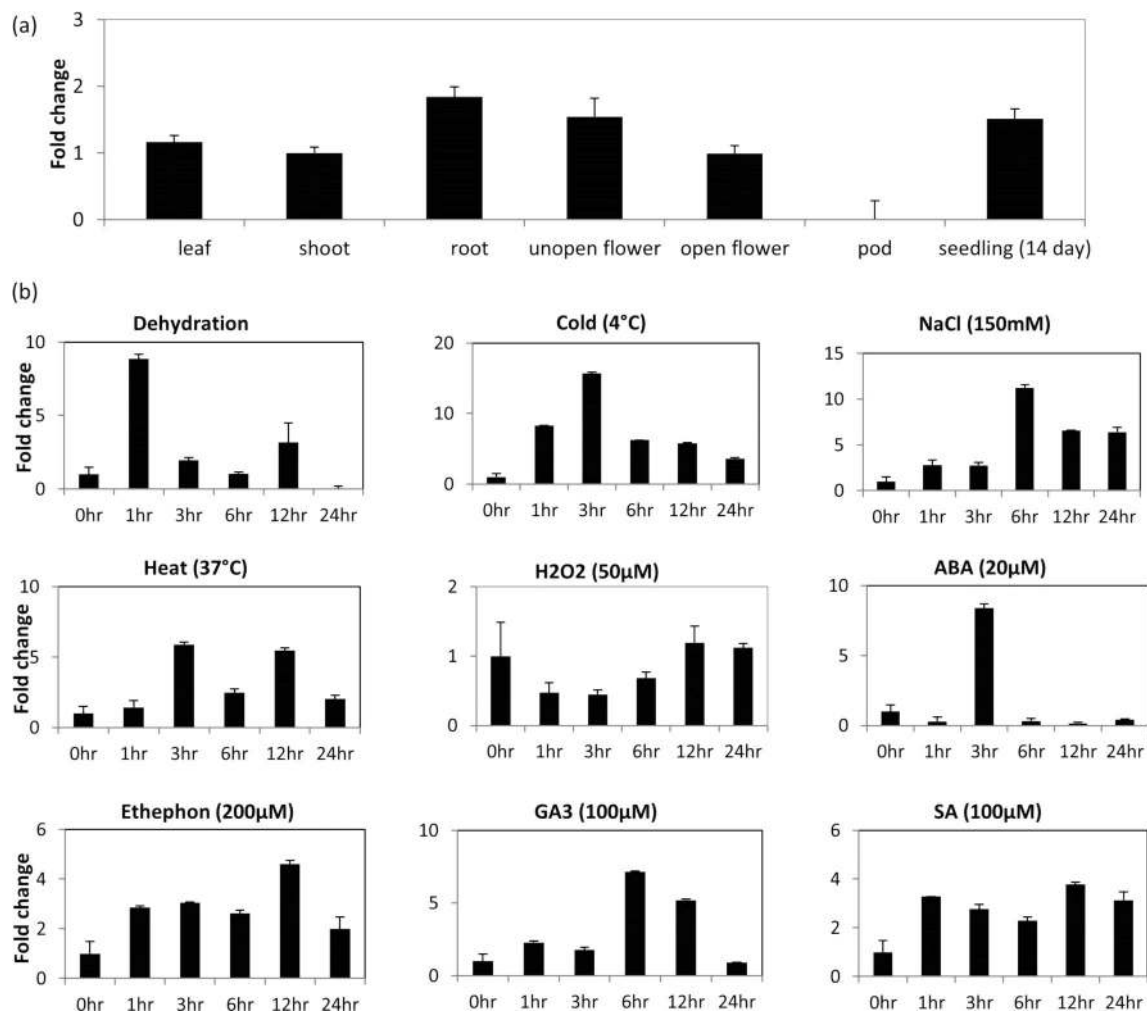
Fig. 5 Quantitative real time PCR analysis of *CarERF116* gene. (a) Expression pattern of *CarERF116* in various tissues of chickpea ICC 4958. Total RNA was extracted from leaves, shoot, roots, unopened and open flowers. (b) *CarERF116* gene under different abiotic stress conditions (dehydration, cold, NaCl and heat) and phytohormones (ethephon, gibberellic acid, abscisic acid and salicylic acid). Relative fold change was calculated by considering 0 h (control sample) as a calibrator. In the graphs, the X-axis represents relative fold change and Y-axis represents time points of stress treatments. Chickpea GAPDH gene was used as the internal control

and *Xba*I and blotted onto nylon membrane. The blots were hybridized with the α-^{32}P-labelled full-length *CarERF116* cDNA as a probe. The restriction enzyme *Xba*I, having one site in the probe region, yielded two bands, whereas with *Eco*RI or *Hin*dIII having no sites, only a single band was obtained (Fig. 6a). This result strongly suggests that chickpea genome contains a single copy of *CarERF116* gene. The BLASTN search of the full-length *CarERF116* (the probe sequence) against chickpea whole genome sequences, also indicates the presence of a single copy of the *CarERF116* gene in the chickpea genome (Fig. 2).

