Genome wide analysis of 14-3-3 proteins in *Cicer arietinum* L. and identification of isoforms responsive to *Fusarium oxysporum*

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In the present study, we have identified and characterized two 14-3-3 isoforms, namely isoform A and C from chickpea (*Cicer arietinum* L.), that might play a crucial role during disease resistance. Further, *in silico* analysis of these 14-3-3 proteins was accomplished, including motif identification and structure prediction from deduced amino acid sequences. Expression profiling of the two representative 14-3-3 isoforms in the roots of wilt resistant and susceptible chickpea varieties upon *Fusarium oxysporum* f. sp *ciceri* race 1 (FOC1) challenge, revealed time dependent isoform specific differential expression in induced chickpea roots upon FOC1 colonization.

Keywords: Bioinformatics, chickpea 14-3-3s, pathogen responsive, transcriptional analysis.

ONE of the notable components of the plant defense network is the 14-3-3 proteins, which have been shown to be upregulated during pathogen attack[1]. The 14-3-3 protein family is widespread across several organisms and comprises multiple genes and protein isoforms. These 14-3-3 proteins are involved in many critical physiological pathways and play key functional roles in the signal transduction mechanism by binding to their phosphorylated targets[2]. This functional aspect is ingrained in the conserved structural core of the 14-3-3 dimer, that depicts grooves for its attachment with two phosphorylated peptides. The primary diversity among 14-3-3 isoforms lies in the N and C termini, with the C-terminal region forming a flexible hinge guarding access to the central core region[2].

Chickpea (*Cicer arietinum* L.), a globally important food legume crop, suffers immense damages due to wilt disease, caused by the soil-borne fungus *Fusarium oxysporum* f. sp. *ciceri* (FOC). Present study was aimed to understand the 14-3-3 expression during chickpea – *Fusarium* interactions. *In silico* analysis of 14-3-3 proteins in chickpea including their motif identification and phylogenetic analysis were performed, which recognized the conserved 14-3-3 family specific and isoform specific motifs. We isolated and cloned two full-length 14-3-3 isoforms (A and C) and studied their expression pattern in roots of chickpea plants when challenged with FOC race 1 (FOC1). Expression kinetics of these two 14-3-3 genes was compared between two chickpea varieties established for their susceptibility (JG-62) or resistance (Vijay) to FOC1 at 1, 2, 4 and 8 days after inoculation (DAI).

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Materials and methods

Plant material, growth conditions, stress treatment and cDNA preparation

Chickpea plants (50 each) of wilt-susceptible (JG-62) and resistant (Vijay) cultivars were grown hydroponically under sterile conditions and inoculated with the pathogen, FOC1, after 7 days of growth of the seedlings as described[3]. Five seeds of JG-62 were sown in each tray as an indicator of successful infection and wilting. Seedlings grown in similar trays with no pathogen inoculation served as control plants. At harvest, the seedlings were removed and the infected roots were briefly rinsed with sterile diethyl pyrocarbonate (DEPC) treated water, to free off the adhering fungal mycelia and quickly frozen in liquid nitrogen. Samples were collected in duplicates for Vijay and JG-62 after 1, 2, 4 and 8 DAI. Total RNA was extracted from each sample using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA was treated with RNase free DNaseI (0.1 U per µg RNA) at 37°C for 1 h in the presence of RNasin (0.4U), and the reaction was terminated by heating at 65°C for 15 min. cDNA synthesis was performed using Powerscript RT III (Clontech, USA).

Cloning and sequencing of 14-3-3 isoforms from chickpea roots

Our earlier studies identified 273 differentially expressed genes among the 2000 transcript-derived fragments (TDFs) in chickpea during root infection by FOC1, using

cDNA-AFLP approach. One of these TDFs was homologous to transcription factor 14-3-3 (ref. 3). This 262 bp long 14-3-3-like TDF (Genbank accession DR749492) was used to design 14-3-3 specific primers for amplification in chickpea using Fast PCR software[4] based on homology with other legume sequences in the NCBI database (Supplementary Table 1). Polymerase chain reaction (PCR) amplifications in triplicate were performed in PTC-200 (MJ Research Inc., USA) from susceptible control (SC), susceptible infected (SI), resistant control (RC) and resistant infected (RI) chickpea cultivar's root cDNAs. The program conditions were: 94°C for 1 min followed by 35 cycles each comprising 30 sec at 94°C, 1 min at 55°C and 1.30 min at 72°C with a final extension of 5 min at 72°C. The amplified fragments were ligated into pGEMT-Easy vector (Promega, USA) and transformed into competent *E. coli* DH5α cells. The insert in each case was sequenced bi-directionally from four representative clones in two independent replicates in an automated fluorescent sequence analyzer using DYEnamic terminator chemistry (Amersham Biosciences, USA). These four sequences from JG-62 and Vijay varieties were deposited to the NCBI database (GenBank accessions EF565383, EF585384, EF643372 and EF643373).

