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ORIGINAL ARTICLE

Molecular characterization of *EcCIPK24* gene of finger millet (*Eleusine coracana*) for investigating its regulatory role in calcium transport

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Abstract Finger millet grains contain exceptionally high levels of calcium which is much higher compared to other cereals and millets. Since calcium is an important macronutrient in human diet, it is necessary to explore the molecular basis of calcium accumulation in the seeds of finger millet. *CIPK* is a calcium sensor gene, having role in activating Ca^{2+} exchanger protein by interaction with CBL proteins. To know the role of *EcCIPK24* gene in seed Ca^{2+} accumulation, sequence is retrieved from the transcriptome data of two finger millet genotypes GP1 (low Ca^{2+}) and GP45 (high Ca^{2+}), and the expression was determined through qRT-PCR. The higher expression was found in root, shoot, leaf and developing spike tissue of GP45 compared to GP1; structural analysis showed difference of nine SNPs and one extra beta sheet domain as well as differences in vacuolar localization was predicted; besides, the variation in amino acid composition among both the genotypes was also investigated. Molecular modeling and docking studies revealed that both *EcCBL4* and *EcCBL10* showed strong binding affinity with *EcCIPK24* (GP1) compared to *EcCIPK24* (GP45). It indicates a genotypic structural variation, which not only affects the affinity but also calcium transport efficiency after interaction of CIPK-CBL with calcium exchanger (*Ec*CAX1b) to pull calcium in the vacuole. Based on the expression and in silico study, it can be suggested that by activating *EcCAX1b* protein, *EcCIPK24* plays an important role in high seed Ca^{2+} accumulation.

Keywords Finger millet · Transcriptome data · CIPK24 · CBL · Modeling · Docking

Introduction

Finger millet (*Eleusine coracana*), an allotetraploid (4X) and annual robust grass, is mainly grown as a grain cereal in the semi-arid tropics and subtropics of the world under rain-fed conditions (Fakrudin et al. 2004). As compared to other cereals, calcium (Ca^{2+}) content is very high in finger millet grains that varies from 100 to 450 mg/100 g of seed, and is amazingly 10–30 times higher than that found in the grains of rice and wheat (National Research Council 1996; Panwar et al. 2010). It can be used in formulating diets for pregnant and lactating women and growing children. Also, high calcium supplements can help in controlling osteoporosis occurring during menopause (Kumar et al. 2012). Molecular characterization of finger millet genotypes revealed that high calcium accumulation in finger millet grain is mainly genetically determined and less is environmentally influenced (Panwar et al. 2010; Kumar et al. 2012; Nath et al. 2013; Singh et al. 2014a).

Ca^{2+} signaling and transport gene family has very important role in seed Ca^{2+} accumulation (Kumar et al. 2015a, b; Singh et al. 2014b, 2015; Sharma et al. 2017). The role of Ca^{2+} exchanger protein is well studied and also used for making transgenic plant for higher seed Ca^{2+} accumulation but study about its regulatory sensor protein, especially CIPK (CBL-interaction protein kinase) is not well studied. CIPK(s) is a type of Ca sensor protein,

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playing an important role in regulation of ion transport, especially Ca exchanger protein. The structure of CIPKs is related to sucrose non-fermenting kinase (SNF1) from yeast and AMP-activated protein kinase (AMPK) from animals (Hrabak et al. 2003). The typical CIPK consists of the conserved N-terminal SNF1-type kinase domain (24 amino acid), which is fused, via a junction domain, to a highly variable C-terminal regulatory domain (Batistic and Kudla 2004). This domain is required for interaction with *CBL* proteins (Albrecht et al. 2001).

It is noteworthy that, during salinity stress, CIPK24 appears to target other ion transporters at the tonoplast including an H^+ pump and a Ca^{2+}/H^+ exchanger (Verslues et al. 2007). CIPK24 interacts with CBL10 to the vacuolar membrane (Kim et al. 2007). The CIPK24/CAX1 interaction is Ca^{2+} dependent and SOS2 must be recruited to the tonoplast to activate cation exchanger (CAX) (Cheng et al. 2004). Although the role of CIPK24 in CAX activation during stresses is well defined, but its role in Ca^{2+} accumulation by activating CAX is not studied so far. Therefore, it was speculated that there might be specific interactions between CIPK24 and CBL complexes with vacuolar calcium exchanger (CAX) in the seed that regulate action of CAX to pump calcium into vacuole of developing seed.

In the present investigation, attempts were made to characterize and define the role of CIPK24 gene homologue of finger millet to explore its role in high grain calcium accumulation. Transcript profiling of CIPK24 gene homologue was done in developing spikes for investigating its role in differential expression in finger millet genotypes differing in grain calcium content. Full-length structural annotation of *CIPK24* was done from the developing spike transcriptome data of two finger millet genotype. In silico characterization and interaction studies of *CIPK24* gene homologues isolated from finger millet genotypes with *CBLs* of finger millet were performed using bioinformatics tools.

