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Unraveling the genomic reorganization of polygalacturonase-inhibiting proteins in chickpea

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Polygalacturonase-inhibiting proteins (PGIPs) are cell wall proteins that inhibit pathogen polygalacturonases (PGs). PGIPs, like other defense-related proteins, contain extracellular leucine-rich repeats (eLRRs), which are required for pathogen PG recognition. The importance of these PGIPs in plant defense has been well documented. This study focuses on chickpea (*Cicer arietinum*) PGIPs (CaPGIPs) owing to the limited information available on this important crop. This study identified two novel CaPGIPs (CaPGIP3 and CaPGIP4) and computationally characterized all four CaPGIPs in the gene family, including the previously reported CaPGIP1 and CaPGIP2. The findings suggest that CaPGIP1, CaPGIP3, and CaPGIP4 proteins possess N-terminal signal peptides, ten LRRs, theoretical molecular mass, and isoelectric points comparable to other legume PGIPs. Phylogenetic analysis and multiple sequence alignment revealed that the CaPGIP1, CaPGIP3, and CaPGIP4 amino acid sequences are similar to the other PGIPs reported in legumes. In addition, several cis-acting elements that are typical of pathogen response, tissue-specific activity, hormone response, and abiotic stress-related are present in the promoters of *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* genes. Localization experiments showed that CaPGIP1, CaPGIP3, and CaPGIP4 are located in the cell wall or membrane. Transcript levels of *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* genes analyzed at untreated conditions show varied expression patterns analogous to other defense-related gene families. Interestingly, CaPGIP2 lacked a signal peptide, more than half of the LRRs, and other characteristics of a typical PGIP and subcellular localization indicated it is not located in the cell wall or membrane. The study's findings demonstrate CaPGIP1, CaPGIP3, and CaPGIP4's similarity to other legume PGIPs and suggest they might possess the potential to combat chickpea pathogens.

KEYWORDS

polygalacturonase inhibitory proteins (PGIPs), gene family, defense-related gene, biotic stress response, leucine-rich repeats (LRRs), promoter analysis, constitutive gene expression, subcellular localization

1 Introduction

Plants deploy a variety of barriers to withstand numerous pathogenic stresses, one of which is the cell wall, a physical barrier that serves as the first line of defense. Pathogens produce enzymes known as cell wall-degrading enzymes (CWDEs) to overcome this plant barrier (Kubicek et al., 2014). Pectin-degrading enzymes called polygalacturonases are among the most important CWDEs. The middle lamella is the plant cell's outermost layer that connects the primary cell walls of adjacent cells (Daher and Braybrook, 2015). Middle lamella is rich in pectin, which primarily constitutes homogalacturonan, a linear homopolymer of D-galacturonic acid monomers linked by *a*-(1–4) glycosidic linkage (Mohnen, 1999). Pectin determines the integrity and rigidity of plant tissue (Voragen et al., 2009), and degrading pectin enables quick access to the components within the cell. By breaking down glycosidic linkages between D-galacturonic acid residues, PGs degrade homogalacturonan and subsequently pectin causing cell separation and maceration of the host tissue (Kalunke et al., 2015; Mojsov, 2016). Polygalacturonases (PG) are secreted at the early stages of infection (De Lorenzo et al., 2001). In defense, plants use polygalacturonase inhibiting proteins (PGIPs) to impede PGs' pectin-depolymerizing activity. Plant PGIPs are located on the cell wall and their potential to suppress PG activity is correlated with plant disease resistance (Ge et al., 2019).

PGIPs are highly conserved proteins (Di Matteoet al., 2003). So far, PGIPs have been reported in every characterized plant species or mutant (Kalunke et al., 2015). Most PGIPs are generally intronless, except a few that include a short intron (Kalunke et al., 2015). PGIPs, like many other resistance gene products, contain extracellular type leucine-rich repeats (eLRRs) (Di Matteo et al., 2003; Kalunke et al., 2014). PGIPs are composed of 10 incomplete LRRs of approximately 24 residues each, which are arranged into two *ß*-sheets. β1 occupies the inner concave side of the molecules, while β2 occupies the outer convex side. These repeats form *ß*-sheet/β-turn/α-helix containing LRR motifs. Motifs that occupy the β1 inner concave side are critical for interaction with PGs.

