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# The role of tripartite interaction of calcium sensors and transporters in the accumulation of calcium in finger millet grain

S.B. KOKANE, R.K. PATHAK, M. SINGH, and A. KUMAR*

*Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar-263145, Uttarakhand, India*

## Abstract

Finger millet (*Eleusine coracana*) is one of important crops, and its grains contain an exceptionally high content of calcium. In order to investigate the molecular mechanism by which it orchestrate the accumulation of $Ca^{2+}$ during grain filling, some candidate genes encoding calcium transporters [calcium exchangers (*CAX1*, *CAX3*)] and sensors [calcineurin-B like (*CBL4* and *10*)], a CBL-interacting protein kinase (*CIPK24*), and calmodulin (*CaM*) were identified using transcriptomics and differential expression analysis in two genotypes of finger millet differing in grain calcium content. These transporters and sensors are highly expressed in leaves and developing spikes of the genotype with a high grain $Ca^{2+}$ indicating their potential role in $Ca^{2+}$ accumulation. Calcium transporters, mainly CAXs, pump $Ca^{2+}$ inside the cell through plasmalemma and tonoplast, and their activities are regulated by CaM dependent and independent $Ca^{2+}$ sensor proteins of CaM and CBL-CIPK networks. Abundance of CaM in a high grain $Ca^{2+}$ genotype is suggestive that CaM might also contribute for grain calcium accumulation by interaction with $Ca^{2+}$ATPase. The up-regulation of *CAX1* in vegetative tissues and developing spikes and *CAX3* only in developing spikes provides the most plausible clue for calcium transport and accumulation regulated by tripartite interaction in finger millet.

*Additional key words*: calcineurin-B like, calcium exchanger, calmodulin, *Eleusine coracana*, protein structure modeling, transcriptomics.

## Introduction

Finger millet represents an important plant species for agriculture and food security of poor farmers, who inhabit arid, infertile, and marginal lands (Gupta *et al.* 2017). It holds an immense economic importance due to a high nutritional quality and richness in grain calcium content (several times more than that found in rice). Calcium content in the grain of finger millet genotypes varies from 1.17 to 4.50 mg $g^{-1}$ (Panwar *et al.* 2010). Uptake, transport, distribution, and accumulation of $Ca^{2+}$ in plants are due to the synchronized activity of different $Ca^{2+}$ transporters and $Ca^{2+}$ sensors. Modulation of cytosolic $Ca^{2+}$ content in plants is achieved through a system of $Ca^{2+}$ transport, signaling, and storage pathway. The $Ca^{2+}$ transporters, such as $Ca^{2+}$ channels, $Ca^{2+}$ ATPases, and $Ca^{2+}$ exchangers, are instruments for signaling and transport.

Calcium, an essential plant nutrient, is taken up by the root system and translocated to the aerial parts of plants including fruits and seeds by symplastic and apoplastic pathways (Kumar *et al*. 2014, Mirza *et al.* 2014). Although a lot of studies have been focused to understand the mechanism of $Ca^{2+}$ signaling in plants, a very little research endeavor has been carried out to understand the mechanism of $Ca^{2+}$ transport into developing grains of cereals. Unfortunately, factors influencing distribution of $Ca^{2+}$ are not known, but it is believed that both environmental factors and genetic factors influence calcium accumulation in seeds.

Transient $Ca^{2+}$ increase in the cytoplasm in response to signals is sensed by an array of $Ca^{2+}$-sensors, which decode a $Ca^{2+}$ signal. A large number of $Ca^{2+}$ sensors

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*Abbreviations*: CaM - calmodulin; CAX - calcium exchanger; CBL - calcineurin B-like protein; CIPK-CBL - calcineurin B-like interacting protein kinase.

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* Corresponding author; e-mail: anilkumar.mbge@gmail.com

have been characterized in plants (Zielinski 1998, Singh *et al.* 2015). Recently, high throughput sequencing has emerged as a powerful tool for non-model organisms, such as finger millet, which has limited genomic information (Kumar *et al*. 2014). Recently, $Ca^{2+}$ sensor and transporter genes were identified in developing spikes of the finger millet transcriptome to elucidate their roles in seed $Ca^{2+}$ accumulation (Singh *et al.* 2015). Earlier studies showed a higher expression of a $Ca^{2+}$ exchanger gene *CAX1* in stems, leaves, and developing spikes indicating its role in $Ca^{2+}$ accumulation (Mirza *et al.* 2014), and similarly, a *CAX3* gene has been found responsive to an increasing concentration of exogenous $Ca^{2+}$ (Singh *et al.* 2015). Calcium-activated calmodulin (CaM) also regulates ion transport through membranes by modulating the activity of ion channels and pumps. The interaction of the $Ca^{2+}$ ion with a sensor protein (decoding mechanism) regulates the number of responses in plants (Reddy *et al* 2004). The cloned full-length *CaM* gene was observed to be highly expressed during the grain filling stages indicating that it is probably involved in regulating $Ca^{2+}$ pumps specifically expressed during grain filling. It helps in activation of $Ca^{2+}$ translocation by water stream *via* xylem vessels (Nath *et al.* 2010, Kumar *et al.* 2014). Similar expression patterns of CaM and $Ca^{2+}$ ATPase in developing spikes of *E. coracana* and not in vegetative tissues indicate that CaM-ATPase interaction might impart its role in $Ca^{2+}$ accumulation in seeds. Another candidate gene calcineurin B-like (CBL) interacting protein kinase (*CIPK*) plays a crucial role in regulation of $Ca^{2+}$ transport through interaction with a unique family of plant CBLs (Cheong *et al.* 2007). It was also reported that the activity of CAX1 protein is regulated by CIPK24 (Mao *et al*. 2016). *In vivo*, CIPK24 is supposed to be activated by $Ca^{2+}$ dependent CBL (Li *et al.* 2009). Although the role of the vacuolar CAX1 in calcium accumulation is well defined, the role of the regulatory kinase, *i.e.*, CIPK24 and its interacting partner(s) CBLs in $Ca^{2+}$ accumulation, has not been studied so far. However, the role of CBL1, CBL4, and CBL10 is depicted in regulation of $Ca^{2+}$ homeostasis during salt stress, where CBLs interact with Son of Sevenless (SOS2)/CIPK24 and form a CBLs-CIPK24 complex, which further interacts with the plasma membrane-localized $H^+/Ca^{2+}$ antiporter (CAX1) and so regulates intracellular $Ca^{2+}$ homeostasis (Pittman *et al.* 2002, 2004, Cheng *et al.* 2005, Li *et al.* 2009, Conn *et al.* 2011). Validation of differentially expressed genes, such as *CAX1, CAX3, CaM, CIPK*, and *CBLs*, identified in finger millet, using the transcriptome of developing spikes, needs to be investigated in more detail. In order to prove the regulatory roles of CIPK proteins in modulating the activity of CAXs and subsequently the $Ca^{2+}$ movements, attempts were made in the present investigation to dissect the role of CAX1 and CAX3 in relation to CIPK24, CBL4, CBL10, and CaM in calcium accumulation in finger millet grains.