In silico analysis of chickpea 14-3-3 proteins

Till date 19 protein sequences including, four of our entries from JG-62 and Vijay (GenBank accessions ABQ95991.1 to ABQ95994.1) belonging to chickpea 14-3-3s have been deposited in NCBI database. To determine the theoretical isoelectric point (pI) and molecular weight (Mw) of these 19 chickpea 14-3-3 proteins, Compute pI/Mw tool was used (https://web.expasy.org/compute_pi/)[5]. Using Pepstats (http://emboss.bioinformatics.nl/cgibin/emboss/pepstats) the amino acid composition of all these 14-3-3 proteins was determined. Further, these sequences were subjected to motif recognition using MEME tool[6]. The parameters were defined such that the minimum motif width was 10 and maximum 50, with a limit of 25 motifs per sequence; wherein any number of motif repetitions in an individual sequence were allowed. Sequences were aligned using CLustalW option of MEGA V 5.0 software; phylogenetic analysis was performed through neighbor-joining using 1000 bootstrap and phylogenetic tree was build using MEGA software[7].

Semi-quantitative reverse transcription-polymerase chain reaction (sq-RT-PCR)

Sq-RT-PCR of SC, SI, RC and RI chickpea root cDNAs was performed using 14-3-3 primer sets at 1, 2, 4 and 8 DAI. The PCR consisted of an initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 30 sec (for 14-3-3), 50°C (for 18S rRNA), extension at 72°C for 1 min; and final extension at 72°C for 5 min. PCRs were carried out in triplicates and 18S rRNA was used as a control for integrity and normalization of RNA quantity. The amplicons were visualized on gel documentation system (Syngene, USA) and identities of the amplified PCR products were confirmed by sequencing all the samples.

Structural predictions of 14-3-3 isoforms of chickpea

Amino acid sequences of 14-3-3 isoforms A and C identified in this gene expression study of chickpea, were used for structure prediction using i-Tasser server[8]. 3D models were built based on multiple-threading alignments by LOMETS and iterative template fragment assembly simulations and function insights were derived by matching the 3D models with BioLiP protein function database (http://zhanglab.ccmb.med.umich.edu/BioLiP/). Quality score for individual input structure was scored using ProSA[9]. The protein stereology of the model produced was assessed by Ramachandran plot analysis using RAMPAGE[10] by determining the amino acid residues located in ≥ favoured, allowed and outlier Φ and Ψ regions.

Motif recognition and phylogenetic analysis of 14-3-3 isoforms A and C across legume family

Motif recognition using MEME was individually performed for representative 14-3-3 isoforms A and C of chickpea with reported 14-3-3 sequences belonging to family Fabaceae showing percent identities of ≥ 70% with respect to corresponding 14-3-3 isoform. The parameters were defined such that the minimum motif width was 10 and maximum 50, with a limit of 25 motifs per sequence; wherein any number of motif repetitions in an individual sequence was allowed. Similarly, phylogenetic analysis was performed for both the 14-3-3 isoforms of chickpea with all the Fabaceae 14-3-3 sequences as described earlier[7].

Results

Isolated two 14-3-3 isoforms in chickpea belong to A and C type

Earlier transcript analysis of chickpea roots upon FOC1 infection[3] prompted us to isolate and characterize the full-length 14-3-3 genes. Based on the 14-3-3 gene sequence information on legumes, we designed primers (Supplementary Table 1) to clone candidate transcripts from chickpea. The amplicon Ca1433-1 from Vijay and JG-62 encoded a 780 bp open reading frame (ORF) for a putative polypeptide of 259 amino acids (Genbank accessions EF565383 and EF643372 from Vijay and JG-62, respectively); while the amplicon Ca1433-2 representing a 786 bp

