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RESEARCH ARTICLE

Genomic characterization of ZIP genes in pigeonpea (*CcZIP*) and their expression analysis among the genotypes with contrasting host response to pod borer

Atul Nag[1,2] · Kapil Gupta[3,5] · Neeraj Dubey[3] · Sujit K. Mishra[2,4] · Jogeswar Panigrahi[1,2,3]

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Abstract Zinc (Zn) is a vital micronutrient from the perspective of biofortification and biotic stress endurance in pigeonpea. The ZIP transporters with domain (Pfam: PF02535) regulate uptake and transport of metal ions, including Zn, in consonance with plant metal homeostasis. Genome-wide analysis in pigeonpea identified 19 non-redundant members of ZIP family (*CcZIP*) that were analyzed for gene structure, conserved motifs and homology besides other structural and biochemical parameters. Intra-specific as well as the inter-specific phylogenetic relationships of these 19 *CcZIP*s were elucidated by comparison with ZIP proteins of *Arabidopsis thaliana, Medicago truncatula, Phaseolus vulgaris* and *Glycine max*. In addition to gene structure, the *cis*-regulatory elements (CREs) in the promoter region were also identified. It revealed several stress responsive CREs that might be regulatory for differential expression of *CcZIP* proteins. Expression analysis showed that both *CcZIP3* and *CcZIP15,* having zinc deficiency responsive element, up-regulated in the reproductive leaf tissues and down-regulated in matured green pods of the pod borer resistant genotypes with higher zinc content. Alternately, the expression of *CcZIP6* and *CcZIP13* was higher in matured green pods than reproductive leaves of the resistant genotypes. These findings on differential expression indicate the possible role of these *CcZIP*s on the mobilization of Zn from leaves to pods, phloem loading and unloading, and higher accumulation of seed zinc in pod borer resistant genotypes used in this study. Further functional characterization of *CcZIP* genes could shed light on their role in bio-fortification and genetic improvement to inhibit the pod borer herbivory in pigeonpea.

Keywords Bio-fortification · Pod borer herbivory · Differential expression · ZIP genes · *Cajanus cajan*

Abbreviations

ABRE	ABA responsive elements
ARE	Auxin responsive elements
CDF	Cation diffusion facilitator
CRE	*Cis* Regulatory elements
ERE	Ethylene responsive elements
GRAVY	Grand average of hydropathicity
HMA	Heavy metal ATPase
HMM	Hidden Markov model
LRE	Light regulatory elements
LTRE	Low-temperature responsive elements
MRE	Metal regulatory element
MUSCLE	Multiple sequence comparison by log-expectation
PCR	Plant cadmium resistance
pI	Isoelectric point
PLH	Pore line helices

✉ Jogeswar Panigrahi
jpanigrahi@kub.ac.in; drjpanigrahi@gmail.com

[1] Department of Biosciences and Bioinformatics, Berhampur University, Bhanja Bihar, Berhampur, Odisha 760007, India

[2] Department of Biotechnology and Bioinformatics, Sambalpur University, Jyoti vihar, Odisha 768019, India

[3] Department of Biotechnology, School of Life Sciences, Central University of Rajasthan, NH-8, Bandarsindri, Ajmer, Rajasthan 305817, India

[4] Department of Zoology, Centurion University of Technology and Management, R. Sitapur, Odisha, India

[5] Department of Biotechnology, Sidhharth University, Kapilvastu, Siddharth Nagar, UP 272202, India

qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species
TMH	Trans-membrane helices
VIT	Vacuolar iron transporter
ZDRE	Zinc deficiency-related elements
ZIP	Zinc-regulated transporter (ZRT) and iron-regulated transporter (IRT) like protein

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.; Family: Fabaceae] is a grain legume crop, and this crop has been growing predominantly in India with an annual production of 3.3 million tons which is more than 70% of global production (FAOSTAT 2017). The yield of pigeonpea is compromised because of the losses incurred due to several biotic and abiotic stresses, and among the biotic stresses pod borer complex (a group of pod borers) causes huge loss in the net production (Grover and Pental 2003; Sharma et al. 2010). An earlier study has reported that pigeonpea cultivars with high zinc and iron content could confer tolerance to pod borer infestation and was also a deterrent to pod borer herbivory (Kaur et al. 2014). The dehulled split grain of dried matured seeds and tender green pods are consumed along with cereal foods as a source of dietary proteins in many parts of the world, including India. Nearly 48% of the human population, those dependent upon vegetarian diets, suffer from zinc deficiency. Most of them showed diverse symptoms, including low immunity, retarded brain development, abnormal child growth, and delayed cognitive development (Hambidge 2000; Clemens 2014). Thus, bio-fortification of zinc in the edible parts, including seeds of pigeonpea is quite important not only to combat the malnourishment of zinc (Zn) micronutrient but also to increase the plant immunity against biotic stresses.

Plant growth and development requires an optimal amount of Zn supply from the soil to drive several metabolic processes, such as photosynthesis, cellular respiration, growth and differentiation etc., as this micronutrient serves both structural and catalytic roles either by acting as a structural component of several proteins and enzymes or catalyzing cellular functions by binding as cations and transporting across the membranes (Sinclair and Krämer 2012). Zn is the important structural component of zinc finger proteins, and these transcription factors were upregulated in response to both abiotic and biotic stresses in several plant systems, including *Arabidopsis thaliana*, wheat and potato, and in turn, their expressions make them stress-tolerant (Schweizer et al. 2013; Lawrence et al. 2014; Zang et al. 2016; Wang et al. 2017). Moreover, Zn as a cofactor also acts as a protective agent against the oxidation of several cell components, including membrane proteins, metalloproteins and lipids, and has defensive properties against several biotic and abiotic stresses in different crop plants (Cabot et al. 2019). One such metalloprotein and the anti-oxidative enzyme 'superoxide dismutase' contain Zn as a cofactor. It catalyzes the dismutation of superoxide radicals to molecular oxygen and H_2O_2 to induce the plant primary immune response which further regulates several intra- and inter-cellular signaling pathways required for plant defense system under zinc stress conditions (Cabot et al. 2019). Even after insect and pathogen attack, the plants with high and low zinc content respond differently by altering alcohol dehydrogenase and carbonic anhydrase activities, and overexpression of metallothionein and proteins for reactive oxygen species (ROS) detoxification (Wongpia and Lomthaisong 2010; Shivashankar et al. 2015; Zang et al. 2016; Ćalić et al. 2017). Zinc content in edible plant parts also inhibits gut amylase of the pod borer (*Helicoverpa armigera*) in pigenopea. The content of Zn was also reported to be higher in the seeds and pods of moderately resistant cultivars than those of susceptible cultivars in pigeonpea (Kaur et al. 2014).

The plants obtain Zn from the soil as divalent cations and transport to the seeds through a cascade of intercellular symplast to symplast movement via various apoplastic spaces, and it involves solubilization of Zn in the soil, Zn uptake into roots, xylem loading in roots and unloading in leaves, phloem loading and phloem unloading in the developing seeds (Olsen and Palmgren 2014; Lira-Morales et al. 2019). Zinc homeostasis in both strategy-I and -II model plants, *A. thaliana* and *Oryza sativa*, had been already documented (Ishimaru et al. 2011; Bashir et al. 2012; Sinclair and Krämer 2012; Olsen and Palmgren 2014; Ricachenevsky et al. 2015; Kawakami and Bhullar 2018; Pita-Barbosa et al. 2019; Lira-Morales et al. 2019). Although the molecular mechanisms and genes involved during solubilization and uptake of zinc, xylem loading in roots and phloem loading for transport and distribution into plant organs and subcellular compartments have been unfolded in model plants, the genes responsive to post phloem loading and unloading of zinc to developing seeds in dicots have not been elucidated till date (Ricachenevsky et al. 2015; Kawakami and Bhullar 2018; Pita-Barbosa et al. 2019). As with other metal ions, the acquisition and cellular movement of zinc require various chelators and membrane-bound transporters to import and export across the biological membranes, including tonoplast and chloroplast (Bashir et al. 2016; Vigani and Hanikenne 2018). Several such membrane proteins capable of uptake and transport of metal ions in consonance with plant metal homeostasis have been characterized in *A. thaliana* and *O. sativa* (Ricachenevsky et al. 2015; Kawakami and Bhullar

2018; Lira-Morales et al. 2019), which include members of the zinc-regulated transporter (ZRT) and iron-regulated transporter (IRT) like protein (ZIP), cation diffusion facilitator (CDF), plant cadmium resistance (PCR), vacuolar iron transporter (VIT), and heavy metal ATPase (HMA) gene families. Lira-Morales et al. (2019) also proposed a ZIP protein regulation pathway in *A. thaliana* under different conditions with emphasis on the basic region of leucine zipper (bZIP) transcription factors, the response of bZIP to Zn availability and occurrence of zinc-deficiency-related *cis*-elements (ZDRE).