## Materials and methods

**Plants and cultivation:** To study tissue wide expression of potential candidate genes involved in calcium signaling and transport, two finger millet [*Eleucine coracana* (L.) Gaertn.] genotypes differing in grain calcium content [GPHCPB1 with a low calcium content (GP1) and GPHCPB45 with a high calcium content (GP45)] were selected. The seeds were sown in earthen pots containing soil and vermin compost in the ratio 1:1 for all the pots. The plants were maintained in a naturally lit greenhouse at a day temperature of 37 to 40 °C and a relative humidity of about 40 %. Irrigation was done as necessary. Geographically, the sites lie in Tarai plains at 29 °N and 79 °E and an altitude of 243.8 m a.s.l. Sampling vegetative tissues (roots, stems, and leaves) was done 60 d after sowing, and of spikes at 4 different developmental stages (S1, S2, S3, and S4) according to Mirza *et al*. (2014). After selection of a sample, the specific tissue of each sample was used for RNA isolation, *i.e*., the upper portion (1/3) of spikes and leaves, 1 - 2 cm of root tips, and 1 - 1.5 cm from the top of a stem (including the shoot apical meristem).

**Total RNA isolation, cDNA synthesis, and PCR amplification:** Total RNA was isolated using the *iRIS* system from *CSIR-IHBT* (Palampur, India) according to the manufacturer's instructions. The total RNA was purified by treating RNA with RNase free DNaseI according to the manufacturer's instruction (*Fermentas*, USA). The first strand cDNA was synthesized from DNase treated total RNA (2 µg) of both genotypes (GP1 and GP45) using an oligo(dT) 18 primer with *RevertAid H Minus M-MuLV RT* (200 units per $mm^3$, *Fermentas*) for real time PCR. The synthesized single stranded cDNA was used to amplify the genes using gene specific primers (Table 1 Suppl.). For transcript profiling the potential candidate gene, a minimum of four replicates of RNA samples were selected and pooled. The finger millet housekeeping gene (*tubulin*) specific primers were used with total cellular cDNA as templates (in duplicate) in a separate tube as a positive control. The negative control in the PCR amplification included everything that was present in the *tubulin* gene amplification except cDNA templates.

**Expression profiling potential candidate genes:** Different sets of primers were designed for the potential candidate genes fetched from the developing spikes transcriptome data of the two finger millet genotypes generated through the next generation sequencing platform (Kumar *et al*. 2015, Singh *et al.* 2015) by the *Primer3* tool (http://bioinfo.ut.ee/primer3-0.4.0/). These primers were used for real-time quantitative PCR analysis

(Table 1 Suppl.).

Real-time quantitative PCR was carried out using the *Maxima™ SYBR Green/ROX qPCR Mastermix* (*Fermentas*) according to the manufacturer's instructions in an *Eppendorf* (Germany) thermocycler *ep Realplex 4*. Two-step real-time PCR was carried out with cDNA from different tissues using gene specific primers (Table 1 Suppl.). The expressions of *CAX1, CAX3, CIPK, CBL4, CBL10*, and *CaM* genes were normalized to *tubulin* expression. The efficiencies of all the genes and *tubulin* were almost equal as analyzed by comparing the $C_T$ values at different dilutions of cDNA (Livak and Schmittgen 2001). The amplification program was: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s; with a melting curve inserted as default. The relative value obtained for quantitation was expressed as $2^{-\Delta\Delta Ct}$, where $\Delta C_T$ represents the difference between the $C_T$ value of the sample and that of *tubulin* in the same sample, and $\Delta\Delta C_T$ is the difference between the $\Delta C_T$ value of a sample and that of its respective control (Livak and Schmittgen 2001).

The samples were amplified in duplicate, and means as well as standard errors were calculated to plot a heat map depicting relative expression using the *R v.3.1.2* (https://cran.r-project.org/). The real-time PCR data were analyzed as a four factorial analysis of variance (*ANOVA*) with genotypes, tissues, and genes as factors. Correlation coefficients were also calculated.