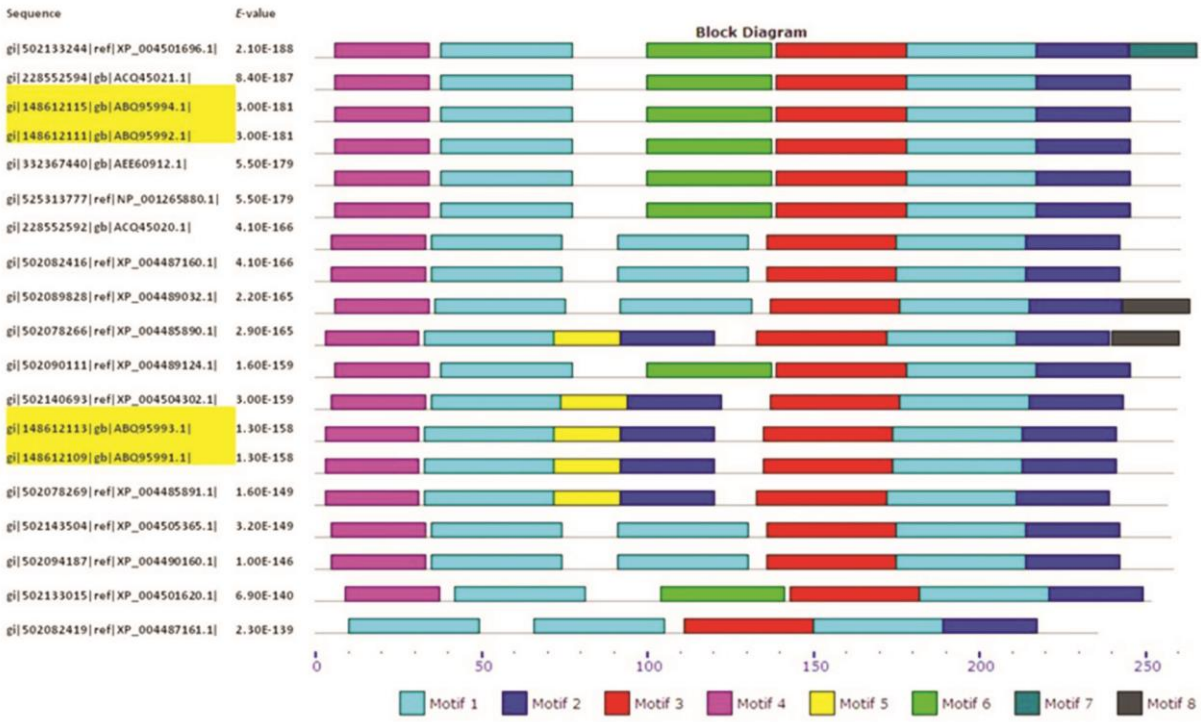

Figure 1. MEME analysis: conserved motifs in chickpea 14-3-3 amino acid sequences**.** Highlighted in yellow are 14-3-3 accessions from present study of chickpea submitted to NCBI.

ORF coded for a putative polypeptide of 261 amino acids (Genbank accessions EF643373 and EF565384 from Vijay and JG-62 respectively). The homology search with reported chickpea 14-3-3 sequences in the NCBI database indicated Ca1433-1 as 14-3-3 C type while Ca1433-2 as 14-3-3 A type respectively.

Genome-wide Architectural features of chickpea 14-3-3 proteins

Mw and pI of all 14-3-3s belonging to genus *Cicer* were determined along with their amino acid compositions. Chickpea 14-3-3 isoforms A and C had Mw of 29.33 and 29.26 kDa respectively, with pI of 4.7 for both. Amongst the other 14-3-3 proteins from *C. arietinum*, XP_004489124.1 had pI of 4.6, while isoform XP_004501696.1 had pI of 5.1. The isoform XP_004487161.1 had Mw of 26.5, while the isoforms XP_004489032.1 and XP_004501696.1 had Mw of 30.01 kDa. Supplementary Figure 1 shows the pI, Mw and amino acid composition of all the chickpea 14-3-3 proteins represented as bar graphs.