The *CarERF116* functions as a potential transcriptional activator

Several classes of transcription activation domains have been identified in plants and yeast, but *in silico* analyses of

CarERF116 protein failed to detect a clear stretch of amino acids that can act as a transcriptional activator. Therefore, we used the yeast one hybrid system to evaluate the transcriptional activation ability of *CarERF116*. The full-length *CarERF116* and a series of deletion constructs of the *CarERF116* gene were fused to the GAL4 BD domain in pGBKT7 and introduced into the yeast Y2H Gold strain. The GAL4 DNA-binding domain alone (empty vector pGBKT7), used as a negative control, was also used to transform theY2HGold strain. The transformed yeast colonies were selected on SD/-Trp and SD/-Trp/X-α-Gal/AbA agar plates. Transformed Y2H gold strain containing a recombinant plasmid (effector plasmid) or empty pGBKT7 were able to grow on SD/-Trp medium. While, the transformed yeast cells containing empty vector pGBKT7, GAL4-A and Gal4-B plasmid were unable to grow on medium containing SD/-Trp/X-α-Gal/AbA the GAL-F (full-length *CarERF116*), GAL4-C
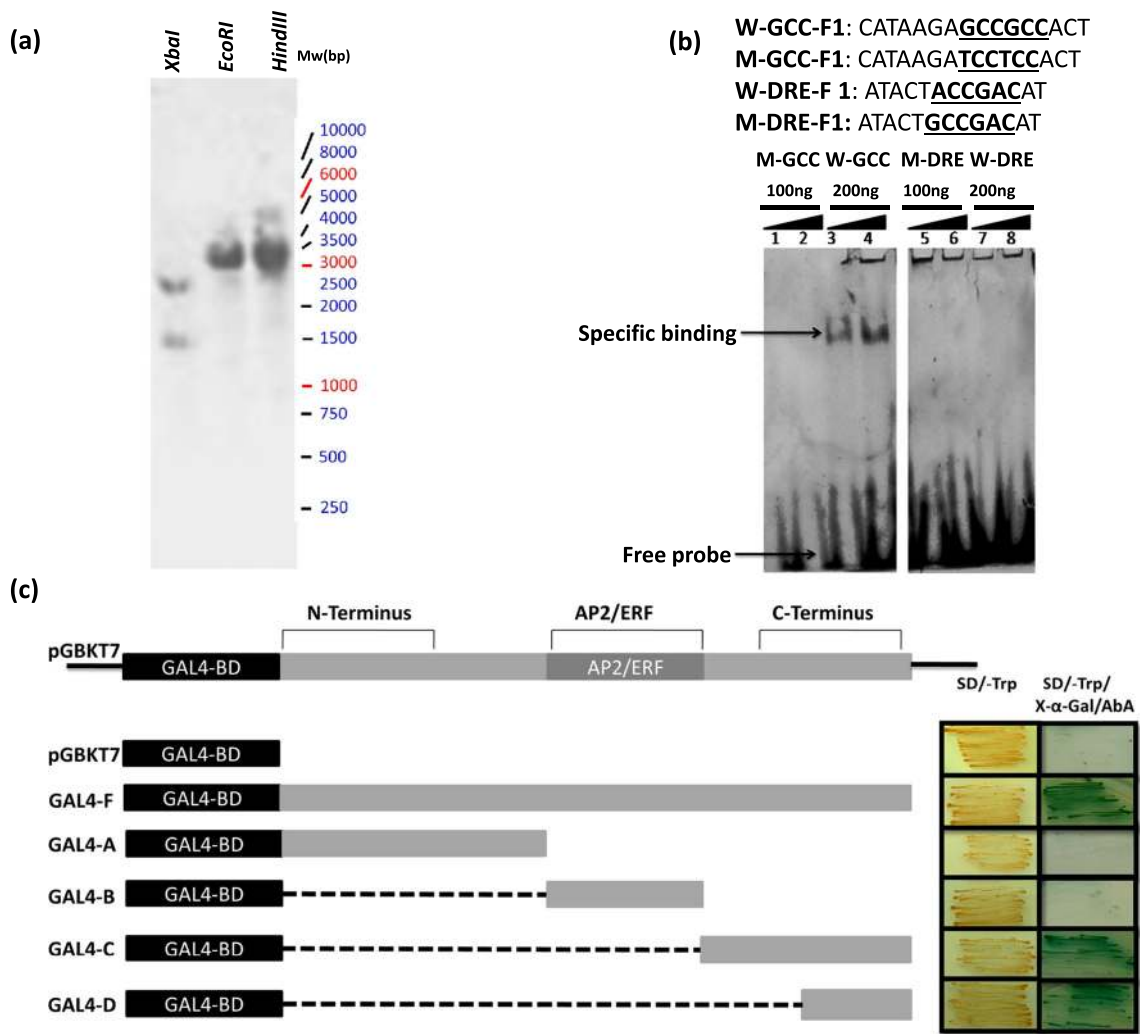
Fig. 6 Molecular characterization of CarERF116. (a) Determination of *CarERF116* copy number by Southern blot hybridization. Southern blot analysis of the *CarERF116* gene. 15 μg genomic DNA (cv. ICC 4958); digested with *Xba*I (lane a), *Eco*RI (lane b), *Hin*dIII (lane c); blotted onto a nylon membrane; and then hybridized with the α-^{32}P-labelled full length *CarERF116* cDNA as a probe. Only one band was obtained when DNA was digested by restriction enzymes that do not cut in the probe (*Eco*RI and *Hin*dIII) and two bands when it was digested by enzymes that cut inside the probe (*Xba*I). (b) EMSAs showing sequence-specific binding of the *CarERF116* fusion protein to the GCC box. Lane 1 and 2: mutated GCC oligonucleotide incubated with 100 and 200 ng of *CarERF116* protein, respectively. Lane 3 and 4: wild-type GCC oligonucleotide incubated with 100 and 200 ng of *CarERF116* protein