**Protein structure modeling and validation:** Three dimensional structures of CAX1, CAX3, CBL4, CBL10, and CIPK24 were made by *SWISS-MODEL* (http://swissmodel.expasy.org/). In order to construct the structure of each protein, a template for homology modeling was searched by the *BLASTp* (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program against the *RCSB* protein data bank (http://www.rcsb.org/pdb/home/home.do). The predicted models were subjected to further refinement through energy minimization by the *ModRefiner* server (http://zhanglab.ccmb.med.umich.edu/ModRefiner/). The Ramachandran plot and protein stability analysis of each protein model were done by the *Rampage* (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) server and *ProsaA* (https://prosa.services.came.sbg.ac.at/prosa.php). The *Discovery Studio 4.1* visualizer (http://accelrys.com/products/discovery-studio/) was used for visualization of modeled protein structures (Pathak *et al.* 2017).

**Protein-protein docking and construction of a molecular signaling network:** Protein-protein docking studies were performed using *ClusPro* (https://cluspro.bu.edu/login.php). It is one of the protein docking tools for prediction of molecular interaction between macromolecular structures. It requires the three dimensional structure of a receptor and ligand for molecular docking. Protein-protein docking CBL4 and CBL10 with CIPK24 were performed to investigate binding affinity between them. Further, the docked files of CIPK24 with CBL4 and CBL10 were docked with CAX1 and CAX3 and constructed a molecular network of calcium signaling using a system biology graphical notation by *CellDesigner4.4* based on the obtained results (Funahashi *et al.* 2003, Pathak *et al.* 2017).

## Results

Tissue wide transcript profiling and *in silico* analysis were carried out in order to prove the regulatory roles of CIPK proteins in modulating the activity of CAX and subsequently $Ca^{2+}$ movements. The expressions of the respective genes were studied in seven different tissues of both genotypes. Transcript profiling each candidate gene in various tissues is depicted in a heat map (Fig. 1). All these genes were expressed in all tissues of both genotypes but showed different expression patterns. Statistical analysis reveals that variations in the expressions in the different tissues were significant ($P < 0.05$) for all the potential candidate genes. An effort was made to correlate the expression pattern of each potential gene with $Ca^{2+}$ accumulation and transport in various tissues reported by Mirza *et al*. (2014). Interestingly, positive correlations of gene expression with $Ca^{2+}$ content in vegetative and reproductive tissues were observed. The highest and continuously increasing $Ca^{2+}$ content was observed in developing spikes followed by leaf tissue, and a scanty $Ca^{2+}$ content was found in roots and stems (Mirza *et al.* 2014).

Expressions of both calcium transporter genes (*CAX1* and *CAX3*) were slightly higher in GP45 roots as compared to those in GP1, which corresponds to its higher $Ca^{2+}$ content. However, the abundance of transcripts of both the transporters in roots was much lower as compared to spikes. The transcription of *CIPK24* was also negligible in roots of both the genotypes. This shows a possible co-expression of CIPK24 with the CAX antiporter. Further, down regulation of transcription of CaM and $Ca^{2+}$ ATPase in root tissue (present in tonoplast) is suggestive of low sequestration of calcium in vacuole of roots while comparing the transcription in developing spikes of finger millet.

Negligible transcriptions of *CAX1*, *CAX3*, *CIPK24*, and *CaM* were observed in stem tissue of both the genotypes, however, a higher expression of *CIPK24* was recorded in GP45 than in GP1.

Expression of *CAX1* in leaves was 2.7-fold higher in GP45 as compared to GP1, but no significant difference of *CAX3* expression was observed. Interestingly, the expression pattern of the *CIPK24* gene was similar to the *CAX1* gene in leaf tissue. Transcription of *CIPK24* was

rather high in leaf tissue of both the genotypes, whereas a negligible *CaM* transcription was observed with a slightly higher expression in GP45.

Expression of *CAX1* in spikes increased continuously from the stage S1 to stage S4 in both the genotypes with a maximum expression of 6.3-fold at stage S4 in GP45. Likewise, transcription of the *CAX3* gene increased continuously from S1 to S3 and after that decreased in S4 in both the genotypes with a maximum expression (45.8-fold) in the S3 stage of GP45. Mirza *et al*. (2014) reported an increasing $Ca^{2+}$ content from the S1 to S4, which correlates with the expression pattern of respective genes including a higher expression in GP45. Expressions of *CIPK24* were rather high at later stages of developing spikes (S3 and S4) in both the genotypes. In GP45 expression increased continuously from the S1 stage to a maximum at the S3 stage (10.9-fold) and then slightly decreased and was much higher than in GP1 at the S3 stage (3.6-fold). Transcript profiling *CaM* in developing spikes exhibited a similar pattern to the calcium transporter.