Further, motif recognition using MEME software[6] revealed a total of 8 motifs out of which the longest motif was of 39 residues and the smallest was 20 residues long among all the chickpea 14-3-3 proteins (Supplementary Table 2). Motifs 1 to 3 showed the most frequent occurrence, being present in all the 19 sequences followed by motif 4 (in 18 out of 19 sequences), while motifs 7 and 8 occurred the least number of times (only in 1 or 2 sequences out of 19 sequences). Thus, 1 to 4 were the most conserved, whereas 7 and 8 were the least conserved motifs in chickpea 14-3-3s (Figure 1). MEME analysis further depicted the most conserved region, motif 1, across all chickpea 14-3-3 isoforms located at two positions, i.e. 35 to 80 and 150 to 220 amino acids respectively (Figure 1).

Chickpea 14-3-3s which harboured motifs 1 (occurring at two locations), 2, 3, 4 and 6 had pI values ranging from 4.65 to 4.81. Isoform XP_004501696.1 with similar motif organization depicting an exceptional presence of motif 7 had the highest pI value (5.1). On the other hand isoform XP_004485890.1 had pI of 4.91, similar to that of other chickpea 14-3-3 isoforms (which housed motifs 1, 2, 3, 4 and 5) in spite of the presence of an additional motif 8. The third type of pattern included presence of the motifs 1, 2, 3 and 4; and their pI values ranged from 4.75 to 4.88. The 14-3-3 accession XP_004487161.1 which depicted an exception in this pattern had only motifs 1, 2 and 3 and the lowest Mw of 26 kDa.

Phylogenetic analysis carried out for the chickpea 14-3-3 proteins segregated them into two main groups wherein 14-3-3A-like proteins formed an exclusive group (group I) except accession XP_004501620.1; while 14-3-3C-like proteins shared the second group (group II) with 14-3-3B and D-like isoforms (Figure 2). All the chickpea 14-3-3 protein sequences, which had motif 1 to 5 grouped together with 14-3-3C isoform into group II. On the other hand 14-3-3 protein sequences containing motifs 1 to 6

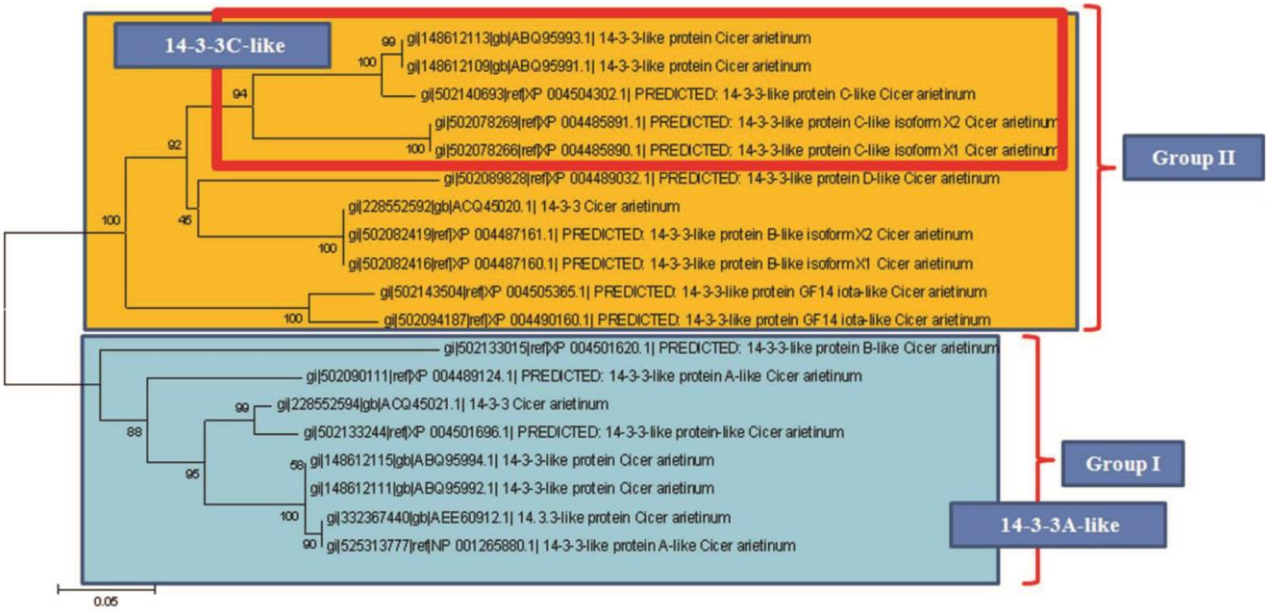

Figure 2. Phylogenetic analysis of chickpea 14-3-3 proteins. Sequences were aligned using ClustalX software of MEGA5.2 package and neighbor-joining tree with 1000 bootstrapping was generated. Phylogenetic group I and group II include 14-3-3A and C like sequences from chickpea.