Among all the members, ZIP gene family are the first heavy metal transporters reported in plants, and their expression is mainly regulated by the Zn content in the plant parts and soil (Grotz et al. 1998; Ricachenevsky et al. 2015). The plant ZIP proteins have 6–9 transmembrane domains, with eight being the predominant ones, histidine-rich variable loop between III and IV transmembrane domains, and their carboxyand amino terminal ends are mainly at the outer surface of the plasma membrane (Guerinot 2000). The genes with ZIP domain have been identified and characterized from several plants, including *A. thaliana*, *O. sativa*, *Medicago truncatula*, *Phaseolus vulgaris*, *Zea mays*, *Setaria italica* and *Poncirus trifoliata* (Grotz et al. 1998; Chen et al. 2008; Stephens et al. 2011; Astudillo et al. 2013; Li et al. 2013; Jain et al. 2013; Mondal et al. 2014; Alagarasan et al. 2017; Fu et al. 2017). The possible role of these ZIP proteins have been elucidated in uptake and transport of Zn cations to the cytoplasm and their translocation to different plant parts (Sinclair and Krämer 2012; Kawakami and Bhullar 2018). Such functions of ZIP genes are also affirmed through gene expression analysis and yeast complementation studies (Krishna et al. 2020). Even the analysis of ZIP gene family in different species also demonstrated their importance in Zn uptake, transport and accumulation under zinc-deficient conditions (Assunção et al. 2010; Stephens et al. 2011; Jain et al. 2013; Astudillo-Reyes et al. 2015; Lilay et al. 2020). One member of the ZIP family (*PvZIP12*) was overexpressed in the *P. vulgaris* genotypes with higher zinc content (Astudillo-Reyes et al. 2015). In *M. truncatula*, three genes (*MtZIP1, MtZIP5* and *MtZIP7*) regulating the zinc transport and homeostasis have also been identified (Stephens et al. 2011). Jain et al. (2013) also hypothesized the transcriptional regulation of *AtZIP4, AtZIP9* and *AtZIP12* by Zn in soil and their role in zinc homeostasis in *A. thaliana*.

As reported earlier, the decoded genome sequence is quite essential for functional genomics research including in silico characterization of various gene families in different plant systems. The draft genome sequence of the *C. cajan* also provides a valuable resource for in silico analysis of the gene families in this species. Although several gene families have already been characterized in *C. cajan* (Malviya et al. 2015; Singh et al. 2019), the genome-wide analysis of the ZIP gene family is not yet reported in pigeonpea. Therefore, we describe here the in silico identification of non-redundant members of genes containing ZIP domain; characterization of their biochemical properties, genomic organization and motif analysis; elucidation of intra- and inter-specific phylogenetic relationship; and expression analysis of ZIP genes in the genotypes with contrasting host response to pod borer and seed zinc content.

Materials and methods

In silico identification and characterization of CcZIP genes

The complete sequence assembly, coding sequence and protein sequence of pigeonpea were downloaded from the public database (http://gigadb.org/dataset/100028), and a total of 48,680 predicted genes in the *C. cajan* Gene Model *V5.0* along with their location on the genome (chromosomes and scaffolds) were loaded to a local MySQL database for easy retrieval during the present study. The Hidden Markov Model (HMM) profile of the ZIP domain (PF02535) from the Pfam database was used as a query for the identification of *ZIP* proteins in *C. cajan* and their corresponding genes (*CcZIP*) were also obtained from Gene Model *V5.0* using the HMMER 3.3 programme with E-value $\leq 10^{-3}$ (El-Gebali et al. 2018). Further, the identified *CcZIP* proteins were also searched against the conserved domain database (CDD) and ensured that each *CcZIP* must have the ZIP domain PF02535 as described earlier (Marchler-Bauer et al. 2015). Various biochemical parameters, such as length of the protein sequence, molecular weight, isoelectric point (*pI*) and grand average of hydropathicity (GRAVY) values of these *CcZIP* proteins were determined using protein analysis module in BioPython (Chapman and Chang 2000). All 19 predicted *CcZIP* proteins were aligned using the multiple sequence comparison by log-expectation (MUSCLE) programme in MEGA-X software to exclude overlapping *CcZIP* genes, if any (Edgar 2004; Kumar et al. 2018). Multiple sequence alignment was further carried out using MCOFFE to explore the relationship between the *CcZIP* paralogs (Moretti et al. 2007), and subsequently, TMCOFFE was used to align the transmembrane domains with discovering the regions present in the helix, outside and inside of the plasma-membrane (Floden et al. 2016). The topology and distribution of transmembrane helices (TMHs), pore-lining helices (PLHs) and signal peptides were determined by using MEMPACK of PSIPRED protein analysis workbench (Nugent et al. 2011), and the findings were also

affirmed by reanalyzing the occurrence of TMHs and signal peptides using TMHMM and SignalP 4.1 server, respectively (Krogh et al. 2001; Petersen et al. 2011). The post-translational modification signature sequences of the *CcZIP* proteins were also determined using ScanProsite (de Castro et al. 2006). Subcellular localization of each *CcZIP* gene were also predicted using ProtComp v9.0 programme. The nomenclature of *CcZIP* genes (*CcZIP1* to *CcZIP19*) was given as per their occurrence on pigeonpea chromosome (CcLG01 to CcLG11) followed by sequence scaffolds (Scaffold-000020 to Scaffold-137616) in ascending order following the genome information (Varshney et al. 2012).

Determination of CcZIP gene structure

The predicted coding sequences (CDS) of *CcZIP* genes were submitted to the gene structure display server, GSDS 2.0, along with the phylogenetic tree of the *CcZIP* paralogs to determine the genomic organization of exon and intron (Hu et al. 2014). Further, the promoter sequences from −1000 to +1 bp of each of the *CcZIP* genes were extracted from the pigeonpea sequence assembly, and were analyzed for *cis*-regulatory elements (CREs) and functional motifs in the promoter using the Softberry Nsite-PL programme along with RegSite PL database of plant regulatory elements (Shahmuradov and Solovyev 2015). The position of significant stress-responsive CREs were graphically represented using GSDS 2.0.

Analysis of conserved motifs in CcZIP proteins

The conserved motifs present in 19 *CcZIP* proteins were identified using the MEME 4.11.2 Suite server considering the parameters, viz. occurrence of motifs with any number of repetitions, the length of motifs should be between 6 and 60 amino acids, only motifs with E-value $\leq 10^{-20}$ and a maximum of 10 motifs per sequence (Bailey et al. 2009). The occurrence of individual motifs in *CcZIP* sequences was also obtained using MAST module of MEME 4.11.2 Suite. The functional annotations of these motifs were carried out using the InterProScan (Quevillon et al. 2005), and the sequence logos of conserved motifs across 19 *CcZIPs* were also generated using WebLogo (Crooks et al. 2004).

Phylogenetic analysis of CcZIP proteins

Non-redundant ZIP proteins with domain PF02535 of three legumes viz. *M. truncatula, G. max* and *P. vulgaris* were retrieved from the Phytozyme database (www.phytozome.net; Supplementary Table 1). Subsequently, the conserved sequences of these retrieved proteins along with the *A. thaliana* ZIP proteins and 19 *CcZIPs* were aligned using the MUSCLE programme in MEGA X software with default parameters (Kumar et al. 2018). Based on their conserved sequence alignment, the rooted phylogenetic tree was also constructed following maximum likelihood method using MEGA-X software (Kumar et al. 2018). The interspecific evolutionary relationships of 19 *CcZIP*s with the ZIP family of *A. thaliana* and three legumes mentioned above were also delineated. In contrast, the intra-specific phylogenetic relationships among 19 *CcZIP* genes were established on the basis of the ZIP domain only, keeping the rest of the parameters unchanged.

In silico expression patterns for CcZIP

In sillico tissue-specific expression patterns of *CcZIPs*, their transcript abundance data (expressed as $\log_2$ transformed FPKM) were retrieved from the *C. cajan* gene expression atlas (CcGEA; Pazhamala et al. 2017). The expression profile of all *CcZIP* transcripts, except *CcZIP10* (due to the unavailability of its expression data in CcGEA), in thirty different tissues of *C. cajan* were visualized as the heat map.