The heat map analysis shows that expression of

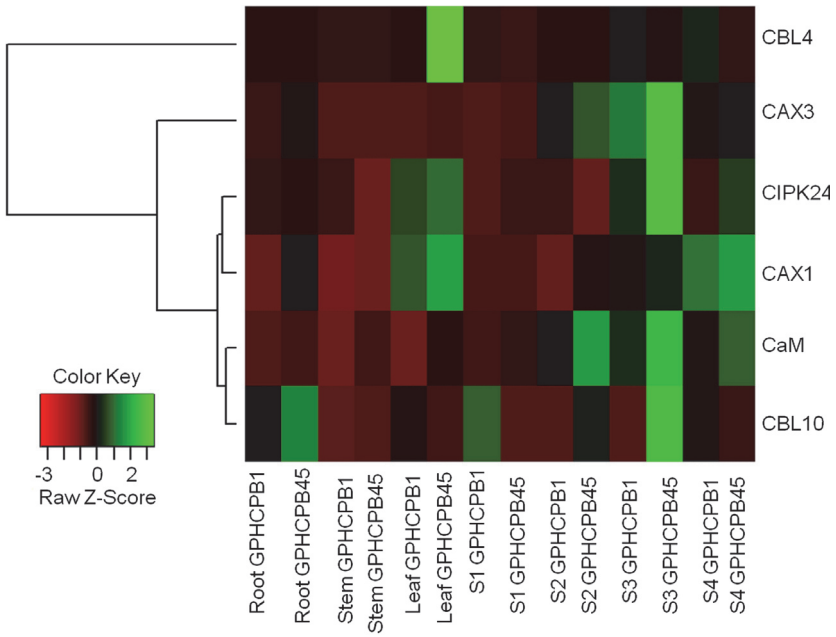


Fig. 1. A heat map generated from real-time qPCR data reflecting expressions of potential candidate genes (*CBL4*, *CAX3*, *CIPK24*, *CAX1*, *CaM*, and *CBL10*) in roots, stems, leaves, and spikes at different stages (S1 - S4) of genotypes GPHCPB1 and GPHCPB45.

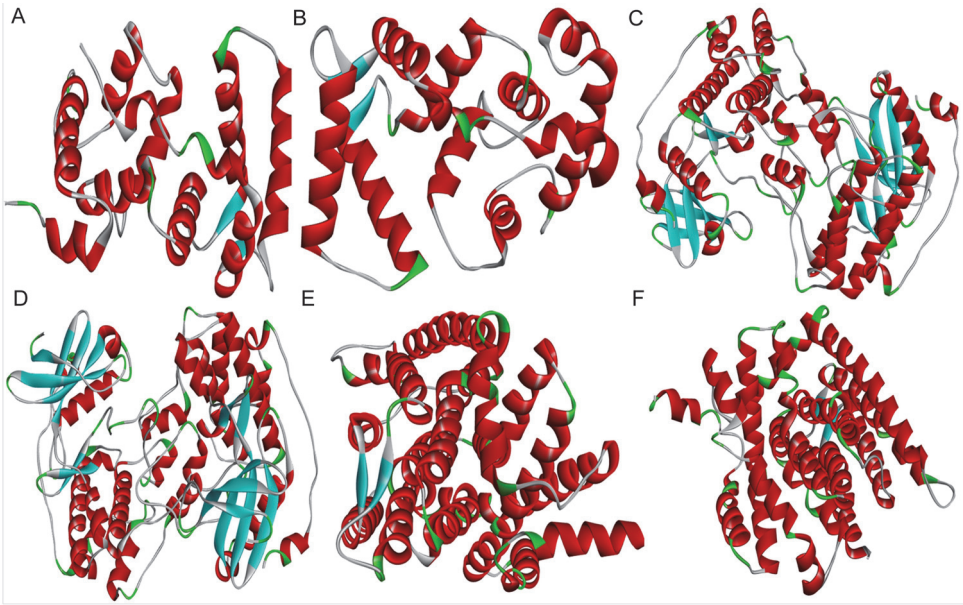


Fig. 2. A predicted structure of target proteins: *A* - CBL4 (GP45), *B* - CBL10 (GP45), *C* - CIPK24 (GP45), *D* - CIPK24 (GP1), *E* - CAX1 (GP45), and *F* - CAX3 (GP45).

*CIPK24* correlated well with *CAX1* in leaves and a moderate positive correlation ($r$ = 0.53) was found between *CIPK24* and both *CAX1* and *CAX3* in developing spikes. The positive correlation was found between *CIPK24* and CAX transporters (*CAX1* and *CAX3)* with correlation coefficients of 0.27 and 0.83, respectively, at developing spike of GP45. A strong positive correlation was observed between *CAX3* and

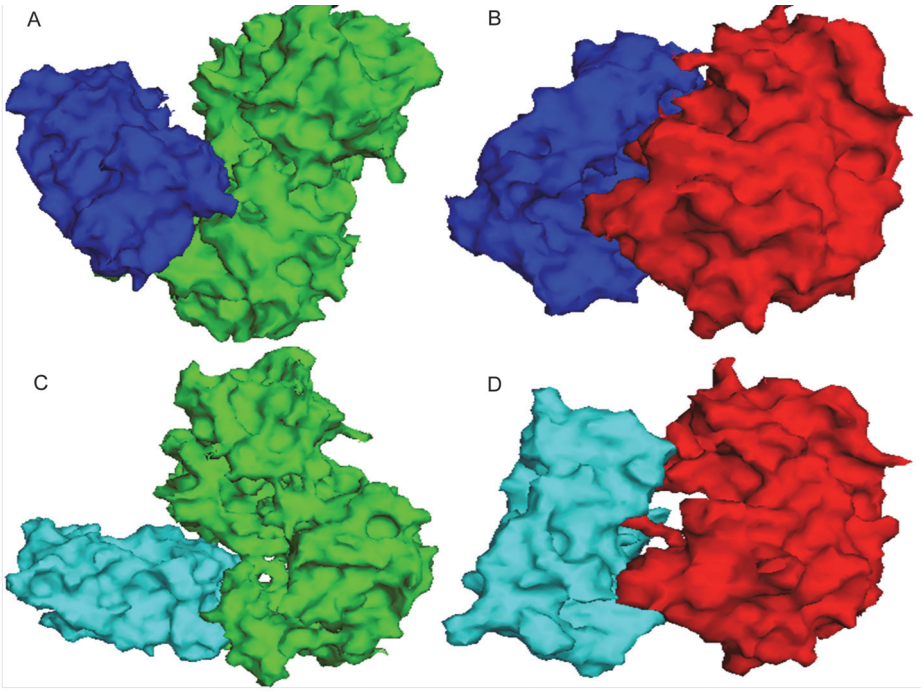


Fig. 3. Protein-protein interaction among CIPK24 [GP45] (*green*)-CBL4 (*blue*) (*A*), CIPK24 [GP1] (*red*)-CBL4 (*blue*) (*B*), CIPK24 [GP45] (*green*)-CBL10(*cyans*) (*C*), and CIPK24[GP1] (*red*)-CBL10 (*cyans*) (*D*).