Materials and methods

Isolation and molecular cloning of partial sequence of CIPK24 gene from finger millet

Primer designing

The homologs of CIPK24 gene of rice, sorghum and maize were retrieved from NCBI database (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignment of these sequences was done using MEGA5 to determine the conserved regions, and these conserved regions were subjected to Primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to obtained primers for expression analysis using qRT-PCR (Tamura et al. 2011).

Genomic DNA extraction and PCR amplification

In this investigation, CTAB method of DNA isolation described by Murray and Thompson (1980) was applied for obtaining good quality genomic DNA which was used as template DNA for subsequent PCR amplification. PCR amplification was performed using 50–100 ng of template DNA, 30 ng of primer, 0.1 mM dNTPs, 1.5 U Taq DNA polymerase (Bangalore Genei Pvt. Bangalore, India), 1X PCR buffer (10 Mm Tris pH 8.0, 50 mM KCL and 1.8 mM MgCl2) in volume of 25 µl. PCR amplified products of all the primers were subjected to gel electrophoresis and were documented using Alpha Imager 1200TM (Alpha Innotech Corporation, USA).

Cloning and sequencing of partial CIPK24 gene in pGEMT vector

The amplified product was analyzed on agarose gel and the expected size amplicon was gel; eluted using QIAquick Gel Extraction Kit (Qiagen, USA) and cloned in pGEM-Teasy vector (Promega, USA) as per the kit instructions. Putative cloned CIPK24 gene was sequenced using M13 universal Primer present in pGEM-Teasy vector. The confirmation of CIPK24 sequence was done through BLAST analysis.

Transcript profiling of *EcCIPK24* gene in vegetative and reproductive tissues

Plant materials

Seeds of finger millet genotypes were collected from Uttarakhand and obtained from Uttarakhand University of Horticulture and Forestry, Ranichauri, India. Two genotypes of finger millet GP1 (low) and GP45 (high) were selected in the present study due to the difference in their total grain calcium contents (Panwar et al. 2010).

Surface sterilized finger millet seeds were germinated on wet paper and were planted on commercial soil mix. Plants were grown at 37 °C in a glass house. The developing spikes of different stages (S1 to S4) were cut off, frozen in liquid nitrogen and kept at 80 °C until further use.

Preparation of RNA and first strand cDNA synthesis

Total RNA was isolated from different developmental stages of finger millet using total RNA isolation *iRIS* system from IHBT Palampur. Total RNA was treated with RNase free DNaseI according to manufacturer's instruction

(Fermentas, Germany). The first strand cDNA was synthesized with 2 μg of purified total RNA (pre-treated with DNase I) using the RT-PCR system (Promega, USA) according to the manufacturer's protocol.

Quantitative real-time PCR

Real-time polymerase chain reaction (RT-PCR) was used to quantitatively determine the expression profile of the *EcCIPK24* genes in vegetative and reproductive tissues of finger millet genotypes. Gene-specific primers were designed to amplify the specific cDNA fragment of gene. Tubulin (CX265249) was used as internal control. RT-PCR was performed in the reaction volume of 20 μl containing 2.5 × Real Master Mix SYBR ROX/20 × SYBR solution, 100 ng of cDNA, 100 nM of forward and reverse primers. The ratio of the target band intensity to the tubulin was used to investigate the relative expression level of the target gene.

Isolation of full-length *EcCIPK24* gene from finger millet transcriptome data

Partial nucleotide sequence of *EcCIPK24* obtained after sequencing was used as a query sequence to perform local BLAST search against our local assembled transcriptome database (TSA accession SRR1151079 and SRR1151080) constructed from transcript sequencing of spike tissues of low and high finger millet genotypes. The contigs sequences showed maximum similarity with *CIPK24* of finger millet were retrieved and assembled as well as its further characterizations were done through computational approaches.

SNP analysis of *EcCIPK24* of both genotypes was performed using clustal W alignment tool. Domain analysis of *EcCIPK24* gene homologue sequences of both genotypes were performed using SMART on line tool (http://www.smart.co.in) with default parameters. Motif analyses of sequences of both genotypes were done with default parameter using MEME (http://meme.nbcr.net) (Schultz et al. 1998; Bailey et al. 2009). ProtParam analysis was done using *EcCIPK24* gene homologue sequences of both genotypes. The protein secondary structure prediction, GOR algorithm based tool GORIV (http://abs.cit.nih.gov/gor/) was used to determined the amino acid residues involved in the formation of helix, sheet and turn (Sen et al. 2005). Secondary structures of proteins were predicted using *EcCIPK24* gene homologue sequences of both genotypes with default parameter. Prediction of protein sub-cellular localization of *EcCIPK24* sequences of GP1 and GP45 was done using CELLO V. 2.5 sub-cellular localization predictor tool (http://cello.life.nctu.edu.tw/).