Differential expression analysis of CcZIP genes by qRT PCR

The expression levels of *CcZIP* transcripts were determined by quantitative real-time polymerase chain reaction (*qRT*-PCR) analysis among the genotypes with contrasting host response to pod borer and seed zinc content, viz. *C. cajan cv.* ICPL87 (susceptible control), *C. cajan acc.* ICP28, *C. cajan acc.* ICP-26, *C. scarabaeoides acc.* ICPW90 and *C. scarabaeoides acc.* ICPW94. The matured green pods and reproductive apical leaves (during the first flush of flowering) were sampled using liquid nitrogen from the plants grown in the greenhouse with identical soil and environmental conditions. Total RNA was extracted from 100 mg of matured green pod and reproductive leaf tissues using G-Sure RNA extraction kit (GCC Biotech, India) following manufacturer's instructions. The quality and yield of RNA (DNAase-treated) were determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, USA), and was validated by agarose gel (1.4% v/v) electrophoresis in 3-*N*-morpholino-propane sulfonic acid (MOPS) buffer. The purified RNA (1 µg) was used for the synthesis of c-DNA using 1 µl of Verso reverse transcriptase enzyme (200 U l^{-1}), dNTP mix (2.5 mM each dNTP) and 250 ng oligo(dT) primer following the instructions of Verso cDNA synthesis kit (Thermo Scientific, USA). The integrity and quality of cDNA were affirmed by tubulin (*TUB6, Gene ID: B9R897*) gene amplification using master mix (Qiagen India Pvt. Ltd,

India) and primers (F: 5′GCCCTGACAACTTCGTCTTC3′ and R: 5′GCAGTTTTCAGCCTCTTTGC3′ (Sinha et al. 2015). The resulting cDNA samples were diluted in nuclease-free water (1:10), and 2 µl of the diluted cDNA sample was used for quantitative real-time assay using Quanti-Fast SYBR green PCR master mix (Qiagen India Pvt. Ltd, India) and the primers described in the Supplementary Table 2 to determine the relative expression of *CcZIP* genes among the genotypes with contrasting host response to *H. armigera* along with susceptible control. The quantitative RT-PCR analysis was performed in 96-well plate using CFX connect Real-Time PCR detection system (Biorad, USA) with a total reaction volume of 10 µl (2 µl of cDNA, 5 µl of SYBR Green PCR master mix, 0.5 µl of each forward and reverse primer, and 2 µl of ddH_2O). Thermal cycling conditions involved a pre-incubation at 95 °C for 7 min followed by 35 cycles of 3-step amplification at 95 °C for 10 s, 55 °C for 20 s and 72 °C for 20 s. The expression of *CcZIP* genes in the samples was normalized with that of *TUB6* as an endogenous control (Sinha et al. 2015). The *qRT* PCR analysis was performed with three technical and three biological replicates, and the specificity of *qRT* PCR assay was confirmed by melting curve analysis. The relative expression of *CcZIP* transcripts in each sample was calculated by measuring ΔC_T value for each of the *CcZIP* genes with respect to the endogenous control '*TUB6*' and was represented as $2^{-\Delta CT}$ value (Livak and Schmittgen 2001).

Estimation of zinc content

Matured green pods and reproductive apical leaves from the five genotypes were harvested in triplicate and air-dried. The dried samples were ground to a fine powder using bio-homogenizer (Pelican Equipment Ltd., Chennai, India), and 0.5 g of the powder of each sample was digested in an infra digestion system (KES12IL, Pelican Equipment, Chennai, India) using triple acid mix (9 nitric acid: 2 sulphuric acid: 1 perchloric acid) till the colourless solution was obtained. The colourless solution was made up to 100 ml using deionized water and filtered through Whatman filter paper (No. 40). The absorbance of the solution was measured using Atomic Absorption Spectrophotometer (Varian AA240, Varian, Palo Alto, CA, USA), and the content of zinc was estimated using the standard curve method (Kundu et al. 2017).

Results and discussion

The micronutrient zinc either acts as the cofactor or as a structural component in functional subunits of several proteins and enzymes essential for the growth and development of plants (Sinclair and Krämer 2012). In addition, zinc protects plant cells from oxidative stress-mediated by scavenging ROS and plays a significant role in plant signalling as an intracellular secondary messenger (Yamasaki et al. 2007; Cabot et al. 2019). The acquisition, uptake, distribution and accumulation of Zn in dicot plants involves several members of heavy metal transporter gene families. Thus characterization of these gene families is essential to understand the homeostasis as well as biofortification of zinc in plant parts. This study reports the identification and characterization of 19 members of ZIP family transporters (*CcZIP*s) in *C. cajan*, and their possible role on seed Zn content.

In silico identification of CcZIP genes and their characterization

The genome-wide search using the ZIP domain (PF02535) as query against the 48,680 *C. cajan* protein sequences (Gene Model *V5.0*) identified 19 non-redundant *CcZIP* genes containing the ZIP domain with an E-value 10^{-3} or less (Table 1). These were numbered as *CcZIP1* to *CcZIP19* based on their location in the chromosomes, followed by sequence scaffolds in ascending order. Among the *CcZIP* genes, six were localized in four chromosomes of pigeonpea (CcLG-01, -03, -09 and -10), and the rest 13 *CcZIP* genes were identified in the sequence scaffolds (Table 1). The occurrence of *CcZIP* genes is attributed to the non-inclusion of several sequence scaffolds to pigeonpea chromosomes (CcLG01 to -11) due to coverage of 72.6% of genomic sequence in the first draft genome sequence of pigeonpea (Varshney et al. 2012).

The full-length CDS of *CcZIP* genes containing open reading frames (ORF) were ranged between 252 to 598 amino acids, and the molecular weight of *CcZIP* proteins also varied from 26.5 kDa (*CcZIP11*) to 62.1 kDa (*CcZIP8*). Among them, size of only three *CcZIP* proteins (*CcZIP2, CcZIP4* and *CcZIP8*) appeared to be quite large beyond the molecular weight of 50 KDa (Table 1). The predicted length of 19 *CcZIP* genes was found to be varied between 795 bp (*CcZIP11*) and 15,312 bp (*CcZIP6*), and their ORF length varied between 759 bp (*CcZIP11*) and 1797 bp (*CcZIP8*). All 19 *CcZIP* genes were predicted to be functional in pigeonpea because they all have an initiation codon and culminate with a stop codon. The theoretical *pI* values of *CcZIP* proteins were estimated to be within the range of 5.82 (*CcZIP12*) to 9.39 (*CcZIP07*), and these acidic or basic properties of *CcZIP* proteins might influence the differential response of pigeonpea to the abiotic and biotic stresses encountered during growth and development (Allagulova et al. 2003). The amino acid composition analysis revealed that all the *CcZIP* proteins have a positive GRAVY value (0.239–0.821) except

Table 1 Properties of 19 ZIP genes identified in pigeonpea (*CcZIP*) and their proteins

ZIP ID	Sequence ID	Location	Gene length (bp)	No. of introns	ORF length (bp)	Polypeptide length (aa)	MW (kDa)	pI	GRAVY
CcZIP1	C.cajan_04710	CcLG02:2,033,309:2,037,230:+	3922	1	963	320	34.5	6.23	0.581
CcZIP2	C.cajan_07435	CcLG02:31,049,287:31,059,180:+	9894	4	1770	589	61.4	7.15	0.721
CcZIP3	C.cajan_09287	CcLG03:12,867,192:12,868,363:+	1172	1	1086	361	38.5	6.15	0.384
CcZIP4	C.cajan_23017	CcLG09:9,846,879:9,862,161:+	15,283	10	1482	493	53.3	6.1	-0.082
CcZIP5	C.cajan_13477	CcLG10:1,494,482:1,498,321:+	3840	11	831	276	29.2	6.51	0.738
CcZIP6	C.cajan_13516	CcLG10:1,801,366:1,803,329:+	1964	1	1011	336	36.9	6.35	0.52
CcZIP7	C.cajan_26486	Scaffold000020:248,574:251,686:-	3113	10	792	263	27.9	9.39	0.678
CcZIP8	C.cajan_37925	Scaffold000077:52,691:58,339:-	5649	4	1797	598	62.1	6.56	0.781
CcZIP9	C.cajan_29763	Scaffold000112:31,275:32,920:-	1646	2	897	298	32	6.69	0.615
CcZIP10	C.cajan_35552	Scaffold000139:144,426:149,655:+	5230	4	885	294	32.4	6.94	0.239
CcZIP11	C.cajan_31656	Scaffold125976:182,116:182,874:-	759	0	759	252	26.5	6.69	0.821
CcZIP12	C.cajan_33570	Scaffold132340:216,344:219,066:-	2723	2	987	328	34.9	5.82	0.665
CcZIP13	C.cajan_40078	Scaffold132759:50,498:52,223:+	1726	2	1014	337	35.8	6.13	0.419
CcZIP14	C.cajan_36394	Scaffold132932:28,466:31,089:+	2624	2	1044	347	37.1	6.03	0.631
CcZIP15	C.cajan_47587	Scaffold134054:4259:8193:+	3935	2	1071	356	38.4	6.59	0.426
CcZIP16	C.cajan_26822	Scaffold134757:100,385:101,259:+	875	1	789	262	28.3	8.18	0.491
CcZIP17	C.cajan_27143	Scaffold135298:59,118:61,460:+	2343	2	1056	351	37.4	6.18	0.531
CcZIP18	C.cajan_46675	Scaffold135342:11,316:13,137:-	1822	2	1014	337	36	6.91	0.414
CcZIP19	C.cajan_42212	Scaffold137616:48,461:51,993:+	3533	3	1068	355	38.3	7.66	0.597
		Average	3792.26	3.368	1069.26	355.42	37.94	6.75	0.535

*pI—Isoelectric point

CcZIP4 (− 0.082; Table 1) which could be attributed to the presence of hydrophobic amino acids in the membrane-spanning ZIP proteins as reported in several plant species (Grotz et al. 1998; Astudillo et al. 2013; Mondal et al. 2014; Krishna et al. 2020).