respectively. Lane 5 and 6: mutated DRE oligonucleotide incubated with 100 and 200 ng of *CarERF116* protein, respectively. Lane 7 and 8: wild type DRE oligonucleotide incubated with 100 and 200 ng of *CarERF116* protein, respectively. Two arrows indicate specific binding of wild type GCC-CarERF116 complex and the unused free probes respectively. (c) Transcription activation analysis of *CarERF116* protein using Yeast I hybrid system. Full length *CarERF116* and truncated cDNA fragments were cloned into the *Nde*I-*Pst*I sites of pGBKT7. Numbers at left, pGBKT7 represent empty pGBKT7 vector, GAL4-F represents full-length *CarERF116* protein and GAL4-A to D represents different truncated *CarERF116* fragments. The transformed yeast cells grew on the SD/-Trp and SD/-Trp/ α-X-Gal/AbA

(481–726) and GAL4-D (607–726) plasmid containing cells grew and turned blue in the presence of the chromogenic substrate X-α-Gal (Fig. 6c). These results indicate that the *CarERF116* possesses transactivation activity and the region close towards the C-terminus (GAL4-C and GAL4-D) is required for the transactivation activity of *CarERF116*. The region towards N-terminal GAL4-A (nucleotide 1–300) and GAL4-B (nucleotide 301–480) did not show any detectable transactivation property.

The transactivation property of CarERF161 protein clearly implicates this protein as a potential transcription factor.

CarERF116 protein binds to GCC box in vitro

To analyze the DNA binding activity of *CarERF116*, the recombinant 6X HIS tagged protein was expressed in *E. coli* BL21and purified for its ability to bind GCC and DRE

sequence in vitro. The electrophoretic mobility shift assay (EMSA) results reveal that the recombinant *CarERF116* fusion protein binds to a 16 bp oligonucleotide probe containing wild type GCC box sequence (GCCGCC) and gave rise to a single, discrete DNA-protein complex that migrated slowly than the free probe (Fig. 6b). The intensity of this shifted band increased upon addition of increasing amounts (100–200 ng) of recombinant *CarERF116* protein. By contrast, CarERF161 did not bind to the mutated oligonucleotide probe, in which G residues within GCC box were substituted by T residues (Fujimoto et al. 2000; Gu et al. 2002). The recombinant *CarERF116* fusion protein could not bind to wild type and mutated DRE box. The results clearly indicate that the chickpea ERF protein in vitro interacts only with the GCC box, but not with the DRE box. These results are similar to the interaction property of the wheat ERF gene, *TaPIEP1*, which interacts in vitro with GCC box element, but not with the DRE element (Dong et al. 2010). Earlier it has been also reported that the *CaERFLP1* gene from hot pepper interacts in vitro with both GCC and DRE box sequences, but with different binding affinities and regulates both biotic and abiotic responses (Lee et al. 2004). Similarly tomato *TERF1* and *JERF3* (Huang et al. 2004; Wang et al. 2004), wheat *TaERF1* (Xu et al. 2007) and barley *HvRAF* (Jung et al. 2007) have been shown to bind to both GCC and DRE elements. Tomato ERF gene *Pti4* was shown to bind the GCC box in vitro and to regulate the expression of several GCC box–containing genes in vivo (Gu et al. 2000). Our EMSA assay suggests that *CarERF116* transcription factor binds specifically to the GCC box *cis*-element and thus could activate the transcription of downstream genes following binding to the GCC box in the promoter.

CarERF116 promoter sequence contain several abiotic stress related elements

To gain a better insight into the signalling function upstream of the *CarERF116*, 5′-upstream sequence of *CarERF116* gene was isolated by genome walking strategy. Two successive genome walking PCR reactions eventually resulted in the identification of 910 bp putative *CarERF116* promoter region (Fig. 7). The transcription start site (TSS) was mapped using 5′ RACE and confirmed by sequencing of five randomly selected positive clones. Analysis of this amplified fragment revealed that the putative transcription start site is 94 bp upstream of the initiation codon (ATG) of the *CarERF116* gene. A putative TATA box (ATTATA) sequence at −44 to −39 (relative to the transcription start site) and a CAAT box (CAAT) at −97 to −94 were located in *CarERF116* promoter. The occurrence of these core promoter elements is consistent with the regular feature of most of the eukaryotic promoters (Zhu et al. 1995). Further analysis of *CarERF116* promoter sequence revealed presence of several conserved *cis*-acting