Protein structure prediction, evaluation and validation

Protein sequence of EcCBL4, EcCBL10 and EcCIPK24 (GP1 and GP45) was subjected to BLASTp against PDB database (http://www.rcsb.org/pdb/home/home.do) for identification of suitable template as well as methods for modeling of 3D model (Altschul et al. 1990). In addition to BLASTp search, SWISS-MODEL was employed to model 3D structure of target protein sequences (Arnold et al. 2006). The structural refinements through energy minimization of each predicted models were performed by SPDB viewer (http://spdbv.vital-it.ch/refs.html). RAMPAGE server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) was used to analyze the stereochemical quality of structure coordinates of the predicted protein models through Ramachandran plot analysis. The overall quality analysis of each models was done by ProSA (Protein Structure Analysis) and ProQ (Protein Quality Predictor) (Wiederstein and Sippl 2007; Wallner and Elofsson 2003), and DS Visualizer was used for the visualization of 3D models (Fig. 4) (Pathak et al. 2016).

Protein–protein docking

The refined predicted structures of EcCIPK24 (GP1 and GP45) were taken as receptor and the structures of EcCBL4 and EcCBL10 were considered as ligands for the protein–protein docking studies. EcCBL4 and EcCBL10 were docked with EcCIPK24 protein of the both high and low calcium genotype using ClusPro (https://cluspro.bu.edu/home.php) server at default parameter. The best docked conformation was taken to analyze the interactions, which are responsible for regulation of the biological processes on the basis of their binding free energy. The docked complex files were visualized and analyzed by PyMol (Fig. 5) (DeLano 2002; Comeau et al. 2004; Pathak et al. 2013).

Results and discussion

Finger millet, an under-utilized cereal crop, contains exceptionally high amounts of calcium in grains. Since calcium is an important macronutrient in human diet, it requires immediate attention to understand the molecular basis of high calcium accumulation in seed of finger millet. It has been reported in our lab that the seeds of high calcium containing finger millet genotype are having higher level expression of *EcCAX1* gene while compared with low calcium containing genotype of finger millet (Mirza et al. 2014; Singh et al. 2015). Further, it has also been reported that *CIPK24* gene regulate vacuolar CAX1 transporter that

leads to pull cytosolic calcium in vacuole. CIPK24 activates transport protein after interaction with CBL proteins, viz. CBL4, CBL10, etc. (Chinnusamy et al. 2005; Kim et al. 2007; Quan et al. 2007). This gives an indication regarding the role of CIPK24 not only in signaling but also in regulation of calcium transport efficiency across the membranes in seed. The present section deals with isolation and characterization of *EcCIPK24* gene in grain Ca^{2+} accumulation.

Isolation and molecular cloning

A prominent single band with expected size 0.25 kb was consistently observed in genomic DNA of finger millet after PCR amplification with set of primer. It showed conserved sequences in pairwise alignment using Clustal W online tool and closely related in phylogenetic tree using neighbor joining (NJ) method for all cereals CIPK24 gene homologue. The PCR amplicon of 0.25 kb was eluted from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, USA), Subsequently, cloned in p^{GEM} T-Easy vector (Promega, USA) as per the kit instructions. Putative cloned *EcCIPK24* gene was sequenced at DNA sequencing facility, University of Delhi, South Campus. The sequence was confirmed by homology alignment with *CIPK24* of other cereals and used for fetching the contigs from transcriptome data of low and high calcium containing genotype of finger millet.

Expression studies of *EcCIPK24* gene in vegetative and reproductive tissues

The expression pattern of *EcCIPK24* gene homologues was investigated in two finger millet genotypes (GP1 and GP45). The abundance of specific mRNA was investigated in root, stem and third leaf at three different vegetative stages, viz. 30, 60, 90 DAS (day after sowing). The expression pattern of *EcCIPK24* gene was also studied in flag leaf and developing spike at four defined reproductive stages, viz., S1, S2, S3 and S4. The relative expression of gene within genotypes and among genotypes was investigated.

The increased expression of *EcCIPK24* gene was observed in root, stem and leaf tissues of GP45 genotype compared to GP1 genotype. It was noticed that the expression of *EcCIPK24* gene was more in GP45 at 60 DAS (root, leaf tissue) and 90 DAS (in root stem and leaf tissue) but less in GP1 at 60 DAS in stem.