(excluding motif 5) clubbed together with 14-3-3A isoform into group I.

Differential expression of 14-3-3 genes in chickpea genotypes upon FOC1 attack suggest their potential role in plant defense

The expression of 14-3-3 A and C genes was analysed in Vijay and JG-62 genotypes, with and without FOC1 inoculation by sq-RT-PCR. A representative expression profile has been depicted in Figure 3 *a* and *b*. The constitutive levels of both 14-3-3 A and C were comparable in the roots of the control (uninoculated) roots of both the cultivars. There was upregulation of both the transcripts at 2 DAI which attenuated further at 4 and 8 DAI in the control plants of both the varieties. However, upon induction by FOC1, 14-3-3C transcripts were significantly upregulated in the roots of the susceptible variety at 2 and 4 DAI as compared to that in the resistant variety roots. This response diminished further at 8 DAI (Figure 3 *a*). On the other hand, 14-3-3A transcripts showed higher gene expression in control resistant variety as compared to the roots of susceptible variety upon FOC1 challenge at all the four stages with the highest expression at 2 and 4 DAI which reduced at 8 DAI. However, it also showed significantly higher level of transcription up to 4 DAI and moderately higher at 8 DAI in the resistant variety as compared to the susceptible variety upon FOC1 challenge (Figure 3 *b*). Thus, an overall gene expression analysis indicated that 14-3-3C (i.e. Ca14-3-3-1) recorded an upregulated expression in the susceptible variety at an early stage upon FOC1 infection. While, 14-3-3A (i.e. Ca14-3-3-2) depicted an upregulated expression pattern in both the susceptible and resistant varieties upon FOC1, as compared to their respective controls; but with much higher expression in the resistant variety as compared to that in the susceptible one at all the stages.

Varied motif architectures of chickpea 14-3-3s with similar structures and functions

The 3D structures of 14-3-3 isoforms A and C identified in this study were predicted using i-Tasser server (Figure 4). Ramchandran plots of isoforms A and C showed 98% of the residues to be located in favoured and 2% in allowed Φ and Ψ regions. Only 1 to 3 residues were located as outliers. ProSA analysis yielded a Z-score of –7.17 and –7.25 for isoform A and C respectively, and negative energy values for all the residues in this study (Supplementary Figure 2). The evaluation indicated a good structure prediction of 14-3-3 isoforms A and C in chickpea.

The 14-3-3 isoform A comprised 9 perfect α helices running in anti-parallel fashion, with 3 disordered regions and 2 strands. It also had 5-protein binding regions located at positions 1 to 5, 20, 40, 41, 45 and 78 as determined using PredictProtein tool[11] (https://www.predictprotein.org/). On the other hand, 14-3-3 isoform C comprised 9 perfect α helices running in anti-parallel fashion, with 2 major disordered regions and 1 strand. It had 5-protein binding regions located at residues 1, 2, 17, 40, 82 and 167. The predicted 3D structures of 14-3-3 A and C isoforms suggested the presence of mini-helices apart from 9 perfect α helices running in anti-parallel fashion. Isoform A contained 3 mini-helices (one between helix H and I