element related to biotic and biotic stress gene expression regulation such as MYCATRD22 (Abe et al. 2003; Busk and Pages 1998), MYCCONSENSUSAT (Agarwal et al. 2006), MYB1AT (Abe et al. 2003), AUXREPSIAA4 (Klinedinst et al. 2000), ATHB6COREAT (Himmelbach et al. 2002), ERELEE4 (Tapia et al. 2005) and RAV1AAT (Hwang et al. 2008). In addition, five *cis*-acting regulatory elements of *Arabidopsis* response regulatory DNA binding elements (ARR1) were also found in the promoter region of *CarERF116* gene. The ARR1 are responsible for early responses to cytokinins (Haberer and Kieber 2002). Cytokinin is a vital phytohormone controlling various events of plant growth and development such as cell division, seed germination, root elongation, leaf senescence, and the transition from vegetative growth to reproductive development. The *CarERF116* promoter also includes several copies of NODCON1GM, NODCON2GM (Stougaard et al. 1990), OSE1ROOTNODULE (Fehlberg et al. 2005) and ROOTMOTIFTAPOX1 (Elmayan and Tepfer 1995) elements associated with nodulin, AM symbiosis and root specific expression (Campos-Soriano et al. 2011).

Overexpression of *CarERF116* enhanced tolerance to osmotic stresses and freezing during seed germination

To investigate the functional role of *CarERF116*, we used transgenic overexpression approach in *Arabidopsis* Col-0 under the control of strong constitutive promoter CaMV 35S. No discernible phenotypic differences were observed between the 35S::CarERF116 overexpression line OE-1, OE-2 and OE-3 and the wild type *Arabidopsis* plants grown on MS agar plates and in the soil. The 35S::CarERF116 overexpression lines were evaluated with different concentration of mannitol included in the media to ascertain whether CarERF116 over expression affected seed germination. There was no difference in seed germination between the wild type and transgenic plants under normal conditions (Fig. 8a). At 50, 100 and 200 mM concentration of mannitol, no significant difference was observed in seed germination between the wild type and the transgenic 35S::CarERF116 plants (data not shown). However, at 400 mM mannitol concentration, more than 75 % seeds of the 35S::CarERF116 lines germinated and only 10 % of the wild type *Arabidopsis* seeds germinated on the 5th day. On the 6th day, the majority of (more than 92 %) 35S::CarERF116 seeds germinated compared with only 22 % of wild type (Fig. 8b). Thus, the overexpression of CarERF116 imparted tolerance to *Arabidopsis* against osmotic stress exerted by mannitol.

We also evaluated the response of CarERF116 overexpressing lines to freezing tolerance. The 35S::CarERF116 and the wild type seeds were subjected to −30 °C for 24 h and later allowed to germinate at 22 °C. We found that freezing treatment delayed germination in both 35S::CarERF116 lines as well as wild type seeds (about
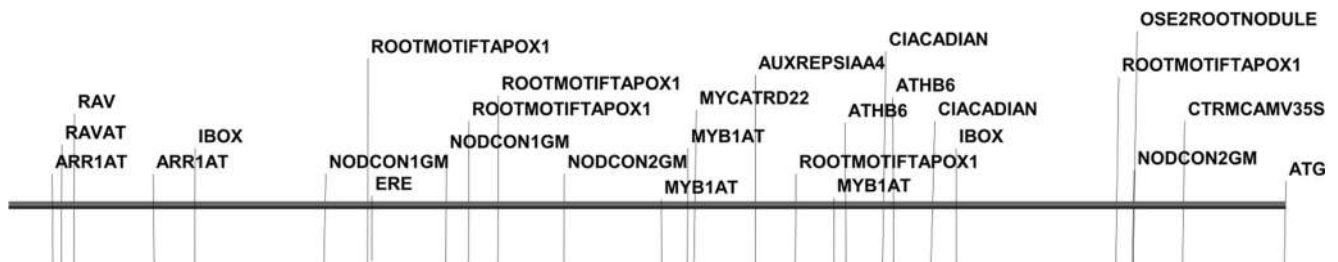
Fig. 7 Putative cis-regulatory elements identified in *CarERF116* promoter sequence. The 5′-upstream sequence of CarERF116 (911 bp) depicting various putative cis-acting element. The possible cis-elements were predicted by scanning *CarERF116* promoter sequence using PlantCARE and PLACE plant promoter analysis tools

5–6 days as compared with control seed germination). However, more than 39 % of 35S::CarERF116 seed germinated as compared to only 4 % seeds of the wild type at 21 days after the freezing treatment (Fig. 8c). These results suggest that CarERF116 makes the *Arabidopsis* plant more tolerant towards osmotic as well as freezing stress at the seed germination stage. Similar observations of improved freezing and osmotic stress tolerance during seed germination have been recorded for transgenic plants overexpressing members of the ERF gene family such as tomato *JERF3* (Wang et al. 2004), tobacco *Tsi1* (Park et al. 2001), hot pepper *CaPF1* (Yi et al. 2004) and tomato *TERF2* (Tian et al. 2011).