Albersheim and Anderson, 1971 were the first to report *PGIP* gene activity in 1971. The first *PGIP* gene, however, was isolated in French beans 20 years later (Toubart et al., 1992). Several *PGIP* genes have been identified in several crops based on sequence identity since 1971. Except for some members belonging to Brassicaceae (Hegedus et al., 2008), most *PGIP* genes do not undergo large expansion and may exist as single gene per genome (Di Giovanni et al., 2008), or clustered into small gene families (Ferrari et al., 2003). In legumes, *PGIP* genes have been characterized in *Glycine max*, *Medicago sativa*, *Medicago truncatula*, *Phaseolus acutifolius*, *Phaseolus coccineus*, *Phaseolus lunatus*, *Phaseolus vulgaris*, *Pisum sativum*, and *Vigna radiata* (Veronico et al., 2011; Gao and Gui, 2015; Kalunke et al., 2015; Matsaunyane et al., 2015; Kaewwongwal et al., 2017). However, only *M. sativa*, *M. truncatula, V. radiata, P. vulgaris,* and *G. max's* genome have more than one *PGIP gene* (Kalunke et al., 2015; Wang et al., 2022).

Pathogen PG inhibition by PGIPs is well established. PGIPs like other defense molecules can be used against pathogens and pests (Hamera et al., 2014; Zhou et al., 2017). The majority of the identified legume PGIPs inhibited fungal infections, such as *G. max's* GmPGIP7 (D'Ovidio et al., 2006; Frati et al., 2006; Kalunke et al., 2014), *M. truncatula's* MtPGIP1, MtPGIP2 (Song and Nam, 2005), *P. vulgaris's* PvPGIP1, PvPGIP 2, PvPGIP3, PvPGIP 4 (Desiderio et al., 1997; D'Ovidio et al., 2006; Frati et al., 2006), *P. acutifolius*'s PaPGIP2, *P. coccineus*'s PcPGIP2, *P. lunatus's* PlPGIP2 (Farina et al., 2009), and *Brassica napus*, BnPGIPs (Wang et al., 2021). However, PGIPs from *V. radiata,* VrPGIP1, and VrPGIP2 (Kaewwongwal et al., 2017; Zhang et al., 2021) and *Brassica rapa* ssp. *pekinensis* BrPGIPs (Haeger et al., 2021) have shown to inhibit insects and *P. sativum* PsPGIP inhibited nematodes (Veronico et al., 2011).

Some *PGIP* genes are expressed in untreated conditions when plants are not stressed, while others respond to external cues. Pathogens and pests such as fungi, oomycetes, insects, and nematodes are known to induce *PGIP* gene expression, as are phytohormones such as abscisic acid (ABA), indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) (Ferrari et al., 2003; Hwang et al., 2010; Hou et al., 2015). *PGIP* gene expression is also triggered by wounding and oligogalacturonic acid treatments (Ferrari et al., 2003; Di Matteo et al., 2006). *PGIP* genes/gene families expression is tissue-specific and developmentally regulated (Li and Smigocki, 2016), studies conducted with basal transcript levels of *B. napus PGIPs* (Hegedus, et al., 2008), *P. vulgaris PGIPs* (Kalunke et al., 2011), and *C. papaya PGIPs* (Broetto et al., 2015) indicate *PGIPs* are expressed in untreated conditions when plants are not stressed.

Currently eighteen PGIPs have been either computationally or biochemically characterized from nine legume species, but major legumes such as chickpeas, peanuts, and lentil PGIPs remain uninvestigated. This study focuses on PGIPs of chickpeas because chickpeas are the world's second most widely produced and consumed leguminous crop, chickpeas have a high protein content (up to 40% protein by weight), are an excellent source of essential vitamins such as riboflavin, niacin, thiamin, folate, and the vitamin A precursor *ß*-carotene, and have other potential health benefits such as lowering cardiovascular, diabetic, and cancer risks (Jukanti et al., 2012; Sharma et al., 2013; Merga and Haji, 2019). Previous publications only report that the chickpea genome harbors two PGIPs (CaPGIP1 and CaPGIP2) on chromosome 6 (Kalunke et al., 2014; Kalunke et al., 2015). Therefore the goal of this study is to investigate PGIPs in chickpeas to gain a better understanding of their structural features, functional domains, regulatory elements, and genomic organization. *CaPGIP* genes were cloned, and their sequence features were evaluated in this study. The basal expression of all *CaPGIPs* was explored. Our findings revealed that *CaPGIPs*, like other legume *PGIPs*, had similar characteristics and can play an essential role in plant resistance against pathogens and pests.