The PROSITE analysis of these *CcZIP* proteins predicted several peptide sequences concerned with post-translational modifications and other specific features, such as *N*-glycosylation site, phosphorylation site, and N-myristoylation, amidation and ATP synthase-A signature sequences (Table 2). In this study, N-myristoylation signature sequence was predominantly present in all *CcZIP* proteins (six in *CcZIP16* to 24 in *CcZIP8*) as the significant post-translational modification sequence. In addition, multiple phosphorylation sites have been documented in *CcZIP* proteins similar to the ZIP genes in several legume plants and model species (Krishna et al. 2020; Tingholm et al. 2020). These signature sequences and post-translation modification sites might act as substrates for several kinases, including casein kinase II, protein kinase-C, cAMP- kinase and cGMP- kinase. The predominance of *N*-myristoylation sequence in *CcZIP* proteins could be attributed to their role in conformational stability and modulation of different functions in many cellular pathways, especially during signal transduction, inter-cellular export and membrane transport of zinc and other heavy metals in pigeonpea as evident in different plants (Zaun et al. 2012; Mondal et al. 2014).

Analysis of gene structure, promoter sequences and motifs of CcZIPs

Similar to other gene families, the structural diversity among the members of the ZIP gene family depends on the number of introns and exons and their length. In terms of the exon–intron arrangement, the members *CcZIPs* were highly diverse, and the number of introns varied from zero in *CcZIP11* to a maximum of 11 introns in *CcZIP5* (Supplementary Fig. 1). Moreover, this variation in gene sequence was also well reflected in the multiple sequence alignment plots among the members *CcZIP* proteins (Fig. 1), whose length was varying between 252 and 598 AA with a minimum similarity of 15.82% between *CcZIP4* and *CcZIP8* to a maximum 94.65% identity between *CcZIP13* to *CcZIP18* protein (Supplementary Table 3). The structural diversity of *CcZIP* proteins were also well corroborated with the earlier report on the diversity of ZIP proteins in *A. thaliana*, *P. vulgaris*, *O. sativa*, *Z. mays* and

Table 2 Post-translational modification sites predicted in 19 *CcZIP* proteins of pigeonpea

	PS00001	PS00004	PS00005	PS00006	PS00008	PS00009	PS00016	PS00017	PS00178	PS00449	PS50079	PS50316
CcZIP1	0	0	4	1	10	0	0	0	0	0	0	0
CcZIP2	1	2	5	8	23	0	0	0	0	0	0	0
CcZIP3	3	1	4	3	10	0	0	0	0	0	0	1
CcZIP4	5	0	3	8	9	0	0	0	0	0	0	1
CcZIP5	0	0	2	4	13	1	0	0	0	0	0	0
CcZIP6	1	0	4	9	7	0	0	0	0	0	0	0
CcZIP7	0	0	6	4	13	1	0	0	0	0	1	0
CcZIP8	1	3	5	9	23	0	0	0	0	0	0	0
CcZIP9	0	0	1	1	9	0	0	0	0	0	0	1
CcZIP10	2	1	2	4	6	0	0	0	0	1	0	1
CcZIP11	0	0	3	1	12	0	1	0	0	0	0	0
CcZIP12	1	2	1	4	16	0	0	1	1	0	0	0
CcZIP13	4	0	2	2	12	0	0	0	0	0	0	1
CcZIP14	2	0	4	8	8	1	0	0	0	0	0	0
CcZIP15	3	1	4	3	11	0	0	0	0	1	0	1
CcZIP16	0	0	2	3	6	0	0	0	0	0	0	1
CcZIP17	0	1	8	4	9	0	0	0	0	0	0	0
CcZIP18	5	0	3	1	12	0	0	0	0	0	0	1
CcZIP19	2	0	7	1	10	0	0	0	0	0	0	0

PS00001: N- glycosylation site (*N-Gly*); ***PS00004***: cAMP- and cGMP-dependent protein kinase phosphorylation site (*cAMP*); ***PS00005***: protein kinase C (*PKC*) phosphorylation site; ***PS00006***: Casein Kinase-II *(CK2)* phsophorylation site*;* ***PS00008***: N- myristoylation (*N-MYR*) site; ***PS00009***: Amidation site (*Amd*); ***PS00016***: Arg-Gly-Asp tripeptide (*RGD*) cell attachment sequence; ***PS00017***: ATP/GTP-binding site motif A (*ATP/GTP-A*); ***PS00178***: Aminoacyl-transfer RNA synthetases class-I signature (*AA-tRNA Ligase*), ***PS00449***: ATP synthase a subunit signature (*ATPase-A*); ***PS50079***: Bipartite nuclear localization signal (*NLS-BP*); ***PS50316***: Histidine-rich region (*His Rich*)

Fig. 1 Multiple sequence alignment of 19 *CcZIP* proteins obtained by MUSCLE programmes. The trans-membrane domains indicated as roman numerals at the top of the alignments and the square indicates the metal binding histidine motif (H-x-H-x-H) in the variable region between TM-III and TM-IV

P. trifoliata (Grotz et al. 1998; Chen et al. 2008; Astudillo et al. 2013; Mondal et al. 2014; Fu et al. 2017; Krishna et al. 2020). Such structural diversity of ZIP genes across species could be attributed to their multifunctional role, particularly in cellular movement and transport of several cations (Krishna et al. 2020). In addition, the diversity of ZIP proteins suggests different responses during adaptation to biotic and abiotic stresses encountered during the growth and development of different plant species (Cabot et al. 2019).

The MEMPACK of PSIPRED, TMHMM server and SignalP 4.1 server delineated the number of transmembrane helices (TMHs), pore line helices (PLHs) and signal peptides present in the *CcZIP* proteins (Table 3; Fig. 2a). The number of TMHs present in the maximum number of *CcZIP* proteins ranged from 6 to 9 as expected (Guerinot 2000; Li et al. 2013) baring three viz. *CcZIP2* (13), *CcZIP8* (13) and *CcZIP10* (5). These increased or decreased number of TMHs in ZIP proteins could be attributed to either sequence duplication or deletion followed by genomic reorganization (D'Ovidio et al. 2004). Similar variation in the number of TMHs had also been reported in maize (Mondal et al. 2014). As expected, most of the TMHs of *CcZIP* protein embedded varying numbers (2–5) of PLHs, which might facilitate the sensing and movement of ions across the membranes in response to different stress

Table 3 Putative localization and functions in 19 CcZIP proteins predicted in Pigeonpea along with their transmembrane helices (TMHs), pore lining helices (PLHs) and signal peptide sequences

ZIP ID	Location	Function(s)	Length (AA)	No. of predicted TMHs	No. of predicted PLHs	Signal peptide
CcZIP1	Plasma membrane	Zinc transporter 8	320	7	4	–
CcZIP2	Plasma membrane	Putative zinc transport	589	13	4	Yes
CcZIP3	Plasma membrane	Fe(2+) transport protein	361	8	5	–
CcZIP4	Plasma membrane	IAA-alanine resistance	493	7	5	Yes
CcZIP5	Endoplasmic reticulum	Zinc transporter	276	8	5	–
CcZIP6	Plasma membrane	Zinc transporter 2	336	8	6	Yes
CcZIP7	Endoplasmic reticulum	Zinc transporter	263	6	4	–
CcZIP8	Plasma membrane	Putative zinc transport	598	13	5	Yes
CcZIP9	Plasma membrane	Fe(2+) transport protein	298	6	5	–
CcZIP10	Plasma membrane	Zinc transporter 8	294	5	2	Yes
CcZIP11	Plasma membrane	Fe(2+) transport protein	252	6	4	–
CcZIP12	Plasma membrane	Zinc transporter 5	328	8	5	–
CcZIP13	Plasma membrane	Zinc transporter 8	337	7	4	–
CcZIP14	Plasma membrane	Zinc transporter 11	347	8	5	Yes
CcZIP15	Plasma membrane	Zinc transporter 5	356	6	3	Yes
CcZIP16	Plasma membrane	Fe(2+) transport protein	262	6	4	Yes
CcZIP17	Plasma membrane	Zinc transporter 8	351	7	3	Yes
CcZIP18	Plasma membrane	Zinc transporter 8	337	6	3	–
CcZIP19	Plasma membrane	Fe(2+) transport protein	355	7	3	Yes

(Maksaev et al. 2018). Moreover, 11 *CcZIPs* (*CcZIP2, CcZIP4, CcZIP6, CcZIP8, CcZIP9, CcZIP10, CcZIP14, CcZIP15, CcZIP16, CcZIP17* and *CcZIP19*) possessed signal peptide sequence (Table 3), which suggest the involvement of these *CcZIP* proteins in the cellular movement of heavy metal ions including Zn^{2+} as reported in several plant species (Krishna et al. 2020). ProtCompv9.0 analysis showed that most of the *CcZIP* proteins are located in the plasma membrane, except for *CcZIP5* and *CcZIP7* proteins, which were predicted to be in endoplasmic reticulum (Table 3). Similar to the present findings, the ZIP transporters were also located in different cell organelles in different species, and this is mostly attributed to their role in zinc homeostasis in plants concerning low and high zinc soils as well as a stress response (Tiong et al. 2015; Bashir et al. 2016; Cabot et al. 2019).