Overexpression of *CarERF116* in *Arabidopsis* alters ABA sensitivity

Plant responses to environmental stresses are controlled by ABA-dependent and independent signaling pathways. In order to determine the role of *CarERF116* in stress signaling, we evaluated the effect of exogenous application of ABA on germination of 35S::CarERF116 seeds. Seeds of wild type and progenies of 35S::CarERF116 were germinated at different concentrations of ABA (0.1, 0.3, 1.0, 3.0 and 10 μM ABA). The exogenous ABA (0.3-3.0 μM) affected the germination of seeds in both wild type and 35S::CarERF116 overexpressed plants (Fig. 8d). The 35S:CarERF116 transgenic *Arabidopsis* plants showed enhanced insensitivity to ABA. For example, on the 6th day, more than 95 % seed of 35S::CarERF116 seeds germinated, whereas only 58 % of wild type seeds germinated in the presence of 0.3 μM ABA. At 1 μM of ABA, more than 74 % of 35S::CarERF116 seeds germinated, whereas most of wild type seeds (less than 94 %) did not germinate. At higher levels of ABA (more than 3.0 μM), none of the seeds of wild type and 35S::CarERF116 transgenic could germinate (Fig. 8e). The reduced ABA sensitivity of CarERF116 overexpressing lines indicate that overexpression of CarERF116 has modified the ABA responsiveness in transgenic *Arabidopsis* lines.

CarERF116 activates the expression of stress responsive genes

In order to identify downstream target genes of *CarERF116*, microarray analysis of *Arabidopsis* 35S::CarERF116 and wild type (WT) plants were carried out using Affymetrix microarray GenChip (Affymetrix). As shown in Additional file 1, 167 genes in 35S::CarERF116 plants were up-regulated and 137 genes showed down-regulation at least two fold higher relative to the wild type plant. These genes were categorized according to their functional category and gene ontology (GO) annotation (according to TAIR database: http://Arabidopsis.org/tools/bulk/go/index.jsp) (Table S3 and Fig. S5). Differentially expressed with GO terms related to biotic and abiotic stresses genes were identified. Genes involved in response to biotic and abiotic stresses categories are summarised in Table 1. Several known biotic and abiotic stress responsive genes were found significantly up-regulated. Eight genes encoding late embryogenesis abundant protein (LEA genes) were found to be highly up-regulated in the *CarERF116* overexpressing lines. Among these up-regulated LEA genes, four genes encoding *dehydrin* (AT2G21490), *ATHVA22B* (AT5G62490), *LEA3* (AT1G02820) and *LEA4.1* (AT1G32560) have been shown to be involved in abiotic stress tolerance. The involvement of LEA genes in abiotic stress tolerance particularly group 1, 2 3, and 4 of the nine different LEA groups has been well documented in *Arabidopsis* (Puhakainen et al. 2004) and Brassica (Dalal et al. 2009).

Similarly, two genes encoding peroxidase 64 (AT5G42180) and peroxidase putative (AT1G44970) were also up-regulated in transgenic 35S::CarERF116 *Arabidopsis* plants. The plant produces ROS by activating peroxidise, which in turn catalyzes the oxido-reduction of various substrates using H_2O_2 (Wasilewska et al. 2008). Under abiotic stress condition, ROS has a dual effect based on cellular amounts. At low levels, ROS likely to function as components of a stress-signaling pathway, whereas at higher levels, ROS become extremely deleterious, initiating uncontrolled oxidative cascades that damage cellular membranes and other cellular components
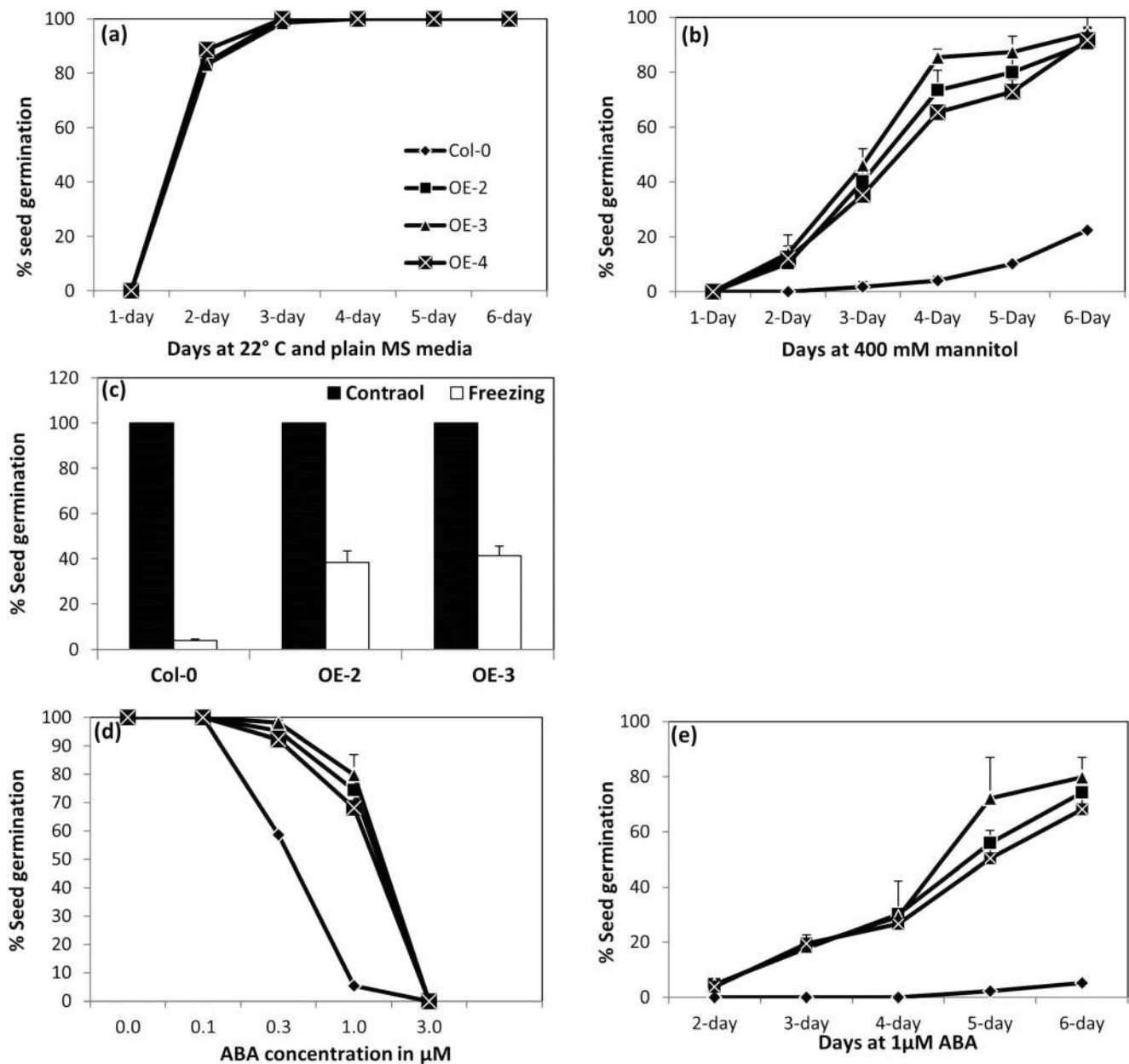
Fig. 8 Effect of mannitol, freezing and ABA on the germination of *Arabidopsis* seeds over-expressing *CarERF116*. Percent seed germination of wild type (*WT*) and 35S::CarERF116 *Arabidopsis* seeds on MS medium and MS media with different concentrations of ABA or mannitol. OE-1, OE-2 and OE-3 represent three independent T3 lines. Percent seed germination of wild type (*WT*) and transgenic plants (OE-1, OE-2 and OE-3) on (**a**) MS agar medium (**b**) MS agar medium containing 400 mM