2 Materials and methods

2.1 Sequence acquisition, phylogeny, and bioinformatics analysis

To identify PGIP homologs in the chickpea genome, a homology search was performed against the NCBI database using the amino acid sequences of previously known legume PGIPs. SignalP 5.0 was used to identify the presence of signal peptides in the candidate genes identified by the NCBI homology search (Armenteros et al., 2019). The molecular

weight and isoelectric point (pI) were determined using ExPASy Server (Gasteiger et al., 2005). NetNGlyc version 1.0 server was used to analyze the putative N-linked glycosylation sites (Gupta & Brunak, 2002). The Swiss-Model server was used to build homology-based 3D models of *CaPGIPs* (Waterhouse et al., 2018). Protein sequences were aligned using ClustalW through the MEGA X program (Kumar et al., 2018). Jalview was used for multiple sequence alignment with a conservation index of 50% (Waterhouse et al., 2009). A phylogenetic tree was generated using MEGA X (Kumar et al., 2018) with the neighbor-joining phylogenetic statistical method, Poisson model and other settings retained at default. The tree was bootstrapped 1000 times for robustness and *Cucumis sativus* PGIPs (CsPGIPs) were used as outgroup. MEGA X generated trees were visualized using the iTOL version 6.1.1 online tool (Letunic and Bork., 2021). The 1,500 bp upstream sequence for all CaPGIP sequences was analyzed for the putative cis-acting regulatory DNA elements using New PLACE (Higo et al., 1999).

2.2 Plant materials

Chickpea (*Cicer arietinum*) cultivar Dwelley was grown in greenhouse conditions. Plants maintained in the greenhouse at 22°C ± 2°C. Leaf, stem, root, flower, pod, and seed tissues were collected at different chickpea growth stages, which are mentioned in Table 1. Tissue samples (100 mg) were collected in three biological replicates and were immediately snap-frozen in liquid nitrogen and stored at −80°C.

2.3 Cloning and sequencing

The total RNA was extracted from the leaves of chickpea cultivar Dwelley using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from 100 mg samples in accordance with the manufacturer's protocol. First strand cDNA synthesis and genomic DNA elimination were performed simultaneously using 5X All-In-One RT MasterMix, containing AccurT Genomic DNA Removal (Applied Biological Materials Inc., Richmond, Canada). cDNA samples were stored at−80 °C until use. Full-length ORFs were amplified with Phusion® High-Fidelity DNA Polymerase (NEB, Ipswich, MA, United States) using gene-specific primer pairs (Supplementary Table S1) using the following protocol: initial denaturation at 98°C for 30 s and 35 cycles of 98°C for 10 s, 60°C and 72°C for 30 s each and followed by a final elongation at 72°C for 8 min. Amplified PCR products with appropriate expected sizes were purified with the Monarch® DNA Gel Extraction Kit (NEB). Purified PCR products were cloned into the pMiniT 2.0 vector (NEB) and transformed into DH10B high-efficiency *E. coli* competent cells (NEB). The plasmids were recovered from *E. coli* using PureYield™ Plasmid Miniprep (Promega, Madison, WI, United States), verified using Sanger sequencing (Laboratory of Biotechnology & Bioanalysis, Pullman, WA, United States), and compared to the GenBank sequences of *CaPGIP1* (XM_004504675), *CaPGIP3* (XM_004493500), and *CaPGIP4* (XM_012713804).

2.4 RNA isolation, cDNA conversion and quantitative real-time-PCR

To determine the absolute expression of *CaPGIP* genes in untreated conditions, total RNA was extracted from various chickpea tissues from the cultivar Dwelley at different growth stages (Table 1). Based on the timing of infection by the major chickpea fungal pathogens, four growth stages (V1, V6, R1, and R4) were selected (Mazur et al., 2002; West et al., 2003; Bretag and

TABLE 1 Chickpea tissues collected during different chickpea growth stages for absolute gene expression quantification.