The expression of *EcCIPK24* gene in GP45 genotype increased continuously in flag leaf tissue and developing spike tissues from S1 to S4 stages (Fig. 1). However, in case of spike tissues of GP1 genotype the expression increased 2.7-fold at S2 stage, and decreased at S3 then it

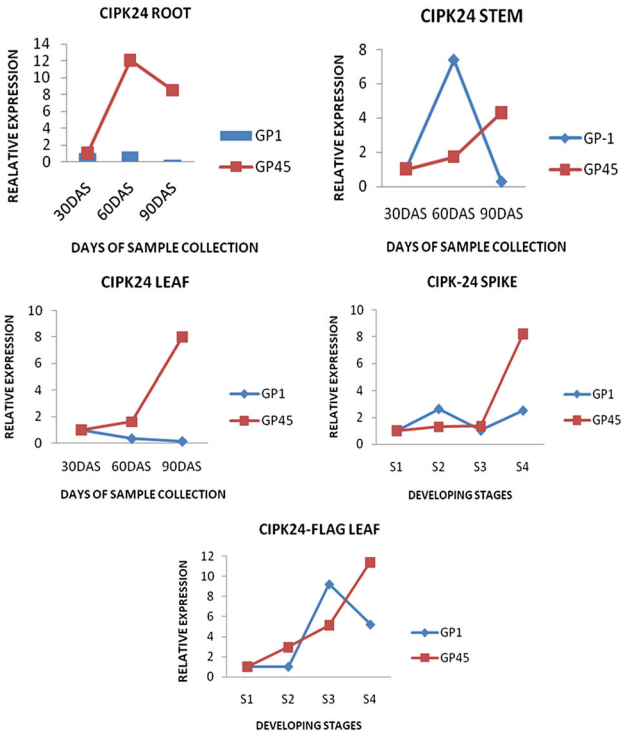

Fig. 1 Relative expression of *EcCIPK24* transcripts in root, stem, leaf tissue at 30, 60 and 90 DAS and in developing stages of Spike, flag leaf among genotypes GP1 (Low calcium genotype) and GP45 (High calcium genotype). *DAS* days after sowing

increased 2.5-fold at S4 stage. In flag leaves expression continuously increased from S1 to S4 stage in GP45, while in GP-1 it drops at S4 stage. The expression of *EcCIPK24* gene is in same increasing pattern in GP45, while decreased in spike and flag leaves tissues of GP1 genotype.

Previous studies conducted in our lab suggested that the expression of *EcCAX1* is higher in developing spikes of GP45 genotype, as compared to GP1. The similar expression patterns of *EcCIPK24* gene were recorded in both vegetative as well as reproductive tissues of GP45 and GP1 genotypes, which indicate the co-regulation of both the genes (Mirza et al. 2014). The result is in agreement with study that CIPK24 after interaction with CBL10 regulate the activity of CAX protein (Cheng et al. 2004; Verslues et al. 2007; Kim et al. 2007).

In silico analysis of *EcCIPK*24 gene homologue with other cereal CIPK24 gene homologue

The contig sequence of *EcCIPK24* and interactive sequences (*EcCBL4*, *EcCBL10*) was retrieved by performing local BLAST against transcriptome local database of finger millet and reassembled by SeqMan Pro gene analysis package (DNASTAR Inc., Madison, WI, USA).

Finger millet *CIPK24* homologue showed more than 90% similarity with available cereal *CIPK* gene

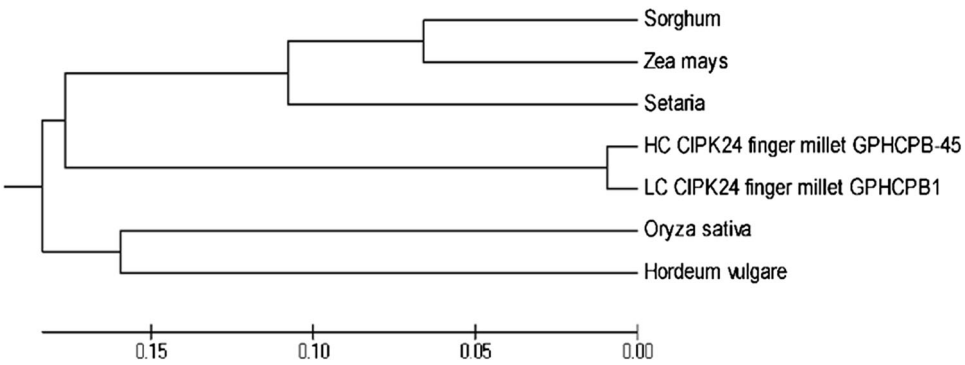

Fig. 2 Phylogenetic tree of CIPK24 genes of cereals constructed using neighbor joining method showing relationship with *EcCIPK* (GP1 and GP45)

homologues, among them with *Oryza sativa* CIPK24 gene homologue gave highest similarity. The result showed that retrieved *CIPK24* gene homologue of finger millet has more conserved sequence among cereals. The result of phylogenetic analysis showed that *CIPK24* gene homologue of both finger millet genotypes was situated in two sub–sub-cluster of a distinct sub-cluster (Fig. 2).