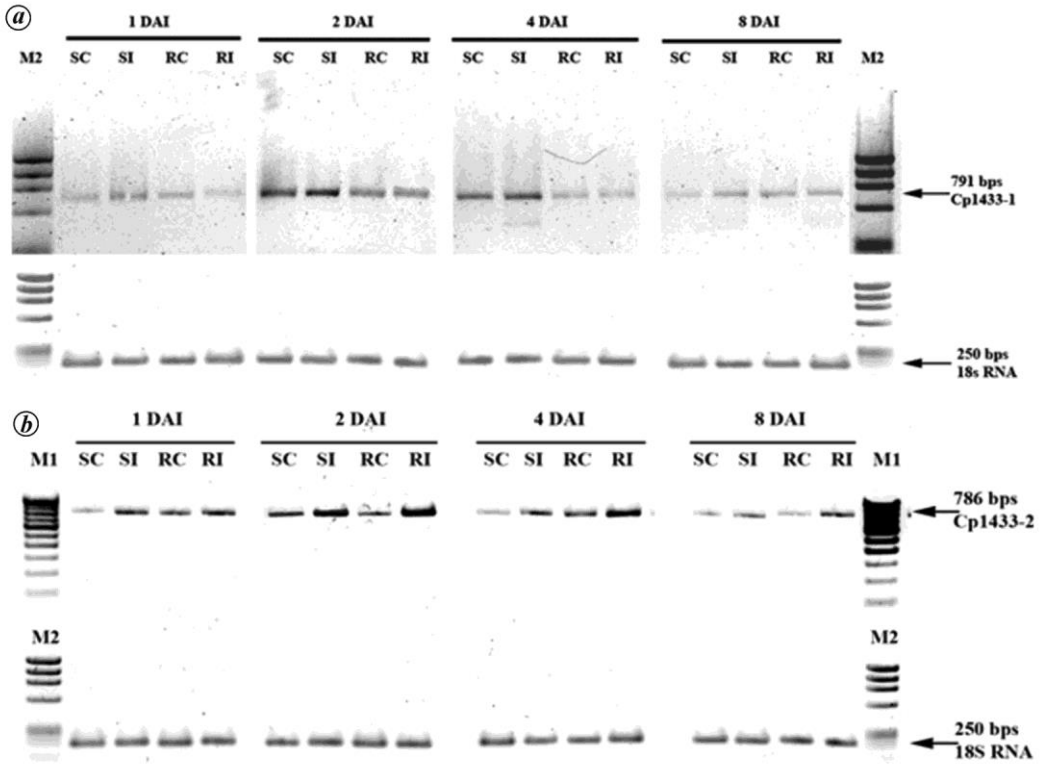

Figure 3. Transcription profiling of the 14-3-3 isoforms. ***a***, Semi-quantitative RT-PCR analysis of 14-3-3C. ***b***, 14-3-3A genes at 1, 2, 4 and 8 DAI in susceptible control (SC), susceptible inoculated (SI), resistant control (RC) and resistant inoculated (RI) cultivars of chickpea. M1 and M2 denote 100 bp and phiX DNA ladder (Bangalore genei, India). Amplification of 18S rRNA gene (normalization) is represented at the bottom of both the images.

and two after helix I) while isoform C comprised only 1 mini helix between helix H and I (Figure 4). These structural differences between 14-3-3A and C might have an effect over their performance upon pathogen attack. Structure of isoform XP_004487161.1 was also generated and was found to be structurally most dissimilar/variable as compared to isoforms A and C. It contained 8α helices running in anti-parallel fashion (Supplementary Figure 3), with 6 major disordered regions and 1 strand. Along with 4-protein binding regions located at positions 1 to 3, 17, 59 and 143; it also contained a polynucleotide-binding region at residue 35.

The amphipathic groove, which is a characteristic of 14-3-3 proteins, was well conserved in both the A and C isoforms. This groove comprised hydrophobic amino acids (V185, L181, W237, L229 and L225 in isoform A and W233, V181, L225, L221 and L177 in isoform C respectively), basic amino acids (R133, R63, R67, K56 and K126 in isoform A and R58, R62, R132, K125 and K51 in isoform C respectively) and acidic amino acids (Y134, D130 in isoform A and Y133, D129 in isoform C respectively). Despite the structural differences, isoform XP_004487161.1 shared the same amphipathic groove residues with other 14-3-3 sequences. The alignment of all 14-3-3 sequences from chickpea along with human 14-3-3 sequence indicated the presence of conserved residues involved in their regulation, self-dimerization, phosphorylation as well as amphipathic groove formation (Supplementary Figure 4).

Motif identification and phylogenetic analysis of chickpea 14-3-3 A and C isoforms across Fabaceae members

MEME based motif analysis and phylogenetic analysis of 14-3-3 isoforms A and C individually, was accomplished for Fabaceae family. A total of 25 motifs were recognized out of which the longest motif was of 50 residues and the smallest was of 10 residues (Supplementary Table 2). Motifs 1 to 6 were the most frequently occurring motifs represented in maximum numbers within the 55 sequences, while few motifs occurred only in 1 or 2 sequences out of 55. Thus, motifs 1 to 6 followed by motif 9 and 10 which occurred in 22 and 14 sequences respectively, were the most conserved motifs in 14-3-3 isoform A; whereas other motifs were the least conserved motifs. The most conserved motif of isoform A (motif 1) across Fabaceae family members was in the region of 175 to 225 amino acids (Supplementary Figure 5). Similar MEME analysis was performed for 14-3-3C isoforms of Fabaceae family. A total of 14 motifs were recognized out of which the longest motif was of 50 residues and the smallest was