Growth stage	Stage	Stage description	Tissue collected	Days after sowing
Vegetative growth stage	V1	First multifoliate leaf has unfolded from the stem	Leaf	10
			Root	10
			Stem	10
	V6	Sixth multifoliate leaf has unfolded from the stem	Leaf	25
			Root	25
			Stem	25
Reproductive growth stage	R1	Early bloom, one open flower on the plant	Flower	55
			Leaf	55
			Root	55
			Stem	55
	R4	Flat pod, pod has reached its full size and is largely flat	Leaf	80
			Pod	80
			Root	80
			Stem	80
			Seed	80

Horsham, 2004; Markel et al., 2008; Jiménez-Fernández et al., 2011; Moore et al., 2011; Khan et al., 2012; Wunsch, 2014; Knights and Hobson, 2016). RNA was extracted from 100 mg samples using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA synthesis and genomic DNA elimination were performed simultaneously using 5X All-In-One RT MasterMix, containing AccurT Genomic DNA Removal (Applied Biological Materials, Inc.). To preserve sample integrity, RNA extraction, genomic DNA removal, and cDNA synthesis were performed on the same day. Samples were stored at −80°C until use. Using Primer3Plus (Untergasser et al., 2007) at the default parameters, quantitative PCR primers (Supplementary Table S1) were generated based on the sequences of *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* and the reference gene 18SrRNA and 25SrRNA. The NCBI Primer-BLAST (ncbi.nlm.nih.gov/tools/primer-blast/) program was used to ensure that primers are unique specifically to the corresponding gene. Primers were referenced against the chickpea genome ASM33114v1. Standard curves generated by serial dilution of cDNA for 18SrRNA, 25SrRNA, CaPGIP1, CaPGIP3, and CaPGIP4 were used to evaluate primer efficiency (Supplementary Figure S1). Transcript levels of chickpea *PGIPs* (*CaPGIP1*, *CaPGIP3*, and *CaPGIP4*) in chickpea at untreated conditions were evaluated following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). Each RT-qPCR reaction consisted of 1x SsoAdvancedTM universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad, Hercules, CA, United States), 2.5 µM of each gene-specific primer, and cDNA converted from 100 ng RNA in a final reaction volume of 10 µL. No template control (NTC), no amplification control (NAC), and negative reverse transcription (NRT) controls were included for each primer pair, and all reactions were performed with three separate biological replicates in technical triplicates. qPCR was carried out in the CFX96TM Real-Time PCR Detection System using a two-step amplification and melt curve method with the following protocol: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s; 60°C for 30 s; and 72°C for 30 s. Melt curve readings were taken from 65.0°C to 95.0°C with an increment of 0.5°C every 5 s. The absolute gene expression assays were performed by constructing standard curves of the corresponding cloned coding region of *CaPGIPs* (Wong and Medrano, 2005). The expression Ct values of *CaPGIPs* were normalized against the expression Ct values of reference genes 18SrRNA and 25SrRNA. Quantification was done using the relative standard curve method (Supplementary Figure S1) (Pfaffl, 2001). The *CaPGIP* expression values are given as the mean of the normalized expression values of *CaPGIPs* normalized against reference genes 18SrRNA and 25SrRNA. Obtained *CaPGIP* data is shown as gene copy number/microgram of RNA (Forlenza et al., 2012). The statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey's *ad hoc* testing using the PROC GLM program in Statistical Analysis System (SAS) version 9.4 (SAS Institute Inc, 2013).

2.5 Subcellular localization

DeepLoc-1.0 (Almagro Armenteroset al., 2017) was used to predict subcellular localization based on CaPGIP protein sequences. The complete coding sequences (CDS) of *CaPGIP1*, *CaPGIP2*, *CaPGIP3*, and *CaPGIP4* genes were cloned into pEarleyGate 103 (ABRC) using the gateway cloning approach to determine their subcellular localization. After sequencing validation, these gateway plant expression vectors were transformed into *Agrobacterium tumefaciens* strain EHA105. Using a blunt syringe, transformed EHA105 cultures harboring *CaPGIP*-mGFP plasmids were infiltrated into 4-week-old *N. benthamiana* leaves, two biological replicates along with two technical replicates per gene were infiltrated and imaged. A laser scanning confocal microscope (Leica SP-8) was used to examine and capture the fluorescence emitted by fusion proteins 72 h after infiltration. GFP fluorescence was excited at 488 nm.