Comparative analysis of SNPs, amino acid composition, and other physico-chemical properties of *EcCIPK24* homologues

Nine SNPs were detected in *EcCIPK24* gene sequence of both genotypes using clustal W (Fig. 3). Domain analysis showed that both sequences have S TKc kinase domain with *E* value 1.23e-105, which is more conserved at C-terminal and variable at N-terminal. The domain starts from the amino acid sequences from 14 and End at 267. Motif analyses of sequences of both genotypes showed the presence of three motifs using MEME (Bailey et al. 2009).

Results of ProtParam analysis are summarized in Tables 1 and 2.

Molecular weight 50860.3 Dalton of EcCIPK24 sequence (GP45) was found to be higher than EcCIPK24 sequence (GP1) 50846.3 Dalton. Isoelectric point and number of amino acids in two sequences are similar with values 7.64 and 451, respectively. Though the number of amino acids is same but change in amino acid composition is observed in both sequences of EcCIPK24. Secondary structure of EcCIPK24 (GP1) showed one extra sheet (161) than that of EcCIPK24 (GP45) (160) using GORIV. The results showed cytoplasmic localization of CIPK24 sequences of both genotypes having 2.456 and 2.357 as an index, respectively, which was predicted by CELLO V. 2.5 sub-cellular localization prediction tool (Yu et al. 2006). Interestingly, it was found that vacuolar localization of EcCIPK24 (GP45) was higher than vacuolar localization of EcCIPK24 (GP1), i.e., 0.044 and 0.043, respectively. Results of ProtParam analysis are summarized in Table 2.

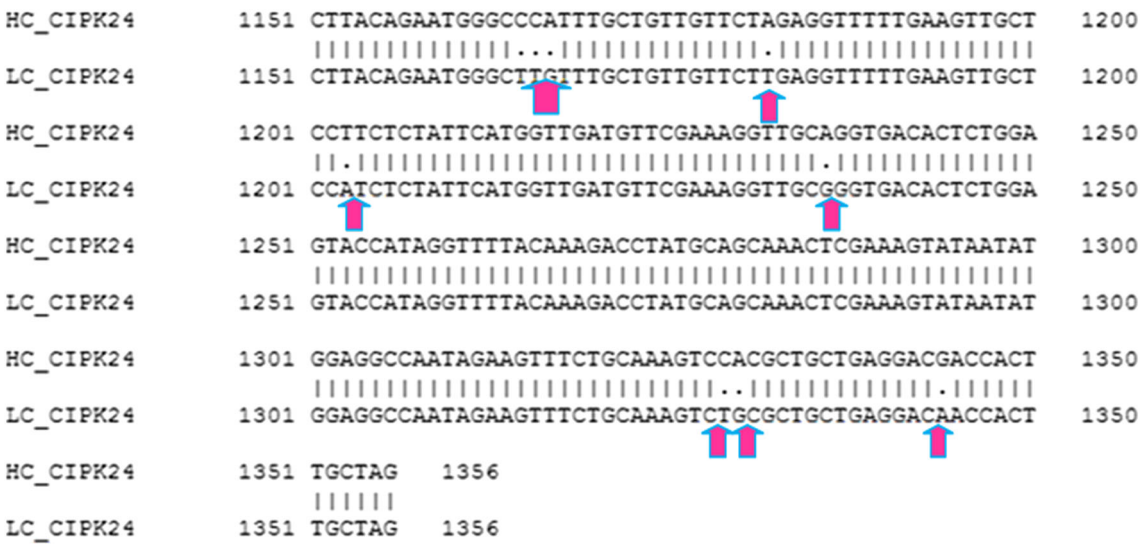

Fig. 3 SNP analysis of *EcCIPK24* gene in GP1 and GP45 genotypes

Table 1 Comparison of physico-chemical properties of *EcCIPK24* sequences isolated from two finger millet genotypes differing grain calcium contents

S. No.	Physico-chemical properties	High calcium genotype (GP45)	Low calcium genotype (GP1)
1.	Molecular weight	50860.3	50846.3
2.	Iso electric point	7.64	7.64
3.	Number of amino acid	451	451
4.	Sub-cellular localization	2.456 (cytoplasmic)	2.357 (cytoplasmic)
		0.044 (vacuolar)	0.043 (vacuolar)
5.	Total number of atoms	7189	7190
6.	Instability index	33.20	32.78
7.	Aliphatic index	92.31	93.39
8.	Grand average of hydropath city (GRAVY)	−0.230	−0.213
9.	Helix	296	296
10.	Sheet	160	161
11.	Turns	47	47