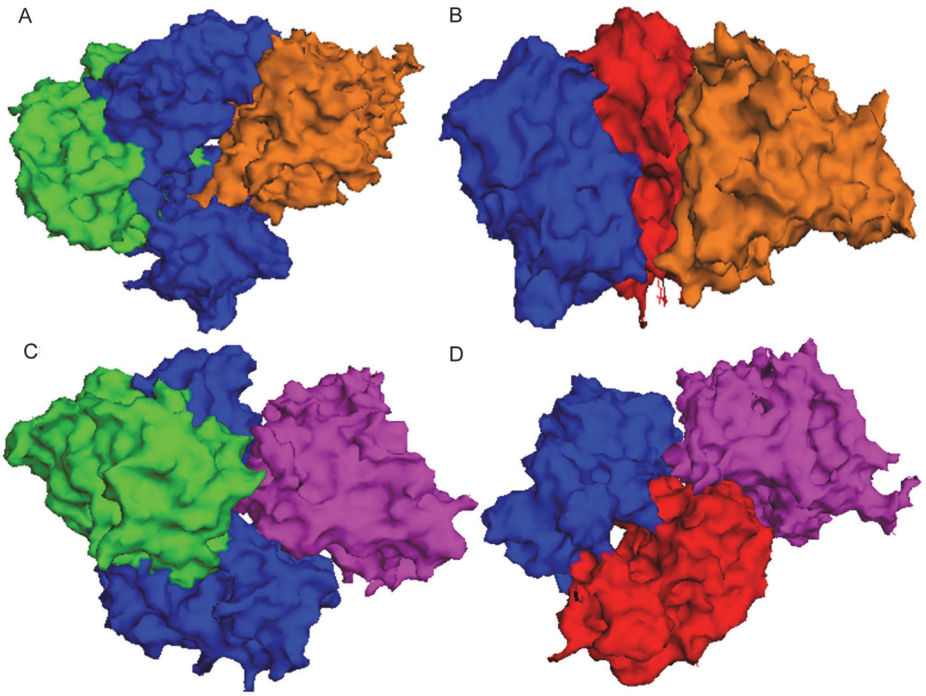


Fig. 4. Protein-protein interaction among CIPK24 [GP45] (*green*), CBL4 (*blue*)-CAX1 (*orange*) (*A*); CIPK24 [GP1] (*red*), CBL4 (*blue*)-*CAX1* (*orange*) (*B*); in GP45 among CIPK24 [GP45] (*green*), CBL4 (*blue*)-CAX3 (*violet*) (*C*); and CIPK24 [GP1] (*red*), CBL4 (*blue*)-CAX3 (*violet*) (*D*).

*CaM* ($r$ = 0.91) in developing spikes, but a negative correlation ($r$ = -0.29) was observed in vegetative tissue. Correlation between CAX1 and CaM was weak ($r$ = 0.14) in developing spikes of GP45.

Statistical analysis of expression data also suggest that the transcriptions of genes *CAX1*, *CAX3*, *CIPK24*, and *CaM* correlated with calcium content measured by Mirza *et al*. (2014). From these data, we can also predict that the *CIPK24* and *CaM* are most important sensors which regulate different transporters CAX and $Ca^{2+}$ ATPase during calcium movement, accumulation, and storage.

In a previous study conducted in our lab, transcriptome sequencing developing spikes in two genotypes of finger millet differing in grain calcium content was completed using *Illumina HisSeq 2000*. The nucleotide sequence of *CBL4, CBL10, CAX1, CAX3*, and *CIPK24* in GP45 and GP1 were obtained from these transcriptome data and subjected to a *Translate* tool (http://web.expasy.org/translate/) to obtain the protein sequence of these genes for further analysis through computational proteomics approaches (Kumar *et al*. 2015, Singh *et al.* 2015).