3 Results

3.1 Insilco characterization of CaPGIPs

CaPGIP1 (LOC101505245) and *CaPGIP2* (LOC101504619) are two previously reported chickpea *PGIPs* (Kalunke et al., 2014; Kalunke et al., 2015). They occupy a 30,150 bp region on chromosome 6. *CaPGIP1* has a single exon with no intron as seen in several legume *PGIPs*. While *CaPGIP2* has two exons separated by a 9,825 bp intron. A homology search against the NCBI chickpea genome assembly ASM33114v1 using the amino acid sequences of known legume PGIPs revealed the presence of additional five candidate *PGIP* sequences (Supplementary Table S2). Only two of them, LOC101499240 and LOC101499557, were suitable for designation as prospective *PGIPs* since they were the appropriate size, had a signal peptide, and had ten LRR sequences. They were named *CaPGIP3* and *CaPGIP4* respectively. *CaPGIP3* and *CaPGIP4* are composed of a single exon with no introns and span a 15,582 bp region on chromosome 3. In addition to the previously known locus of *PGIP* genes (Kalunke et al., 2014; Kalunke et al., 2015), our analysis identifies a new locus. The occurrence of two *PGIP* loci in chickpeas, chromosome 3 and chromosome 6, necessitates a new genomic organization of chickpea *PGIP* genes (Figure 1). Full-length cDNA size (bp), ORF size (bp), predicted protein size (aa), predicted signal peptide size (aa), theoretical molecular mass (kDa), and pI for all four CaPGIPs are presented in Table 2.

Sequence analysis was conducted for all four CaPGIPs. Predicted proteins of CaPGIP1, CaPGIP3, and CaPGIP4 exhibited a typical PGIP sequence identity. SignalP 5.0 (Armenteros et al., 2017) projected 36, 22, and 20 amino acid signal peptides for CaPGIP1, CaPGIP3, and CaPGIP4, respectively (Table 2). As illustrated in Figure 2, the predicted mature protein sequences for CaPGIP1, CaPGIP3, and CaPGIP4 featured an N-terminal domain, a central LRR domain, and a C-terminal domain. These tandemly repeated LRRs fold into a characteristic curved and elongated PGIP shape. As observed in homology 3D models generated using PvPGIP2 as a template for CaPGIP1, CaPGIP3, and CaPGIP4 (Figure 3), the secondary and tertiary structures of CaPGIP1, CaPGIP3, and CaPGIP4 indicate that all 10 LRRs contain an *ß*-turn motif (xxLxLxx) that folds into β1 sheets. β1 sheets occupy the PGIP scaffold's inner concave face, which is the site for PG