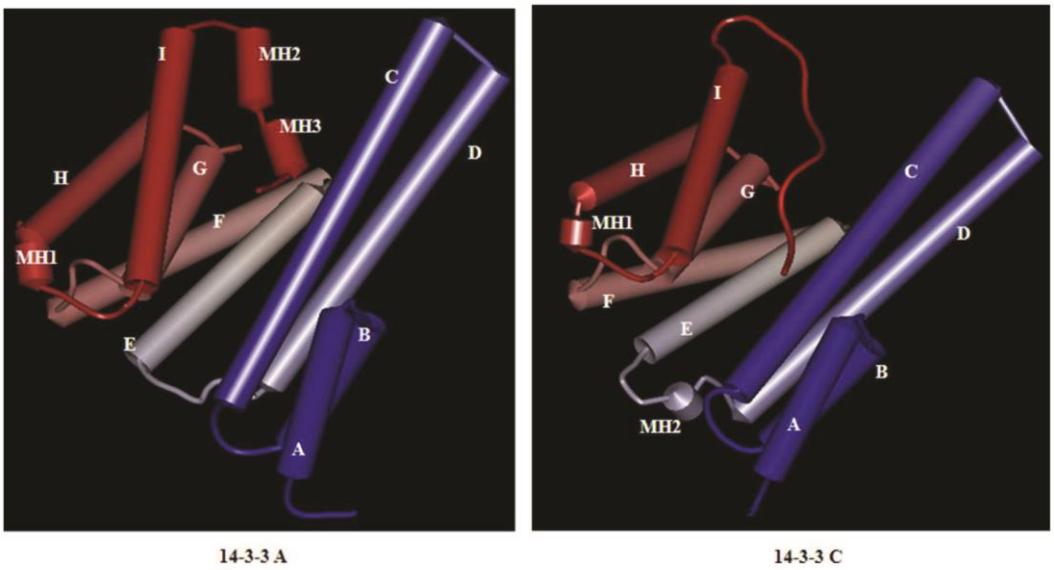

Figure 4. The predicted 3D structures of 14-3-3 isoforms A and C sequences identified in this study using i-Tasser server. The helices in both the structures are labelled alphabetically (as A–I) from N to C terminal. Mini helices are indicated as MH.

of 10 residues (Supplementary Table 2). Motif 4 and 5 were most frequently represented in all the 45 sequences whereas motifs 10 to 14 occurred only in 2 sequences out of 45. Thus, motifs 4 and 5 were the most conserved motifs; whereas motifs 10 to 14 were the least conserved in the 14-3-3 isoform C. The most conserved motifs of isoform C (motif 4 and 5) across the Fabaceae family was in the region of 70 to 150 amino acids (Supplementary Figure 6).

Phylogenetic analysis of 14-3-3 isoforms belonging to Fabaceae family members which aligned with chickpea 14-3-3 isoforms A and C separately, including the ones identified in this study, was accomplished and neighbor-joining trees with 1000 bootstrap values were generated. 14-3-3 isoforms were observed to form groups based on their motif architectures (Supplementary Figures 7 and 8). In case of 14-3-3A phylogenetic tree, two major groups could be identified out of which all the sequences containing motifs 1, 2, 3, 5, 7, 9 and 21 formed a sub-group (group A, except for sequence NP_001236005.1). 14-3-3 sequences of *Glycine max* (accession NP_001236005.1) and *Lupinus albus* (accession AFP43758), although had different combination of motifs, still clubbed together with 14-3-3A isoforms of group A (Supplementary Figure 7). In case of 14-3-3C, the sequences could be categorized into two groups based on the phylogenetic tree, out of which, sequences containing motifs 1 to 7 as generated using MEME tool (Supplementary Figure 6) clubbed together forming sub-group C (including 14-3-3C isoforms identified in this study). However, 14-3-3 sequence of *Vicia faba* (accession number BAB17822), which clubbed with group C sequences, lacked the presence of motif 7 as compared to other group members (Supplementary Figure 8).