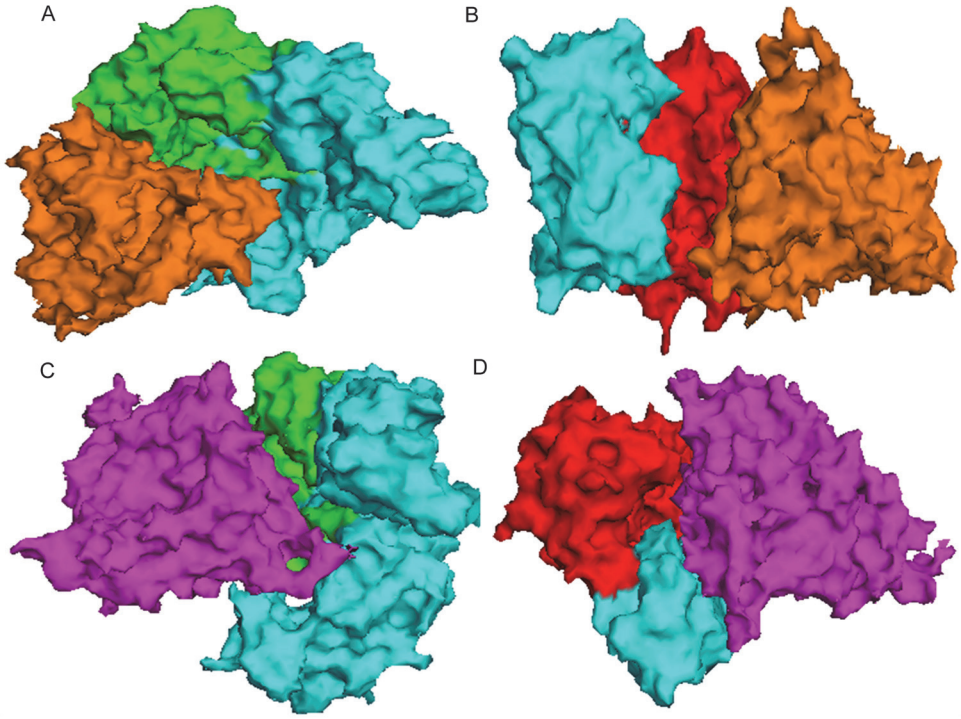


Fig. 5. Protein-protein interaction among CIPK24 [GP45] (*green*), CBL10 (*cyan*)-CAX1 (*orange*) (*A*); CIPK24 [GP1] (*red*), CBL10 (*cyan*)-CAX1 (*orange*) (*B*); CIPK24 [GP45] (*green*), CBL10 (*cyan*)-CAX3 (*violet*) (*C*), and CIPK24 [GP1] (*red*), CBL10 (*cyan*)-CAX3 (*violet*) (*D*).

Table 1. Stereo-chemical properties of predicted protein(s).

| Name of protein | Number of residues in the favored region [%] | Number of residues in the allowed region [%] | Number of residues in the outlier region [%] | Overall model quality (Z score) | |
|---|---|---|---|---|---|
| | | | | template | target |
| CBL4 | 96.6 | 3.4 | 0.0 | -6.24 | -6.12 |
| CBL10 | 95.6 | 3.8 | 0.5 | -6.31 | -6.59 |
| CIPK24(GP45) | 93.9 | 5.6 | 0.5 | -7.15 | -7.31 |
| CIPK24 (GP1) | 94.3 | 5.4 | 0.4 | -7.15 | -7.35 |
| CAX1 | 94.6 | 3.8 | 1.6 | -6.32 | -5.48 |
| CAX3 | 94.1 | 4.8 | 1.1 | -6.32 | -4.93 |

*SwissModel* was used to generate the 3D models of CAX1, CAX3, CBL4, CBL10, and CIPK24 proteins. The *Discovery Studio 4.1* visualizer was used for visualization of each predicted model (Fig. 2). The predicted models were subjected to a further refinement at the *ModRefiner* server. It is based on an algorithm having atomic level, high resolution protein structure refinement, which refine protein structure from initial C-alfa traces based on two steps and atomic level energy minimization and provides accurate side chain positions, good hydrogen boundary

network as well as fever atomic overlaps (Xu and Zhang 2011). The *Rampage* server was used to analyze the reliability of each model backbone of torsion angles φ and ψ to quantify the amino acid residues present in the available zone of the Ramachandran plot. The *ProSA* program was used to determine the energy profile of each model and to calculate the Z-score value to identify overall quality as it measures the total energy of the structures and calculates the interaction energy per residue using a distance-based pair potential. The *ProSA* analysis of the model CAX1 achieved a Z score of -5.48 and that of the template was -6.32; CAX3 Z score -4.93 and its template -6.32; CIPK24 (GP45) -7.08, and its template -8.46; CIPK24 (GP1) -7.74 and its template -8.46; CBL4 -6.51 and its template -6.96; CBL10 -6.48 and its template -6.31. Tthe negative *PROSA* energy reflects reliability of the model for further investigations (Table 1).

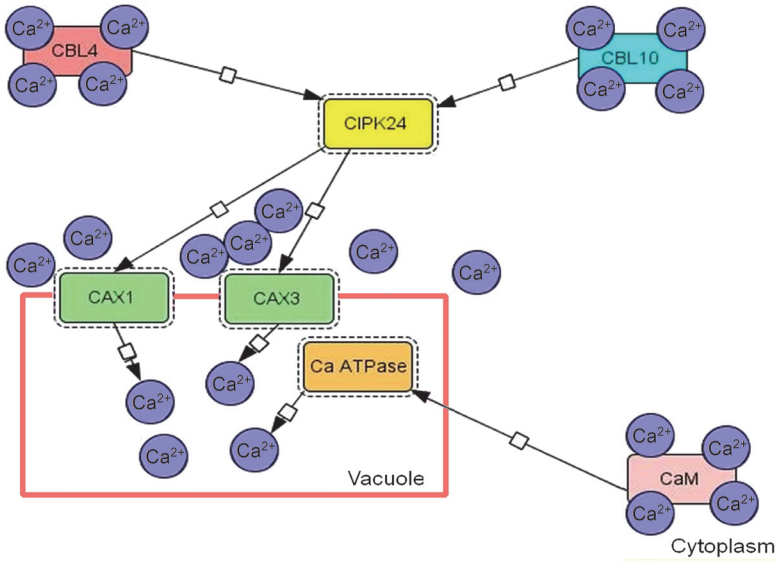


Fig. 6. A map of molecular interactions among calcium exchangers and sensors in different tissues involved in the regulation of calcium transport and accumulation in finger millet seeds (constructed by *CellDesigner4.4*). The CIPK-24 acts as a regulator of CAX1 and CAX3 through calcineurin B-like protein (CBL) interactions and consequently increases calcium storage in vacuoles of seeds. Since *CaM* expressesed abundantly in developing spikes and thus regulated the $Ca^{2+}$ ATPase transporter located in the vacuolar membrane. The $Ca^{2+}$ ATPase transports $Ca^{2+}$ against $Ca^{2+}$ gradient using ATP as an energy source and helps in pumping $Ca^{2+}$ into vacuoles, which act as a storage site of calcium in developing seeds.