The *cis*-regulatory elements (CRE) are important signature sequences involved in transcriptional regulation of genes and remain very important during plant growth and development during several biotic and abiotic stresses in their habitat by modulating gene expression. Analysis of the putative promoter sequence of *CcZIP* genes, 1,000 bp upstream of the transcription initiation site using Nsite-PL detected an array of CREs regulating plant development, plant hormonal response, biotic and abiotic stress induction, and secondary metabolism in addition to basal gene expression (Supplementary Table 4; Supplementary Fig. 2). The promoter of *CcZIP16* gene contains the maximum number (43) of CREs, whereas *CcZIP7* has only eight CREs. Some of these predicted CREs are stress-responsive, such as metal regulatory element (MRE), light regulatory element (LREs), ABA-responsive elements (ABRE), zinc deficiency-related elements (ZDRE), low-temperature responsive elements (LTRE) and TGACG-motif. In addition, some of the hormone signalling stress responsive CREs, such as methyl jasmonate responsive element (MejA-RE), ethylene responsive element (ERE) and auxin responsive element (ARE) (Supplementary Table 4; Fig. 3). The promoter sequences of two members of pigeonpea ZIP family, *CcZIP3* and *CcZIP15*, also have ZDRE *cis*-element as reported earlier in the members of ZIP family of *A. thaliana* and *Z. mays*, and these ZDREs might regulate the transcription of ZIP gene(s) under zinc deficiency stress (Assunção et al. 2010; Jain et al. 2013; Mondal et al. 2014). The putative CREs for biotic and abiotic stress induction were predicted at multiple sites in the promoter regions of *CcZIP* genes reported in soybean. These CREs might have been involved in pathogen

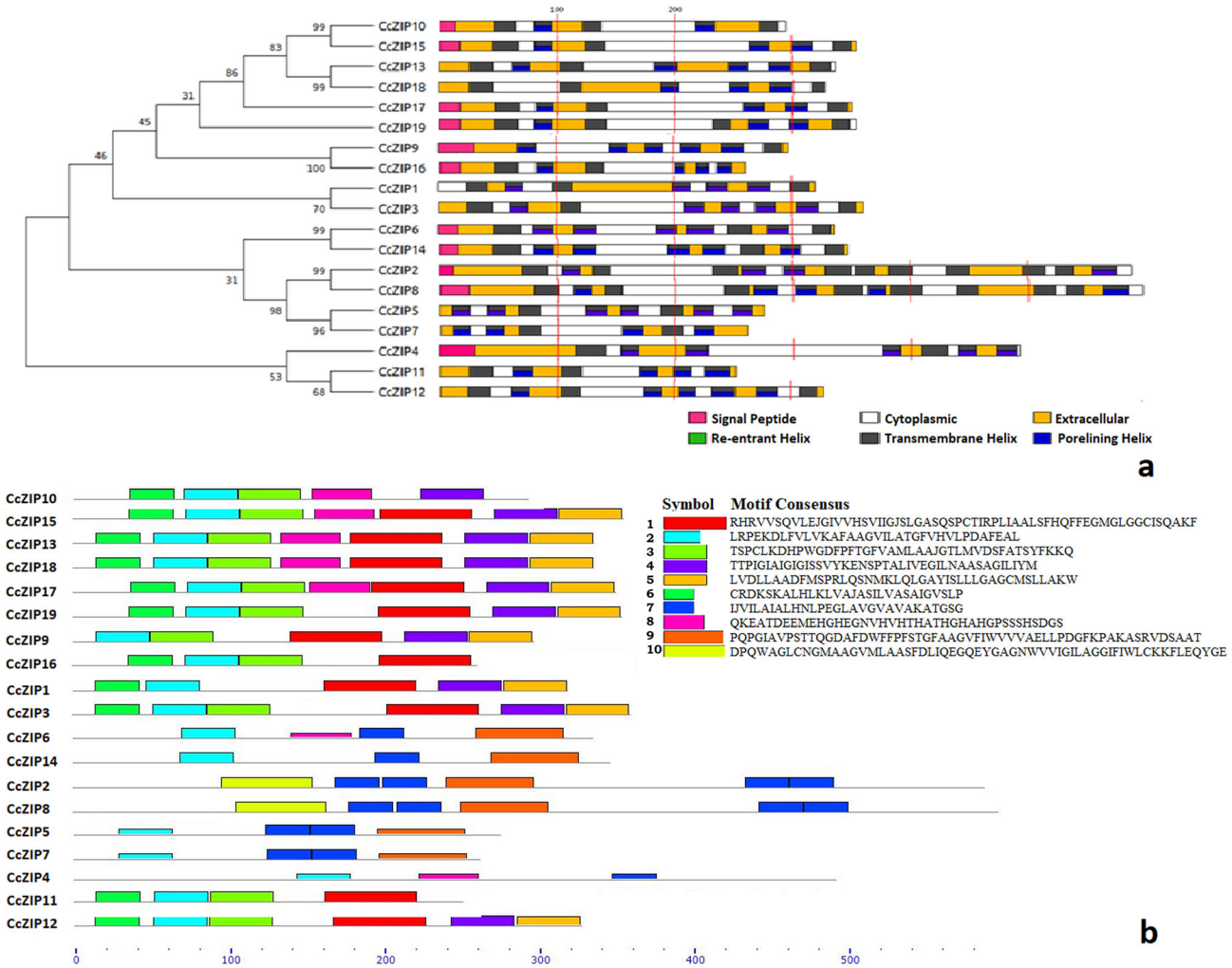

Fig. 2 Intraspecific relationship and structure of *CcZIP* proteins and functional motifs of 19 CcZIPs. (**a**) A phylogenetic tree with 100 bootstrap replications along with schematic protein sequence showing transmembrane helices, pore lining helices and signal peptide sequences, (**b**) Conserved motif composition of each CcZIP protein. Motifs 1–10 are displayed as differently colored boxes with the corresponding sequence information for each motif

infection, herbivory attacks and abiotic stress response (Wang et al. 2015). In addition to stress-responsive CREs, several *cis*-elements associated with cellular development, plant metabolism, cell cycle regulation and hormonal development were also predicted. The presence of these CREs is indicative of their possible involvement in regulating *CcZIP* gene expression during cellular growth and development in response to different biotic and abiotic environments (Cabot et al. 2019). The motif analysis using MEME suite also identified 10 conserved motifs, containing 29–60 amino acid residues, among the *CcZIP* proteins and the prime function of these motifs were annotated to be zinc transporter, iron transporter and a component of TMH (Fig. 2b; Supplementary Table 5). Similar conserved motifs for cation transport are also reported in ZIP proteins of *A. thaliana, P. vulgaris, O. sativa* and *Z. mays* (Chen et al. 2008; Astudillo et al. 2013; Mondal et al. 2014; Krishna et al. 2020). The sequence logo of these conserved motifs generated by WebLogo is presented (Supplementary Fig. 3). As described above, most of the *CcZIPs* were found to have 6–9 TMHs with an average of 7.49, and these predicted TMHs have C-terminal and N-terminal ends present inside and outside the surface of the plasma membrane, respectively. Another important feature of the ZIP proteins is a variable hydrophobic loop located between TMH-III and -IV (Guerinot 2000), and this feature was also quite evident among the 19 members *CcZIP* proteins (Fig. 2). This variable hydrophobic loop is characterized by conserved histidine residues (H-x-H-x-H) and is predicted to be the cytoplasmic metal ion binding site (Eng et al. 1998; Guerinot 2000). Similar to *AtZIP7*, *AtZIP8* and *AtZIP11* in *A. thaliana*, *MtZIP1* and *MtZIP7* in *M. truncatula*, and *PtZIP2* in *P. trifoliata*, *CcZIP11* and *CcZIP12* lack this *His*-rich region between TM-III and

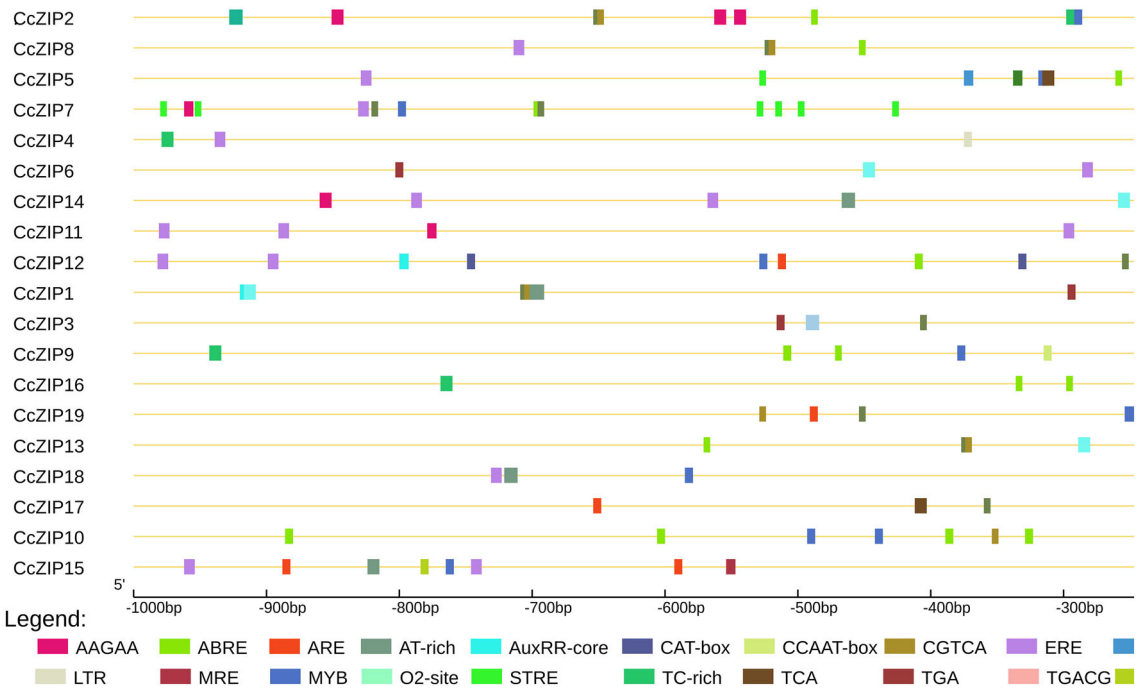

Fig. 3 Promoter analysis of 19 CcZIP genes. The legend shows different stress-response elements located in the 1000 bp upstream region of each CcZIP gene