mannitol (**c**) 0.1–3.0 μM ABA and (**d**) 1 μM ABA. (**e**) In order to check the response of freezing stress on germination of *Arabidopsis* seeds over-expressing CarERF116, Seeds of wild type (*WT*), OE1 and OE2 were stored at −30 °C for 24 h (for freezing stress treatment) and were then allowed to germinate at room temperature. The data on seed germination were recorded at 21 days after plating

resulting in oxidative stress and eventually cell death (Carvalho et al. 2008).

Gene encoding seed specific proteins such as *oleosin* (AT4G25140, AT5G40420, AT3G27660 and AT3G01570) were also up-regulated. GO and functional categorization indicate involvement of these genes in lipid storage, seed germination and response to freezing. Oleosin deficient

mutants showed reduced seed germination when subjected to freezing indicates the role of oleosins in protecting seeds against freeze/thaw-induced damage of their cells (Shimada et al. 2008). Similarly, two low temperature and salt respon-sive genes *RCI2B* (AT3G05890) and *RCI2* (AT1G05260) were also up-regulated in 35S::CarERF116 lines. These genes are not only induced by low temperature, but also by ABA,

Table 1 Stress and defense-related genes up-regulated by CarERF116. Genes up-regulated in CarERF116 transgenic *Arabidopsis* plants (35S::CarERF116) compared with wild-type *Arabidopsis* plants. Genes involved in response to biotic and abiotic stress categories are listed with the fold induction in transgenic 35S::CarERF116 line in comparison to that in the wild-type plants. Complete list of differentially expressed genes is available in Table S3