Of all the 14-3-3A sequences of Fabaceae family used for phylogenetic analysis, only a few have been studied for their functional roles. 14-3-3A accessions P46266 and CAB42546.2 were the only ones which had been well characterized and shared similar motif architecture as chickpea 14-3-3A sequence. Accession P46266 from *P. sativum* was involved in plant responses against wounding[12]. While in another study, accession CAB42546.2 from *P. sativum* was shown to interact with plastidial precursor proteins and not with mitochondrial precursor proteins[13]. However, none of these isoforms were studied for their role in plant–pathogen interactions. The other accessions such as NP_001235679, NP_001238407, BAB17821 and CAA69347 which belonged to the same major phylogenetic cluster and did not completely share motif architecture with our 14-3-3A isoform, also had important biological functions. Accession NP_001235679 was implicated in the early development of soybean nodules[14]. Nodulation is a plant–microbe association with mutualistic benefits for both partners, opposite to what is observed in plant–pathogen interaction. However, initial plant responses like microbe recognition and adherence remain common in both the associations. Saalbach *et al.*[15] reported that broad bean 14-3-3 had a role in ion channel regulation by modulating the kinase activity or binding the channel. Whereas, another broad bean 14-3-3 accession was studied by Emi *et al.*[16] to bind to plasma membrane H+-ATPase, thus activating H+-ATPase guard cells. As with 14-3-3A, only a few 14-3-3Cs belonging to Fabaceae family used for phylogenetic analysis in this study have been assigned functional roles till date. Some of these 14-3-3C proteins shared similar functions with 14-3-3A, such as binding to chloroplast precursor proteins, involvement in plant wounding response and activation of H+-ATPase guard cells in pea and broad bean[13,16].

Discussion

In the present study 14-3-3 isoforms of chickpea responsive to FOC attack were analysed for their gene expression as well as structural features. The sequence characterization indicated no variation in the sequences for 14-3-3A and C of susceptible or resistant chickpea varieties used in the present study. Interestingly, 14-3-3A transcripts showed overexpression upon FOC1 challenge in the resistant cultivar indicating its potential role in plant defense against biotic stress. Similar observations were made in potato wherein 14-3-3 protein involved in signalling was more strongly induced in 2-week-old potato resistant variety than that in the susceptible variety[17]. Also in case of rice at least four 14-3-3 genes, namely *GF14b*, *GF14c*, *GF14e* and *Gf14f* were differentially regulated during interactions with *Magnaporthe grisea* and *Xanthomonas oryzae* pv. *oryzae* – the incompatible interactions showed a stronger induction of the genes than the compatible interactions[1]. While, in case of wheat, 14-3-3 transcripts were undetectable in roots of susceptible cultivar infected by the fungus *Gaeumannomyces graminis*[18]. In our study, expression of 14-3-3C decreased in the resistant cultivar whereas it increased in the susceptible cultivar upon infection, compared to their respective controls, indicating that 14-3-3C might have some different role in plant–pathogen interaction; an aspect that needs further investigation. Furthermore, the structural differences observed between 14-3-3A and C also might have some differential effect over their performance in such interactions.

Bioinformatics based predictions and wet-lab validations have led to the understanding of protein–protein interaction networking in model plants such as *Arabidopsis thaliana* (AtPIN: *Arabidopsis thaliana* Protein Interaction Network)[19]. Though such interaction network has not yet been developed for legumes including chickpea, the model plant information can certainly give us clues about the 14-3-3 interactome. Such legume–pathogen studies in view of 14-3-3 proteins can help decipher their roles in this interaction. This will further enable to build a better understanding of plant response against pathogen attack by developing a global view of 14-3-3 interactome especially in legumes.

Conflict of interest: Authors declare no conflict of interest.

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ACKNOWLEDGEMENTS. Authors acknowledge funding from Council of Scientific and Industrial Research (CSIR), India to CSIR-National Chemical Laboratory under network project (BSC0117). GG acknowledges funding from Department of Biotechnology (DBT), Ministry of Science and Technology, India under BioCARe scheme (GAP308126) and the DBT-Foldscope project (BT/IN/INDO-US/Foldscope/39/2015). SN is thankful to CSIR, India for research fellowship.

Received 14 April 2020; revised accepted 23 August 2021

doi: 10.18520/cs/v121/i8/1039-1045