TM-IV, and in lieu of this *His* residues were found in their TM-III as an alternative metal-binding site as reported in *A. thaliana* and *P. trifoliata* (Eng et al. 1998; Fu et al. 2017). Four *CcZIP* proteins (*CcZIP2, CcZIP5, CcZIP7* and *CcZIP8*) did not have *His* rich region (H-x-H-x-H), and instead polar amino acid residues were present adjacent to either TM-III or TM-IV domains as reported in *Z. mays, P. vulgaris* and *Solanum tuberosum* (Astudillo et al. 2013; Mondal et al. 2014; Li et al. 2020). It was also reported that either deletion or mutation in H-x-H-x-H region of *TjZNT1* (a ZIP transporter) did not affect Zn^{2+} and Cd^{2+} transport activity, rather it increased the specificity for Zn^{2+} in *Thlapsi japonicum* (Nishida et al. 2008). Moreover, Kawachi et al. (2008) showed that yeast cell containing mutant *AtMTP1*, a vacuolar Zn^{2+}/H^{+} antiporter of *A. thaliana* lacking 32 residues in the histidine rich loop, became hyper-resistant to Zn^{2+} and resistant to Co^{2+}. Contrasting to these reports, three ZIP genes in *P. vulgaris* (*PvZIP6*, *PvZIP7* and *PvZIP18*) lacking these *His* rich region (H-x-H-x-H) remained non-functional (Astudillo et al. 2013). *CcZIP4* has more significant number of histidine residues in the variable loop between TM-III and TM-IV, and it implies multiple metal ion binding abilities as reported in the case of *StZIP12* of *S. tuberosum* (Li et al. 2020). Kawachi et al. (2008) also proposed that the histidine-rich loop of *AtMTP1* functions as buffering pocket of Zn^{2+} and acts as a sensor to determine zinc level at the cytoplasmic surface. These structural variations of *CcZIP* proteins are also well corroborated with the earlier studies on ZIP proteins in several dicots and monocot species (Chen et al. 2008; Astudillo et al. 2013; Mondal et al. 2014; Fu et al. 2017; Krishna et al. 2020; Li et al. 2020).

Phylogenetic analysis of CcZIP proteins

The intraspecific genetic relationship among 19 *CcZIP* proteins of pigeonpea depicted in a phylogenetic tree showed the clustering of these *CcZIP* proteins into six major groups, mostly engendered during evolution to the subgroups identified by motif analysis (Fig. 2a). Similar kind of intraspecific diversity of ZIP genes were also made in *P. vulgaris*, *Z. mays* and *S. tuberosum* (Astudillo et al. 2013; Mondal et al. 2014; Li et al. 2020). The interspecific evolutionary relationships of ZIP family members of *C. cajan* along with three other legume species (*G. max*, *P. vulgaris* and *M. truncatula*) and model dicot *A. thaliana* was deduced, and consequently a phylogenetic tree was drawn (Fig. 4). This phylogenetic tree grouped 101 genes containing ZIP domain (PF02535) into three major clusters (A, B and C), and major cluster-A has eight sub-clusters (A_1–A_8) which mostly corroborated with the presence of conserved motifs barring few cases (Fig. 4). In this study, we considered ten conserved motifs (MEME-1 to 10) generated by MEME suite and among them, seven (MEME-1 to -5, MEME-9 and -10) were annotated to be the core component of zinc transporter protein

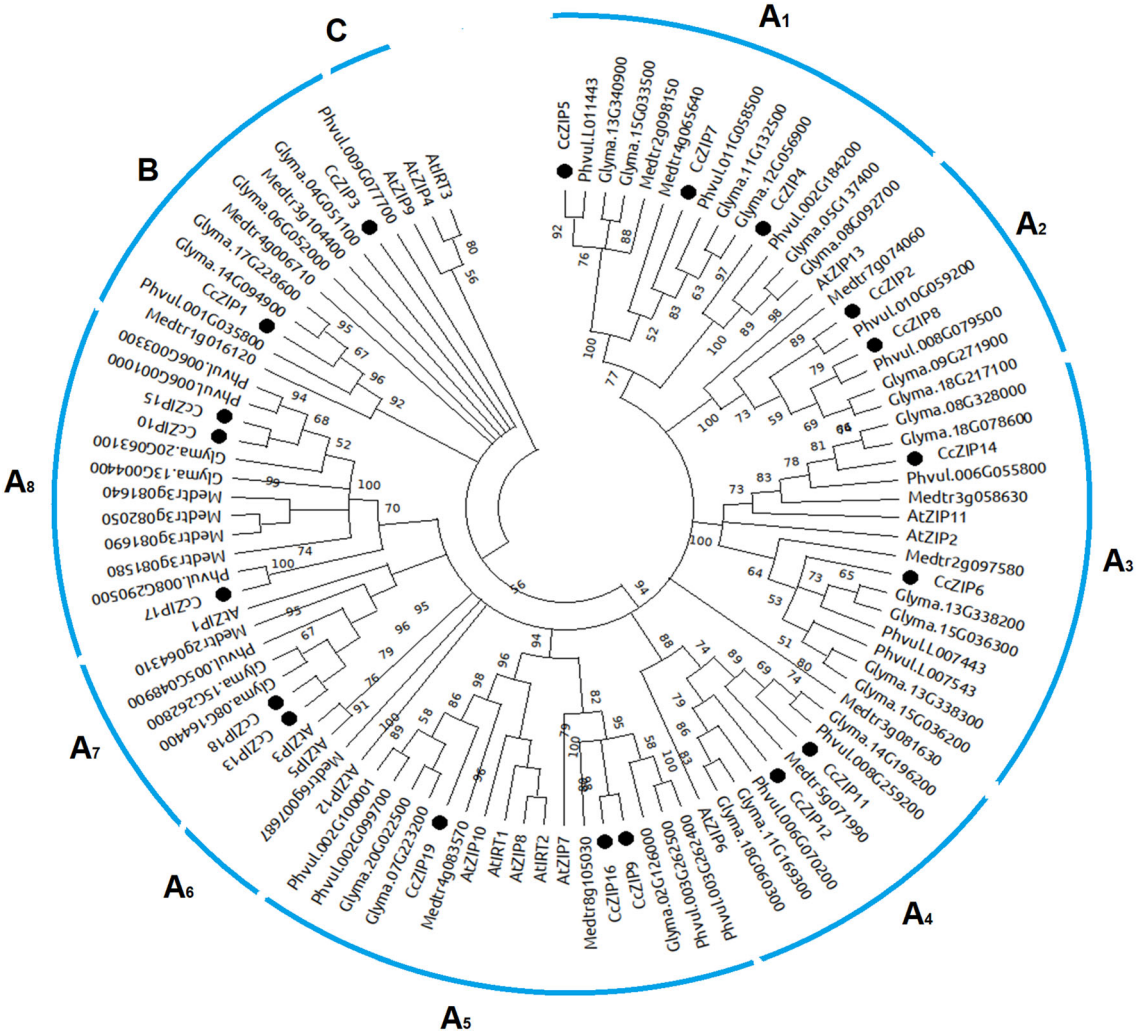

Fig. 4 Interspecific phylogenetic tree (maximum likelihood) of 19 *CcZIP* proteins along with 82 ZIP proteins of three allied species *G. max*, *M. truncatula* and *P. vulgaris*, and model dicot *A. thaliana*

(Supplementary Table 5) because of their involvement in the binding and transport of Zn cations as reported in other plants (Krishna et al. 2020). The 19 *CcZIP* genes fell under two (A and B) out of three clusters in the interspecific phylogenetic tree (Fig. 4). The sub-cluster-A_1 consisted of 14 genes containing ZIP domains, including three CcZIP members (*CcZIP4*, *CcZIP5* and *CcZIP7*), all of which possessed MEME-2 to -7 motif in common. Similarly, cluster-B and sub-cluster-A_2, -A_3, -A_4, -A_5, -A_7 and -A_8 contained three to seven common motifs in different permutations and combinations in corroboration with their grouping under phylogenetic trees, respectively. A similar kind of clustering of zinc transporters was also reported in both dicots and monocot plants (Krishna et al. 2020). In some clusters few ZIP genes possessed different motif(s) in addition to common motifs, and this kind of heterogeneous motif distribution has also been noticed during phylogenetic classification of different gene families in several dicots and monocots, including *P. vulgaris* and *Z. mays* (Astudillo et al. 2013; Mondal et al. 2014; Krishna et al. 2020).