AGI code	Description (TAIR annotation)	Fold change
LEA		
AT3G15670	LEA protein	44.4
AT1G52690	LEA protein, putative	10.8
AT3G17520	LEA domain-containing protein	10.7
AT2G21490	LEA (DEHYDRIN LEA)	8.8
AT5G62490	ATHVA22B (Arabidopsis thaliana HVA22 homologue B)	7.9
AT1G32560	Late embryogenesis abundant group 4.1	7.7
AT4G21020	LEA domain-containing protein	6.2
AT1G02820	Late embryogenesis abundant 3 family protein	3.7
Seed protein		
AT4G25140	OLEO1 (OLEOSIN1)	275.8
AT5G40420	OLEO2 (OLEOSIN 2)	175.8
AT3G27660	OLEO4 (OLEOSIN4)	31.9
AT3G01570	Glycine-rich protein / oleosin	48.9
AT4G26740	ATS1 (Arabidopsis thaliana Seed Gene 1)	43.6
AT5G55240	Caleosin-related family protein / embryo-specific protein	5.9
Cold		
AT3G05890	RCI2B (RARE-COLD-INDUCIBLE 2B)	4.3
AT1G05260	RCI3 (RARE COLD INDUCIBLE GENE 3)	2.4
Pathogenesis related protein		
AT2G15010	Pathogenesis-related protein (PR-13)	56.3
AT2G02120	LCR70/PDF2.1 (Low-molecular-weight cysteine-rich 70)	23.8
AT1G47540	Trypsin inhibitor, putative	23.6
AT1G75830	LCR67/PDF1.1 (Low-molecular-weight cysteine-rich 67)	6.1
AT4G23690	Disease resistance-responsive family protein	2.8
AT1G72260	THI2.1 (THIONIN 2.1)	2.4
Enzymes		
AT1G14950	Polyketidecyclase	27.9
AT1G54870	Oxidoreductase	23.5
AT1G14940	Polyketidecyclase	7.0
AT2G29130	LAC2 (laccase 2)	4.8
AT5G49190	SUS2 (SUCROSE SYNTHASE 2)	4.6
AT3G60140	DIN2 (DARK INDUCIBLE 2)	4.4
AT3G59845	NADP-dependent oxidoreductase	3.3
AT2G29090	CYP707A2. ABA 8′-hydroxylase activity	2.9
AT3G19450	CAD4 (CINNAMYL ALCOHOL DEHYDROGENASE 4)	2.8
AT3G12710	Methyladenineglycosylase family protein	2.1
AT2G37040	PAL1 (PHE AMMONIA LYASE 1)	2.1
AT4G18780	CESA8 (CELLULOSE SYNTHASE 8)	2.0
Detoxification		
AT1G48130	ATPER1 (1-cysteine peroxiredoxin 1)	54.7
AT3G56350	Superoxide dismutase (Mn)	9.7
AT5G42180	Peroxidase 64 (PER64) (P64) (PRXR4)	2.9
AT1G44970	Peroxidase, putative	2.2
Others		
AT1G64780	ATAMT1;2 (AMMONIUM TRANSPORTER 1;2)	3.1
AT1G69530	ATEXPA1 (ARABIDOPSIS EXPANSIN A1)	2.9
AT3G12610	DRT100 (DNA-DAMAGE REPAIR/TOLERATION 100)	2.2
AT5G25830	Zinc finger (GATA type) family protein	2.1

Table 1 (continued)

AGI code	Description (TAIR annotation)	Fold change
AT5G44380	FAD-binding domain-containing protein	2.1
AT1G74660	MIF1 (MINI ZINC FINGER 1); DNA binding	2.1
AT3G22840	ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN)	2.0

dehydration, and high salt concentration (Medina et al. 2007). Expression of several known disease resistance genes such as *PDF1.1* (AT1G75830) *PDF 1.2* (AT2G02120), *PR-13* (AT2G15010), *THI2.1* (AT1G72260) and trypsin inhibitor (AT1G47540) showed up-regulation. Gene members of the PDF, PR and THI (thionin) were found to be regulated by ERF genes (Zarei et al. 2011). The expression analysis CarERF116 under different stress and expression of both freezing and pathogenesis related genes indicates that the *CarERF116* may be playing crucial role in the crosstalk between cold tolerance and pathogen resistance in chickpea. Recently, several other studies also indicated that the freezing signals are closely linked to pathogen resistance in plants (Zhu et al. 2014).

Discussion

Chickpea (*Cicer arietinum* L.) is the Asia's first and world's second-largest cultivated food legume (FAOSTAT 2012). Chickpea is known for its better drought tolerance than most other cool-season food legumes, but the basis of its tolerance is still unknown. Several efforts have been made towards understanding the drought tolerance mechanism in chickpea using a variety of genomics, transcriptomics and Bioinformatics tools (Deokar et al. 2011; Varshney et al. 2009; Varshney et al. 2014). Additionally, using comparative genomics and available EST resources, chickpea homologs for previously characterized dehydration induced genes has been identified and tested for their performance under different stress conditions in the heterologous transgenic plants (Kaur et al. 2013; Shukla et al. 2006).

Transcription factors are master regulators of gene expression and several of them have been reported to involve in stress response and plant development. The homologs of AP2/ERF domain transcription factors are vital candidate for biotic and abiotic stress improvements in plants. Several homologs of AP2/ERF domain genes such as *TaERF* from wheat (Xu et al. 2007), *JERF1* and *JERF3* from tomato (Zhang et al. 2010) and *AP37*, and *AP59* from rice (Oh et al. 2009) impart enhanced tolerance for several abiotic stresses. Similarly, some of the ERF genes (e.g., *GmERF3*) induced by both biotic and abiotic stresses and conferred enhanced resistance

against pathogens and tolerance to high salinity and dehydration stresses (Zhang et al. 2009). The above observation indicate that ERF gens involved in both abiotic and biotic stress responses and also enhances multiple stress tolerance. The *CAP2*, an ortholog of DREB2 gene from chickpea has showed abiotic stress specific expression response and improved drought tolerance in transgenic tobacco plants (Shukla et al. 2006), apart for *CAP2* gene, none of the homologous AP2/ERF genes have been identified and characterized in chickpea.