Table 2. Molecular docking studies illustrates a minimum free energy required for non-covalent interactions with docked protein structures.

| Protein-protein docking | Docking energy [J $mol^{-1}$] |
|---|---|
| CBL4 *vs.* CIPK24 (GP45) | -3599913.6 |
| CBL4 *vs.* CIPK24 (GP1) | -3829196.8 |
| CBL10 *vs.* CIPK24 (GP45) | -3461423.2 |
| CBL10 *vs.* CIPK24 (GP1) | -3806603.2 |
| CIPK24 (GP45)-CBL4 complex *vs.* CAX1 | -6530805.6 |
| CIPK24 (GP1)-CBL4 complex *vs.* CAX1 | -5670156.8 |
| CIPK24 (GP45)-CBL10 complex *vs.* CAX1 | -6781845.6 |
| CIPK24 (GP1)-CBL10 complex *vs.* CAX1 | -5345060.0 |
| CIPK24 (GP45)-CBL4 complex *vs.* CAX3 | -5725385.6 |
| CIPK24 (GP1)-CBL4 complex *vs.* CAX3 | -5342549.6 |
| CIPK24 (GP45)-CBL10 complex *vs.* CAX3 | -6746281.6 |
| CIPK24 (GP1)-CBL10 complex *vs.* CAX3 | -5779777.6 |

The *ClusPro* protein docking program was used to identify binding free energy between selected protein structures using default parameters. The CBL4 was docked with CIPK24 (in GP45 and GP1) showing binding free energy of -3599913.6 and -3829196.8 J $mol^{-1}$, respectively, and CBL10 was docked with CIPK24 showing binding free energy of -3461423.2 and -3806603.2 J $mol^{-1}$, respectively (Fig. 3).

Further, the docked complex file of CIPK24 with CBL4 were docked with CAX1 protein showing binding free energy of -6530805.6 and -5670156.8 J $mol^{-1}$, respectively; CIPK24 with CBL10 were docked by CAX1 showing binding free energy of -6781845.6 and -5345060 J $mol^{-1}$, respectively; CIPK24 with CBL4 were docked with CAX3 showing binding free energy of -5725385.6 and -5342549.6 J $mol^{-1}$, respectively. CIPK24 (GP45 and GP1) with CBL10 were docked with CAX3 showing binding free energy of -6746281.6 and -5779777.6 J $mol^{-1}$ (Table 2).

Based on the expression and protein-protein interaction analysis of candidate gene(s)/protein(s), a tripartite molecular interaction map of calcium signaling, transport, and accumulation was constructed using the system biology graphical notation by *CellDesigner4.4* in finger millet (Fig 6) (Pathak *et al.* 2013b).

**Discussion**

Calcium distribution and accumulation by plants are variable among species, genotypes, and even different tissues (Mirza *et al.* 2014). The crops with increased abilities to acquire $Ca^{2+}$ and accumulate it in edible tissues can be an alternate source of calcium. Generally, $Ca^{2+}$ moves apoplastically with transpiration stream and its concentration in phloem-fed tissues, such as fruits, seeds and tubers, are generally low in many crops (Karley and White 2009, Kumar *et al.* 2014), but finger millet was found to be one of crop plants accumulating a very high seed calcium content (Panwar *et al.* 2010). Therefore, it becomes an important species for elucidation of the genetic and epigenetic basis of $Ca^{2+}$ uptake, transport, and accumulation in plants. Calcium transporters are actively involved in uptake and transport of $Ca^{2+}$ in cells, whereas $Ca^{2+}$ sensors are involved in regulation of these transporters (Singh *et al.* 2015).

The role of different $Ca^{2+}$ channels, $Ca^{2+}$ ATpase, and $Ca^{2+}$ exchangers and their regulation, which are responsible for differential spatial $Ca^{2+}$ accumulation in finger millet seeds, were reported by Nath *et al.* (2013) and Kumar *et al.* (2015). Accumulation of $Ca^{2+}$ in finger millet during development of spikes is regulated by different $Ca^{2+}$ sensors because *CaM* is highly expressed during grain filling (Kumar *et al.* 2014). Another study conducted by Mirza *et al*. (2014) also suggested that *CAX1* expression continuously increases during grain filling in finger millet. Singh *et al.* 2015 has also identified the responsiveness of *CAX3* gene with respect to increasing concentration of exogenous calcium. These genes were identified as potential candidate genes as reported in previous studies using *Arabidopsis thaliana* as a model system. On the cellular level, deletion of CAX1 and CAX3 caused relative increases in $Ca^{2+}$ outside different types of storage vacuoles. The regulation of CAX1 also disrupted the selective $Ca^{2+}$ accumulation abilities of certain cell types (Punshon *et al.* 2012). It is also considered that the CBL-CIPK-24 complex activates a cation exchanger during abiotic stress and controls intracellular calcium homeostasis (Cheng *et al.* 2005, Li *et al*. 2009). To appreciate the result of present studies, it was speculated that there might be a specific interaction amongst different signaling and sensors with different $Ca^{2+}$ transporters during calcium accumulation in finger millet (Chinchole *et al.* 2017).