Expression patterns of CcZIP genes in pigeonpea

In silico expression patterns for 18 *CcZIP* genes (except *CcZIP10*) were determined using the *C. cajan* gene expression atlas (CcGEA; (Pazhamala et al. 2017), and tissue-specific differential expression was observed for all the *CcZIP* genes analyzed (Fig. 5). Among all, the *CcZIP9* and *CcZIP16* meagerly expressed in all tissue except reproductive buds and nodules, whereas the *CcZIP1, CcZIP2, CcZIP3, CcZIP4, CcZIP5, CcZIP8, CcZIP11* and *CcZIP14* showed moderate to high-level expression in both the tissues of *C. cajan* cv. *Asha*. The rest of the eight *CcZIP* genes showed quite heterogeneous and tissue-specific expression. In silico expression analysis of *CcZIP* proteins

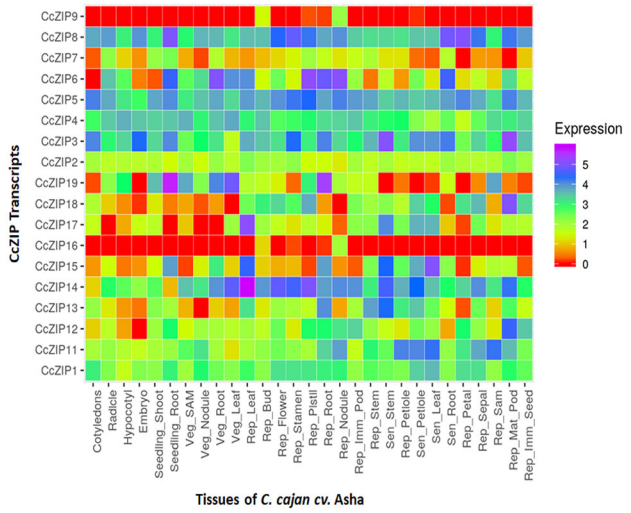

Fig. 5 In silico analysis showing abundance of 18 *CcZIP* transcripts in 30 tissues of pigeonpea on the basis of data obtained from gene expression atlas (CcGEA; Pazhmala et al. 2017)

under a controlled environment revealed that *CcZIP3, CcZIP5, CcZIP6* and *CcZIP8* genes consistently showed higher expression (> 30 Log_2 transformed FPKM) among four kind of roots, viz. seedling roots (SR), vegetative roots (VR), reproductive roots (RR) and senescence roots (SsR). Whereas *CcZIP19* showed higher expression in all three kinds of roots except SsR, *CcZIP15* showed comparatively higher expression in both SR and RR, and *CcZIP14* showed higher expression in VR and RR. *CcZIP4* and *CcZIP13* showed higher expression in VR and RR, respectively. In both vegetative and reproductive leaves *CcZIP6, CcZIP8* and *CcZIP14* showed higher expression, whereas in vegetative leaves, *CcZIP4* and *CcZIP19*, and in reproductive leaves *CcZIP3, CcZIP5, CcZIP13, CcZIP15* and *CcZIP17* showed higher expression, respectively. Similarly, in both vegetative and reproductive shoot apical meristems (SAM) *CcZIP19* is expressed *at par*, whereas in vegetative SAM *CcZIP4* and *CcZIP14*, and in reproductive SAM *CcZIP11* showed higher expression, respectively. Likewise, in both reproductive immature and mature reproductive pods *CcZIP5, CcZIP8, CcZIP12* and *CcZIP14* are expressed *at par*, whereas in mature pods, *CcZIP3* and *CcZIP18* showed higher expression. On comparison of expression of *CcZIP* genes among different kinds of roots, leaves, SAMs and pods, it was found that *CcZIP13* consistently showed higher expression both in reproductive roots and leaves, and *CcZIP18* showed higher expression in reproductive matured pods (Supplementary Table 6). The above findings indicate the putative role of *CcZIP13* in the acquisition of zinc from the soil and its mobilization and distribution at the onset of the reproductive phase (Jain et al. 2013) and the role of *CcZIP18* on phloem unloading in the pods (Olsen and Palmgren 2014). The in silico differential expression of *CcZIPs* were also well correlated with in vivo expression data of reproductive leaves and matured green pods using qRT-PCR analysis. This varied expression of *CcZIPs* in both tissues among different genotypes is likely to be associated with the regulation of transcription factors under abiotic environment, stress and stages of development such as *bZIP19* and *bZIP23* in *A. thaliana* under Zn deficit condition (Assuncao et al. 2010; Lira-Morales et al. 2019).

As erstwhile discussed, zinc is the structural and regulatory component of several inter-related metabolic pathways, and it plays an essential role even in embryo and endosperm development (Vallee and Falchuk 1993). The Zinc transporters proteins, particularly P1-B-ATPase pumps, are essential for the export and accumulation of zinc inside the seeds of *A. thaliana* (Olsen et al. 2016). Even in rice and maize, several ZIP genes regulating cellular movement and phloem unloading of zinc either in the endosperm or in the kernel had been identified (Krishna et al. 2020). Seed zinc content in pigeonpea was also influenced by soil zinc content in a genotype-dependent manner. The higher zinc content in the seeds of *C. cajan* has been reported to inhibit the pod borer herbivory (Kaur et al. 2014; Cabot et al. 2019). In the present study, two pod borer susceptible genotypes (*C. cajan acc.* ICP-28 and *C. cajan acc.* ICP-26) have comparatively lower Zn content in its seeds (11.08 ± 0.42 and 11.51 ± 0.33 μg g^{-1}) at par with susceptible control (10.95 ± 0.4 μg g^{-1}). Whereas the two resistant genotypes (*C. scarabaeoides acc.* ICPW-94 and *C. scarabaeoides acc.* ICPW-90), primarily used as the genetic resources for the introgression of pod borer resistance allele, had higher seed Zn content (16.37 ± 0.57 and 15.65 ± 0.61 μg g^{-1}) than susceptible genotypes tested. This finding was also well corroborated with the earlier report on Zn content in pod borer resistant genotypes in pigeonpea (Kaur et al. 2014). Zn-deficient soybean plants also showed increased aphid colonization, and was attributed to a higher accumulation of amino acids caused by reduced protein synthesis under zinc deficiency (Helfenstein et al. 2015; Cabot et al. 2019). Similarly, low Zn status inhibit the expression of defence-related genes, including PR1, through evolutionary conserved Zn sensing mechanism with respect to plant growth *vis-à-vis* defence (Bouain et al. 2018). The reproductive leaves in dicots play a crucial role in phloem unloading of micronutrients in the seed through pod wall and maternally derived seed coat (Garcia and Grusak 2015) and are also valid for the legumes; thus, it influences the seed nutrition composition. Even the seed development in legumes accomplished by networks of regulatory and metabolic genes of several ontological pathways, which affect its size and composition (Weber et al. 2005), and among them, Zinc finger (CCHC and C_2H_2) type proteins were associated with seed size

(Radkova et al. 2019). Thus, the expression analysis of *CcZIP* genes was carried out in two different plant parts (reproductive leaves and matured green pods) of four different genotypes, with contrasting host responses to pod borer and heterogeneous seed zinc content, and their involvement in seed zinc accumulation and pod borer herbivory has been hypothesized.

The *qRT*-PCR analysis revealed that expression of seven *CcZIP* genes (*CcZIP1, CcZIP2, CcZIP4, CcZIP8, CcZIP12, CcZIP14* and *CcZIP16*) were relatively up-regulated both in the reproductive leaves and matured green pods of susceptible genotypes, whereas *CcZIP17* was up-regulated in both tissues of resistant genotypes (Fig. 6). The expression of *CcZIP3 and CcZIP15* were up-regulated in the reproductive leaves whereas down-regulated in the matured green pods of resistant genotypes (Fig. 7). In contrast, the expression of *CcZIP6* and *CcZIP13* was induced in the matured green pods of the resistant genotypes compared to their reproductive leaves (Fig. 7). On comparison of the relative expression of *CcZIPs* between two tissues of the resistant genotypes, it has been revealed that six *CcZIPs* (*CcZIP4, CcZIP7, CcZIP8, CcZIP15, CcZIP17* and *CcZIP19*) in reproductive leaves, and 11 *CcZIPs* (*CcZIP1, CcZIP2, CcZIP3, CcZIP6, CcZIP9, CcZIP10, CcZIP11, CcZIP12, CcZIP13, CcZIP14* and *CcZIP18*) in matured green pods showed higher expression. Similarly, among the susceptible genotypes four *CcZIPs* (*CcZIP4, CcZIP6, CcZIP7* and *CcZIP8*) showed higher expression in reproductive leaves, whereas 10 *CcZIPs* (*CcZIP1, CcZIP2, CcZIP3, CcZIP9, CcZIP10, CcZIP12, CcZIP14, CcZIP15, CcZIP16* and *CcZIP18*) in matured green pods. Similar heterogeneous expression of the ZIP genes has already been reported in several plants, including *A. thaliana* and *P. vulgaris* (Astudillo et al. 2013; Jain et al. 2013), and this might be attributed to different stress and growth responsive CREs and transcription factors with regards to different genotypes. In *P. vulgaris*, seven genes were characterized among two genotypes (G19833 and DOR364) under different zinc treatments, and four of the genes (*PvZIP12, PvZIP13, PvZIP16* and *PvbZIP1*) showed differential expression depending upon the type of tissues (roots, leaves and pods), genotype and zinc treatment. *PvZIP12* and *PvZIP13* showed more expression in G19833 genotype than *DOR 364*, and *PvZIP01*, *PvZIP12* and *PvZIP16* showed maximum expression under zinc deficit treatment in pods, vegetative leaves and reproductive leaves, respectively. Further, Astudillo et al. (2013) recommended *PvZIP12* as a good candidate gene for enhancing seed zinc concentration through their genetic mapping studies. A similar kind of genotype-specific heterogeneous expression of the zinc transporters genes has also been reported in maize and rice, the strategy II plants (Lee et al. 2010a, b; Ishimaru et al. 2011; Mondal et al. 2014). In *A. thaliana* three members of ZIP family (*AtZIP4, AtZIP9* and *AtZIP12*) also showed higher expression in roots and shoots under Zn deficient seedlings and they were suppressed with the availability of Zn (Jain et al. 2013). During this study, *CcZIP3* and *CcZIP15* (containing ZDRE in its promoter region) were significantly up-regulated in the reproductive leaf tissues and down-regulated in matured green pods of the resistant genotypes with higher zinc content. Thus the role of these two *CcZIP* genes could be attributed to the translocation (cellular movement) of Zn from leaves to pod walls (Garcia