We identified and performed genome-wide analyses of 120 chickpea ERF transcription factors. This analysis is the first comprehensive study of the ERF gene family in chickpea and provides a valuable resource for further study into the functional analysis of these genes in chickpea and also comparative genomics studies of ERF genes in other grain legumes. The number of ERF genes in chickpea (120) is in close agreement to the *Arabidopsis* (122), but is slightly higher than in *Medicago truncatula* (106). The gene family size variation is an important indication of the involvement of the various gene families in the evolution of complex traits, diversification, and adaptation (Jacquemin et al. 2014). Gene families continually undergo expansion (via gene duplication) and contraction (via gene deletions) which affects the gene family size. However, the process of expansion and contraction among different lineage are different (Guo 2013) and also the variable selective pressure on gene family members may also contribute to the diverse gene family size. Based on the above observations, the larger ERF subfamily size over the DREB subfamily in chickpea might suggest a predominant role of ERF members in the better adaptation of chickpea to adverse environmental conditions.

We found several conserved motifs outside of the AP2/ERF domain which are a common feature with several other members of the ERF gene family of *Arabidopsis*, soybean and rice. These motifs with unknown function can be potentially characterised by associating them with functionally known motifs from other plant species. The identification of conserved and functionally relevant motif outside the AP2/ERF domain of chickpea ERF genes helps to classify them into groups, as the functionally important regions in proteins (domains and motifs) tend to be more conserved among the related proteins (Horan et al. 2010). The conserved motif sequences between the proteins may specify the functional

equivalence, and thus can be reliably used to predict functional feature of uncharacterised proteins from the newly sequenced chickpea genome.

The gene duplication analysis within the CarERF gene family indicates that, the chickpea ERF gene family content mainly emerged from segmental duplication. Similarly, the predominant contribution of segmental duplication in expansion of transcription factor families in *Arabidopsis* (Liu et al. 2013), maize (Wei et al. 2012), rice (Sharoni et al. 2011), and soybean (Yin et al. 2013) has been also reported earlier.

Quantitative PCR demonstrated that CarERF116 was induced by multiple stresses, including dehydration, salinity, cold stress, ABA, GA3, SA and ethephon. The response of *CarERF116* to several abiotic stresses implied that *CarERF116* might be involved in different stress signaling pathways as a connection point. In addition, the CarERF116 promoter sequence analysis identified the presence of several biotic and abiotic stress responsive *cis*-elements which further correlate with the pattern of *CarERF116* expression under various biotic and abiotic stresses.

The CarERF116-overexpressing *Arabidopsis* plants show osmotic stress and freezing tolerance during seed germination. *CarERF116* overexpression displayed up-regulation of antioxidants genes which play major roles in preventing oxidative damage under stress. Emerging evidence also suggests that the oxidative stress dependent signaling pathway plays key roles in the crosstalk between different biotic and abiotic stress signaling (Schmidt et al. 2013). Based on our data, we propose a model to explain the potential role of CarERF116 in response to multiple stresses in chickpea (Fig. S6). Our results provide a view that the *CarERF116* might regulate the expression of genes involved in ROS-scavenging and ROS accumulation and in turn contribute towards improved tolerance against osmotic stress. Further, large numbers of genes for the synthesis of the osmo-protectant such as LEA genes were highly expressed in *CarERF116*-overexpressing *Arabidopsis*. Accumulation of LEA protein in CarERF116-overexpressing *Arabidopsis* most likely provides osmotic stress tolerance during seed germination. Plants adapted in temperate and cool temperate areas, with acquired freezing tolerance have been shown to withstand multiple stresses such as mechanical stress, low-temperature stress and drought stress (Shimada et al. 2008). At the molecular level, plants subjected to low temperature and drought stress exhibit very similar responses and several genes induced by drought and low temperature stress are common (Shinozaki and Yamaguchi-Shinozaki 2000). Our expression analysis also shows that *CarERF116* is highly induced in response to both dehydration and cold stress. In the transgenic *Arabidopsis* plant, *CarERF116* might be regulating genes involved in both dehydration as well as cold stress.

The phytohormone abscisic acid (ABA) plays an important role in seed dormancy, embryo development and adaptation to various environmental stresses, including drought, salt, cold, and other abiotic stresses (Xiang et al. 2008). A number of studies have shown that enhanced drought tolerance is associated with hypersensitivity to ABA treatments during seed germination and early seedling development (Ko et al. 2006). Interestingly, in the *Arabidopsis CarERF116* overexpressing lines the osmotic stress tolerance is not associated with ABA sensitivity, rather the overexpression lines showed more insensitivity for the ABA during seed germination. These results provide new insight into the ERF transcription factors as involved in abiotic stress response in chickpea. The precise mechanisms behind increased osmotic tolerance associated with decreased sensitivity towards ABA in the *CarERF116* overexpressing *Arabidopsis* lines is still unknown, but similar phenotype of reduced ABA sensitivity has been reported for the overexpression of *AtTPS1* (Avonce et al. 2004), *CaXTH3* (Cho et al. 2006), *OsMYB3R-2* (Dai et al. 2007), *OsWRKY45* (Qiu and Yu 2009) and *ABO3* (Ren et al. 2010). It may be possible that there are several pathways separately involved in ABA response and stress tolerance in *Arabidopsis*, and the overexpressed *CarERF116* under control of the constitutive CaMV 35S promoter in *Arabidopsis* plants may be regulating the genes involved in both the pathways simultaneously. However, our understanding of the exact role and *CarERF116* in regulating ABA insensitivity and stress tolerance is not yet complete and only further experimentation will be able to clarify the picture. The present study provides new insight into the ERF transcription factor family and CarERF116 as involved in abiotic stress response in chickpea.

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