Table 2 Comparison of amino acid composition of *EcCIPK*24 sequences isolated from two finger millet genotypes differing grain calcium contents

S. No.	Amino acid composition	CIPK24 of GP45 (%)	CIPK24 of GP1 (%)
1.	Ala (A)	7.1	7.3
2.	Arg (R)	6.9	6.9
3.	Asn (N)	3.3	3.3
4.	Asp (D)	6.0	6.0
5.	Cys (C)	1.1	1.1
6.	Gln (Q)	2.7	2.7
7.	Glu (E)	7.1	7.1
8.	Gly (G)	6.9	6.9
9.	His (H)	2.0	2.0
10.	Ile (I)	7.1	7.1
11.	Leu (L)	9.3	9.5
12.	Lys (K)	6.4	6.4
13.	Met (M)	2.4	2.4
14.	Phe (F)	4.0	4.0
15.	Pro (P)	3.3	3.1
16.	Ser (S)	6.9	6.9
17.	Thr (T)	5.3	5.1
18.	Trp (W)	0.9	0.9
19.	Tyr (Y)	4.0	4.0
20.	Val (V)	7.3	7.3
21.	Pyl (O)	0.0	0.0
22.	Sec (U)	0.0	0.0

3D structure prediction and validation

The homology modeling approach was employed to determine a reasonable 3D structure of these proteins based on the known structure available in Protein Data Bank. The 3D structure modeling of EcCPIK24 (GP45), EcCPIK24 (GP1), EcCBL4 and EcCBL10 was done using SWISS-MODEL (http://swissmodel.expasy.org) (Biasini et al. 2014) showed in Fig. 4. The modeled protein structures were subjected to SPDB Viewer for stabilizing their stereochemical properties through energy minimization. It is a computational technique employed to eliminate the unwanted contacts of the macromolecules for the purpose of structure refinements (Vyas et al. 2012). The refined structures were further subjected to RAMPAGE, ProSA (Protein Structure Analysis), and ProQ (Protein Quality Predictor) to validate its overall quality. It was suggested that the predicted model quality to be acceptable and can be utilized for molecular docking studies. The structural template used for modeling of target sequences and physico-chemical properties of modeled protein structures was shown in Table 3 (Wallner and Elofsson 2003; Wiederstein and Sippl 2007).

Molecular interaction prediction of target proteins through molecular docking

ClusPro (https://cluspro.bu.edu/home.php) server was used for the molecular docking studies between EcCIPK and EcCBL proteins to investigate its role in calcium accumulation. It is a fully automated, web-based program for the docking of protein structures. It required the 3D co-ordinate files of receptor and ligand protein structure to predict its binding affinity.

EcCBL4 was docked with EcCIPK24 (both GP45 and GP1) with binding energies −860.4 and −915.2 kcal/mol, respectively (Table 4); the probable range of interacting amino acid residues through non-covalent bonding is found at 17–24 and 101–107 of the EcCIPK24 (GP45) with