The CAX1 protein has also a role in calcium transport, distribution, and accumulation in leaves due to localization of the protein in mesophyll tonoplast. It is accompanied by a lower $Ca^{2+}$ concentration in the vacuole and a higher accumulation of $Ca^{2+}$ in the mesophyll (Cheng *et al.* 2005). An earlier report also showed that expression of *CAX1* is low in root tissue as compared to other tissues (Conn *et al.* 2010). This suggest that during uptake of $Ca^{2+}$ from soil and its transport through root cells, $Ca^{2+}$ is transported in apoplast and further by transpiration stream, rather than it is sequestrated in the vacuole of root cells, however, some storage of $Ca^{2+}$ in root vacuoles might be important for root metabolic activity and for maintaining integrity of root cell membranes. Efflux of $Ca^{2+}$ from cells to apoplast is carried out by different transporters (Hashimoto *et al.* 2005, Mirza *et al.* 2014), and it is transported *via* xylem further to the apical part of plants (White 2000). In stems, a negligible amount of transcripts of target genes were observed in both the genotypes. However, a higher expression of *CIPK24* in GP45 than in GP1was found; this might be important in $Ca^{2+}$ signaling.

Delivery of $Ca^{2+}$, transport, and distribution in leaves are mostly dependent on water flow to and through leaves (Gilliham *et al.* 2011). However, Carter *et al.* (2004) suggested that CAX1 protein has a role in calcium accumulation within a leaf due to its localization in tonoplast of mesophyll cells. An increased content of CIPK24 might concern a high $Ca^{2+}$ because CIPK24 activates the CBL protein. The CIPK24-CBL complex activates CAX1 in leaf tissue, and this indicates that in leaf tissue, CIPK24 is an interacting partner of CAX1 (Cheng *et al.* 2004). The pattern of expression of both *CAX1* and *CAX3* corresponds to $Ca^{2+}$ content in finger millet seeds, *i.e*., higher expressions of *CAX1* and *CAX3* genes in GP45 indicate higher uptake and accumulation of $Ca^{2+}$ in GP45 as compared to GP1.

Ion transporters present in the tonoplast, including an $H^+$ pump and $Ca^{2+}/H^+$ exchanger, are activated by CIPK-24 (Batelli *et al.* 2007, Li *et al*. 2009). A CIPK24-CAX1 interaction is $Ca^{2+}$ dependent, and CIPK must be recruited to the tonoplast to activate CAX1. Interestingly, all regulatory proteins exhibited expression patterns similar to the transporter genes with generally higher transcriptions in GP45 than in GP1. A higher expression of *CIPK24* in GP45 indicates that *CIPK* was responsible for cation homeostasis and was regulated by a $Ca^{2+}$ sensor CBL protein. The CIPK plays an important role in regulation of ion transport and activity of CAX proteins (Cheng *et al.* 2005).

An expression pattern of *CaM* might be helpful for elucidation of the role of *CaM* in $Ca^{2+}$ accumulation and transport in finger millet. The expression pattern of the *CaM* gene was found similar in both the genotypes, with a maximum transcription observed in developing spikes of GP45 indicating an important role of the *CaM* gene in $Ca^{2+}$ transport and accumulation. Generally, the expression pattern of *CaM* is similar to $Ca^{2+}$ ATPase (Mirza *et al* 2014), *CAX1*, and *CAX3*. This indicates that CaM isoforms might be specific to developing spikes, and they might activate $Ca^{2+}$ ATPase by interaction with a CBD domain (Amtmann and Blatt 2009, Mirza *et al.* 2014). The statistical analysis suggests that the CAX transporter was responsible for $Ca^{2+}$ accumulation in seeds and other tissues of finger millet.

Protein structure modeling using protein sequence information is considered as one of the most accurate methods for prediction of 3D structure and generating suitable models for a wide range of applications in

biological sciences (Bodade *et al*. 2010, Pathak *et al*. 2013a, 2017). At least one known structure available in Research Collaboratory for Structural Bioinformatics Protein Data Bank (*RCSB PDB*) plays a significant role in prediction of an unknown structure of investigated protein. This approach would provide reasonable results based on the theory that the tertiary structure of two proteins is similar, when their sequences are closely related to each other. The obtained protein model from homology modeling through *SwissModel* was used for molecular interaction analysis through protein-protein docking because computational approaches that dock one protein into the structure of other proteins and score their potential complementarily to binding sites and visualization of docked complex structures are widely used for molecular investigation in biological systems (Kitchen *et al.* 2004, Kumar *et al.* 2015a, Pathak *et al.* 2016). Expression and protein-protein interaction studies suggest that CBL4-CIPK24 and CAX1 as well as CBL10-CIPK24 and CAX3 are involved in regulation of different calcium transport and accumulation machinery. The CIPK24 may act as a regulator of CAX1 and CAX3 through interaction with a CBL protein and play a vital role in storage of calcium in vacuoles of seeds. All molecular processes were summarized to develop a molecular map for a better understanding of transport and accumulation machinery in crop plants (Kumar *et al.* 2015a).

Based on the results, it can be concluded that all the investigated genes play important roles in $Ca^{2+}$ transport and accumulation in finger millet seeds (Fig 6). *In silico* docking suggests that interaction of $Ca^{2+}$ transport and sensor genes led to activation of transport machinery that pumped $Ca^{2+}$ from the cytosol to the vacuole in developing seeds. It is suggested that (SOS3)/CBL4 and CBL10 strongly interact with CIPK-24. The SOS/CBL4 binds and activates SOS2/CIPK-24 kinase by relieving self-inhibitory folding within the regulatory and kinase domain of the SOS2/CIPK-24 protein. Ion transporters present at the tonoplast, including an $H^+$ pump and $Ca^{2+}/H^+$ exchanger, are the targets of CIPK-24 for their activation.

Despite the knowledge that calcium deficiency is a widespread problem across the globe, very little effort has been put to understand the mechanism(s) of high calcium accumulation in finger millet seeds. Such information would be immensely helpful in genetic engineering common cereals, such as rice, maize, and wheat, for high seed calcium content and in turn, be an important step towards alleviating the problem of calcium deficiency in children and adults who are mainly dependent upon the staple cereals, which contain very low amounts of calcium.

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