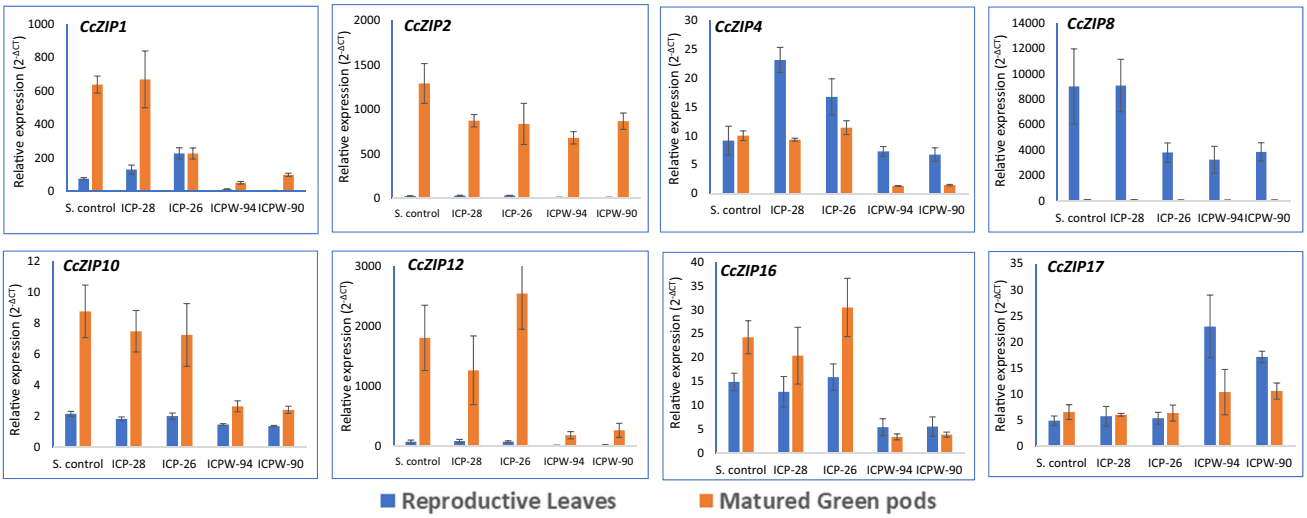

Fig. 6 Relative expression of eight *CcZIPs* in two tissues (reproductive leaves and matured green pods) among the four genotypes of *Cajanus* spp. (on X-axis) with contrasting host response to pod borer and seed zinc content. The expression normalized by measuring ΔC_T value for each of the *CcZIP* gene with respect to the endogenous control '*TUB6*' and was represented as $2^{-\Delta CT}$ on Y-axis. The error bars represent the standard deviation from three biological replicates

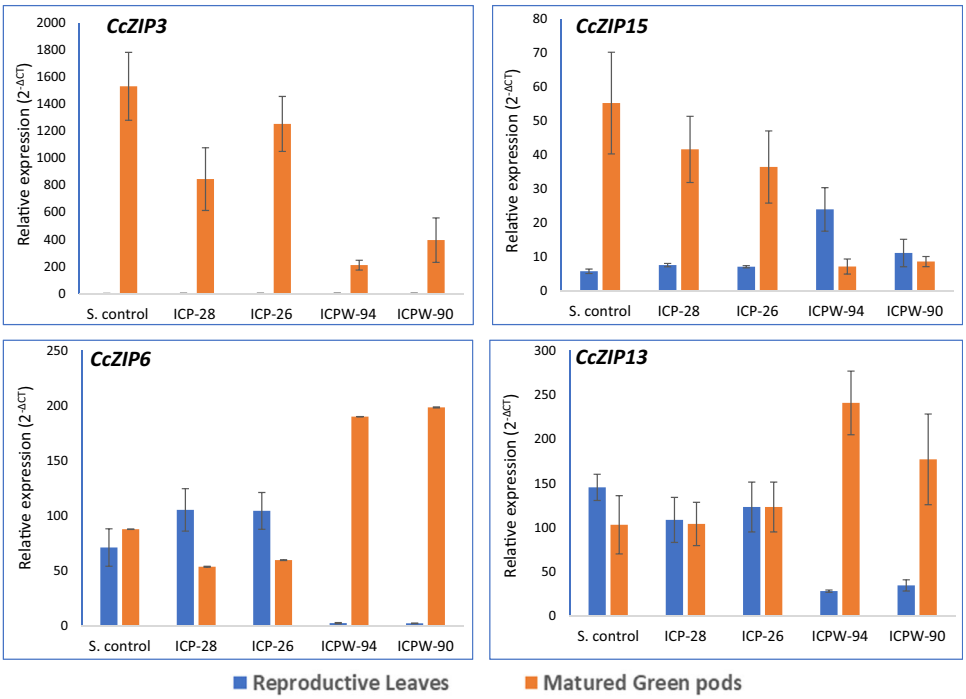

Fig. 7 Differential expression of four *CcZIPs* (*CcZIP3*, *CcZIP6*, *CcZIP13* and *CcZIP15*) in reproductive leaves and matured green pods among the genotypes of *Cajanus* spp. (on X-axis) with contrasting host response to pod borer and seed zinc content. The expression normalized by measuring ΔC_T value for each of the *CcZIP* gene with respect to the endogenous control '*TUB6*' and was represented as $2^{-\Delta CT}$ on Y-axis. The error bars represent the standard deviation from three biological replicates

and Grusak 2015). Alternatively, *CcZIP6,* and *CcZIP13* were up-regulated in the matured green pods, and down-regulated reproductive leaf tissues of the genotypes with higher zinc content; thus these *CcZIP* genes might have played a subsequent role in phloem unloading and accumulation in the seeds (Garcia and Grusak 2015). This kind of tissue specific expression of ZIP genes was reported in several dicots and monocots (Astudillo et al. 2013; Jain et al. 2013; Mondal et al. 2014). Therefore, *CcZIP3*, *CcZIP6, CcZIP13* and *CcZIP15* might be considered probable candidate genes for higher accumulation seed zinc content in the reproductive leaves and matured green pods of the pod borer resistant genotypes atleast used in this study, which is quite relevant for bio-fortification and genetic improvement to inhibit the pod borer herbivory in pigeonpea. Differential tissue-specific expression of these *CcZIP* genes and other allied gene families, *cis*- regulatory elements in the promoter of these genes and their regulatory transcription factors in pigeonpea under both Zn-abundant and Zn-deficient conditions, as well as various plant defence-related enzymes activities are being investigated to support the findings.

Conclusion

This study on identification, characterization and analysis of zinc transporters, including the members of ZIP gene family in *C. cajan* could be used in functional genomic studies on Zn biofortification, stress modulation and plant development. Gene structure characterization and expression analysis showed the possible role of *CcZIP3* and *CcZIP15*, having ZDRE in their promoter region, on the mobilization of Zn from leaves to pods in the pod borer resistant genotypes. In addition, the higher expression of *CcZIP6* and *CcZIP13* in matured green pods of both the resistant genotypes compared to reproductive leaves indicates their possible involvement in phloem unloading, leading to higher seed Zn content in the resistant genotypes. Differential expression of these *CcZIP*s along with other allied gene families and transcription factors could delineate the strategy not only in Zn bio-fortification but also for the genetic improvement to inhibit the pod borer herbivory in pigeonpea.

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Author contributions JP and AN conceived and designed the experiments; AN, SKM, KG, ND performed the experiments and bioinformatics analysis; JP, KG and AN analysed the data and interpreted the results; AN, KG, SKM and JP wrote the manuscript. All authors have reviewed the manuscript and have given consent to the final version submitted.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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