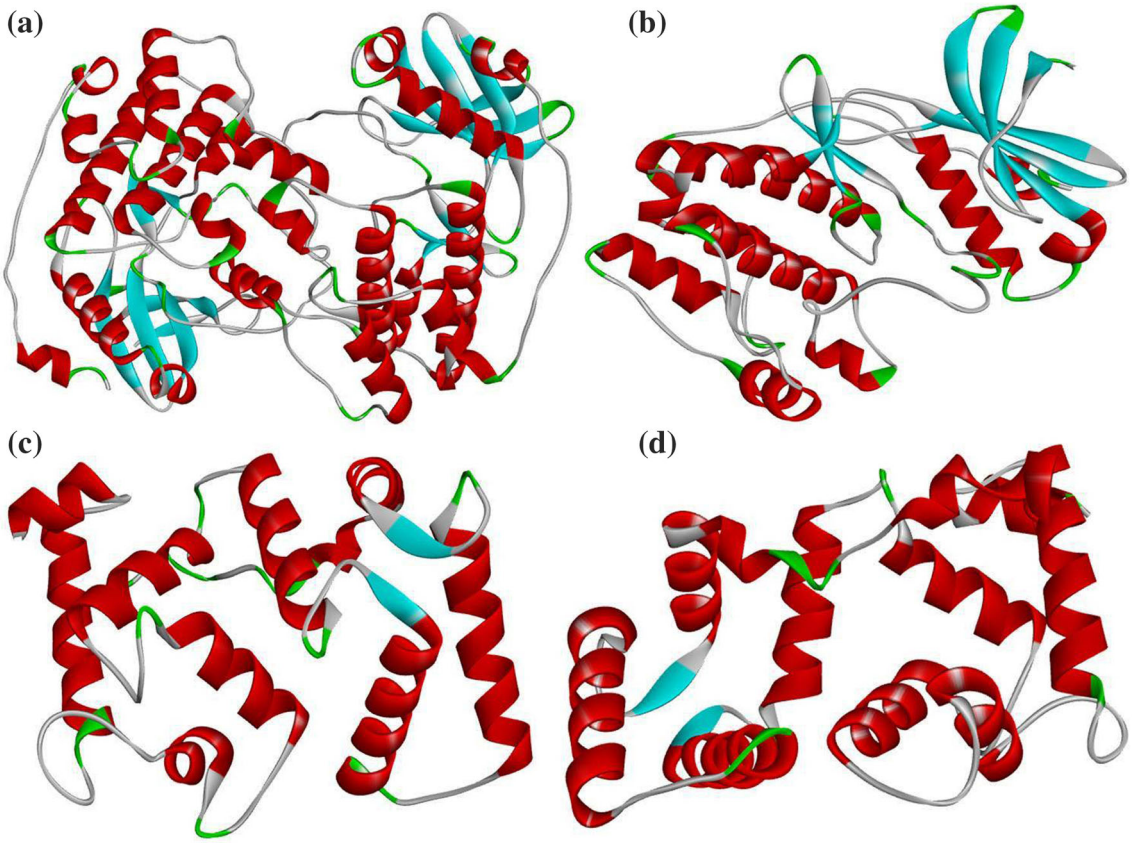

Fig. 4 Modeled 3D structures of finger millet proteins involved in calcium accumulation **a** EcCIPK24 (GP45), **b** EcCIPK24 (GP1), **c** EcCBL4 and **d** EcCBL10

Table 3 Comparative analysis of protein models of *EcCIPK24* gene homologues

S. No.	EcCIPK24 and its interacting proteins of both genotypes	Template	Physico-chemical properties						
			Number of group	Number of atoms	Number of bonds	QMEAN4	Sequence identity in percent	C_beta interaction energy	Torsion angle energy
1	EcCIPK24 (GP45)	4dz8.A	562	4485	4575	−3.90	70.14	−2.14	−2.64
2	EcCIPK24 (GP1)	4dz8.A	282	2248	2293	−2.95	70.14	−0.95	−2.78
3.	EcCBL10 (GP45)	2zfd.A	184	1506	1532	−1.08	54.75	0.28	−0.48
5.	EcCBL4 (GP45)	2ehb.A	181	1471	1496	−0.80	68.32	−0.24	−0.33

Table 4 Protein–protein docking studies: illustrates minimum free binding energy

S. No.	Protein–protein interactions		Docking energy (KCal/mol)	
	Receptor	Ligand	High calcium genotype GP45	Low calcium genotype GP1
1.	EcCIPK24	EcCBL4	−860.4	−915.2
2.	EcCIPK24	EcCBL10	−827.3	−909.8

EcCBL4 at 40–50, 61–66 and 194–197. Moreover, 237–247 and 288–292 of EcCIPK24 (GP1) with 137–139 and 189–197 of CBL4 are found to be interacting. EcCBL10 was docked with EcCIPK24 (GP45) and (GP1) with energy values −827.3 and −909.8 kcal/mol, respectively (Table 4), the probable interacting amino acid

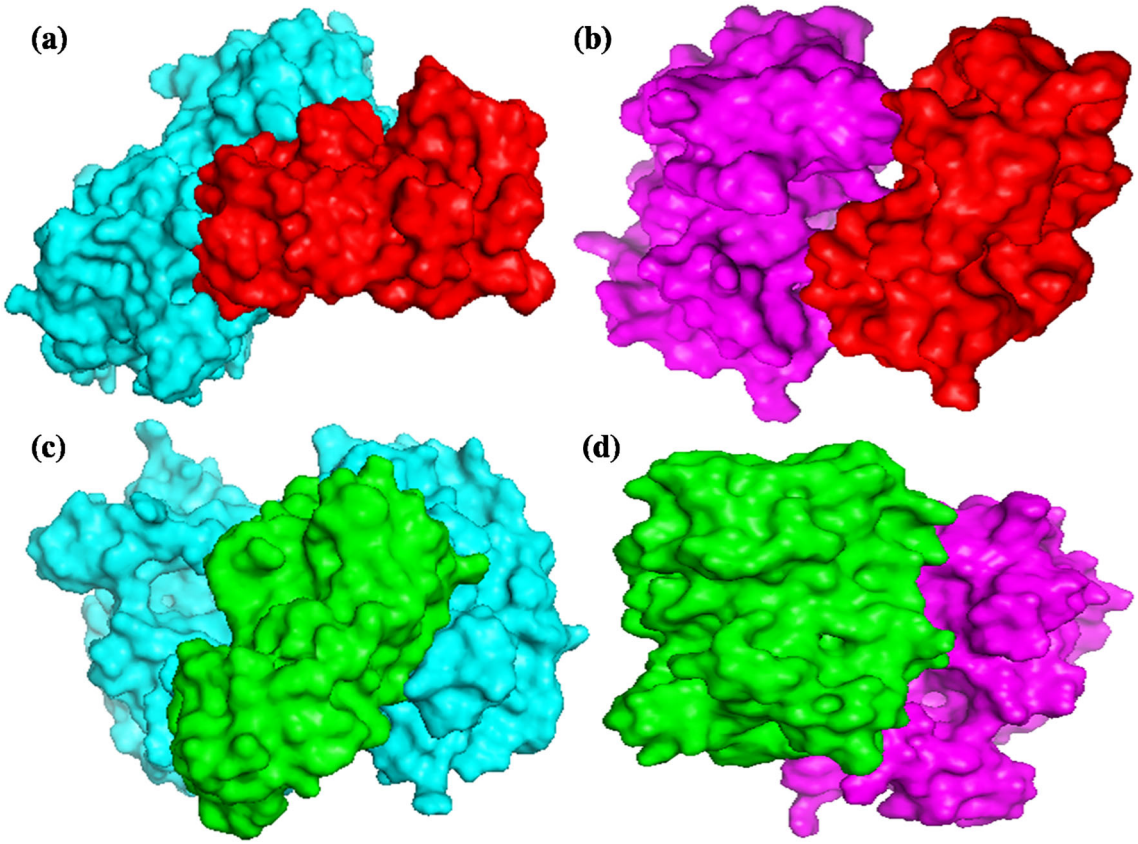

Fig. 5 Protein–protein docking; **a** EcCIPK24 (GP45)$_{(cyan)}$–EcCBL10$_{(red)}$, **b** EcCIPK24(GP1)$_{(magenta)}$–EcCBL10$_{(red)}$, **c** EcCIPK24(GP45)$_{(cyan)}$–EcCBL4$_{(green)}$, **d** EcCIPK24(GP1)$_{(magenta)}$–EcCBL4$_{(green)}$

residues predicted between these proteins at 48–54, 86–88, 105–108 and 167–171 of EcCIPK24 (GP45) with 52–59, 82–90 and 123–126 of EcCBL10. Moreover, 241–247 and 287–290 of EcCIPK24 (GP1) with 175–180 and 226–232 interacted through non-covalent bonding. On the basis of docking energy we have investigated the binding mode of EcCBL proteins, and was predicted that the EcCBL4 has greater affinity with EcCIPK24 proteins compared to CBL10 (Fig. 5).

Based on such protein–protein docking studies, we have found that EcCBL4 might interact with EcCIPK24 at vacuolar membrane and form an EcCIPK24–EcCBL4 complex that might interact and regulate EcCAX1 at vacuolar membrane to pull calcium in vacuole. Probably, this might also be playing a significant role in high accumulation of calcium in finger millet seed.

Conclusions

In the present study, an attempt has been made to isolate partial *EcCIPK24* ORF from finger millet, and the eluted product was cloned in pGEM T-Easy vector and sequenced. In silico analysis of full-length *EcCIPK24* gene homologues identified from transcriptome of both genotypes showed differences of nine SNPs, one extra beta sheet domain, and vacuolar localization and amino acid composition in their sequences. Docking study suggested that EcCBL4 has stronger binding affinity with EcCIPK24 and might play a significant role in the accumulation of calcium in seeds. Based on such studies, elucidation of a probable pathway for exploring differential accumulation of calcium in finger millet is proposed, which suggest that EcCBL4 gets activated from Ca signature and interact with EcCIPK24 at vacuolar membrane. The EcCIPK24–EcCBL4 complex interacted and regulated EcCAX1 at vacuolar membrane to pull calcium in vacuole. The differences in the structures of EcCIPK24, variation in their interaction with interacting proteins, *EcCBL4* and *EcCAX1* and differential expression of *EcCIPK24*, in two genotypes of finger millet genotypes may be a plausible reason for interpreting it for differential accumulation of Ca in finger millet genotypes. Higher expression of *EcCIPK24* in high calcium containing genotype and lower affinity of *EcCIPK24* with *EcCBL4* and *EcCBL10* might regulate *Ec*CaX1B at vacuolar membrane to pull calcium in vacuoles in finger millet seeds. Probably, this interacting pathway may also contribute for differential accumulation

of calcium in finger millet seed. It can be further validated by establishing the relationship of membrane calcium transport efficiency with *EcCIPK–EcCBL* interaction as proposed in the present study using comparative genomics and interactomics studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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