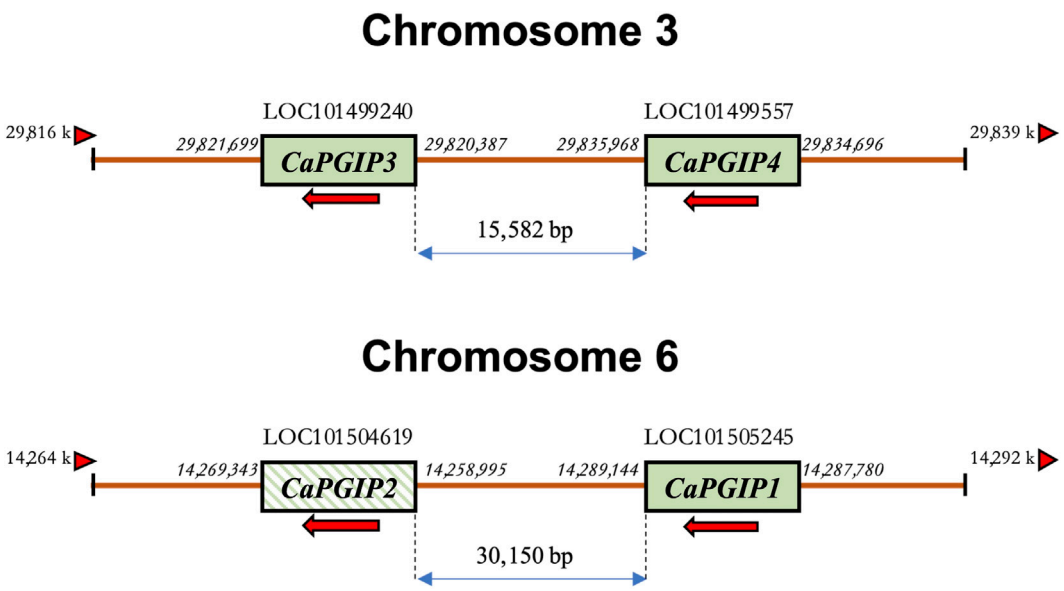

FIGURE 1
Schematic representation of the revised genomic organization of the *PGIP* gene family in *Cicer arietinum* showing two loci for *CaPGIPs*, chromosomes 3 and 6. The numbers on the side of genes represent their start and stop codon location on the genome. The numbers between the genes denote distances in base pairs. Arrows indicate the transcriptional direction of coding regions. The arrowhead triangles indicate the orientation/direction of the DNA strand on which the genes are placed. Crossed box for *CaPGIP2* indicates the presence of an intron within the gene.

interaction. Aside from that, all LRRs have β2 sheets and 3_{10}-helixes. PGIPs are glycoproteins with N-glycosylation sites ((N–x–S/T; where N is asparagine, x can be any amino acid except proline (P), S is serine, and T is threonine). As a result, the NetNGlyc version 1.0 server predicted five, three, and eight N-glycosylation sites in CaPGIP1, CaPGIP3, and CaPGIP4 proteins, respectively. CaPGIPs contain several conserved cysteine residues. CaPGIP1 has eight, CaPGIP3 has ten, and CaPGIP4 has nine cysteine residues.

Interestingly, CaPGIP2 (Figures 2, 3), on the contrary, lacked many of the above sequence identities. The absence of signal peptide suggested it is not a secretory protein. Its secondary structure reveals that it lacked more than half of the LRR modules, with just the 6th to 10th LRRs. This short LRR sequence on CaPGIP2's C-terminal perfectly matches CaPGIP1's C-terminal. The CaPGIP2 homology 3D model indicated the absence of the distinctive concave face that harbors PG interaction sites (Figure 3). CaPGIP2 has one N-glycosylation site and five cysteine residues, fewer than its counterparts.

3.2 Sequence comparison and phylogenetic analysis of theCaPGIP proteins

Multiple sequence alignment showed that CaPGIP amino acid sequences are highly similar to those of other legumes such as soybean, common bean, runner bean, tepary bean, lima bean, barrel clover, peas, mung bean, and alfalfa, with the presence of five conserved cysteine residues shared by all. The higher similarity was observed in the β2-sheet regions, along with variable portions present in both *ß*-sheets, as evident in plant-specific LRR proteins (Figure 4). This sequence alignment demonstrates that PGIPs are highly conserved within the legume family.

In the phylogenetic analysis (Figure 5), CaPGIPs were compared to 45 other known PGIPs from various crop families. The tree (Figure 5) comprises five main branches that are separated into monocots and dicots. The Poaceae family is represented by one cluster, the majority of the legume PGIPs are represented by a second cluster, three PGIPs from *Beta vulgaris* are represented by a third cluster. CaPGIP3, and PGIPs from *V. radiata* are represented

TABLE 2 NCBI accession number, full-length cDNA size (bp), ORF size (bp), predicted protein size (aa), predicted signal peptide size (aa), theoretical molecular mass (kDa), and pI for all four *CaPGIPs*.

Name	NCBI accession	Gene symbol	cDNA (bp)	ORF (bp)	Protein (aa)	Signal peptide (aa)	Molecular mass (kDa)	Isoelectric point (pI)
CaPGIP1	XM_004504675	LOC101505245	1365	1041	347	36	37.65	9.05
CaPGIP2	XM_027335237	LOC101504619	523	492	164	NA	18.18	5.64
CaPGIP3	XM_004493500	LOC101499240	1313	1005	335	22	37.61	8.44
CaPGIP4	XM_012713804	LOC101499557	1273	987	329	20	36.19	6.64

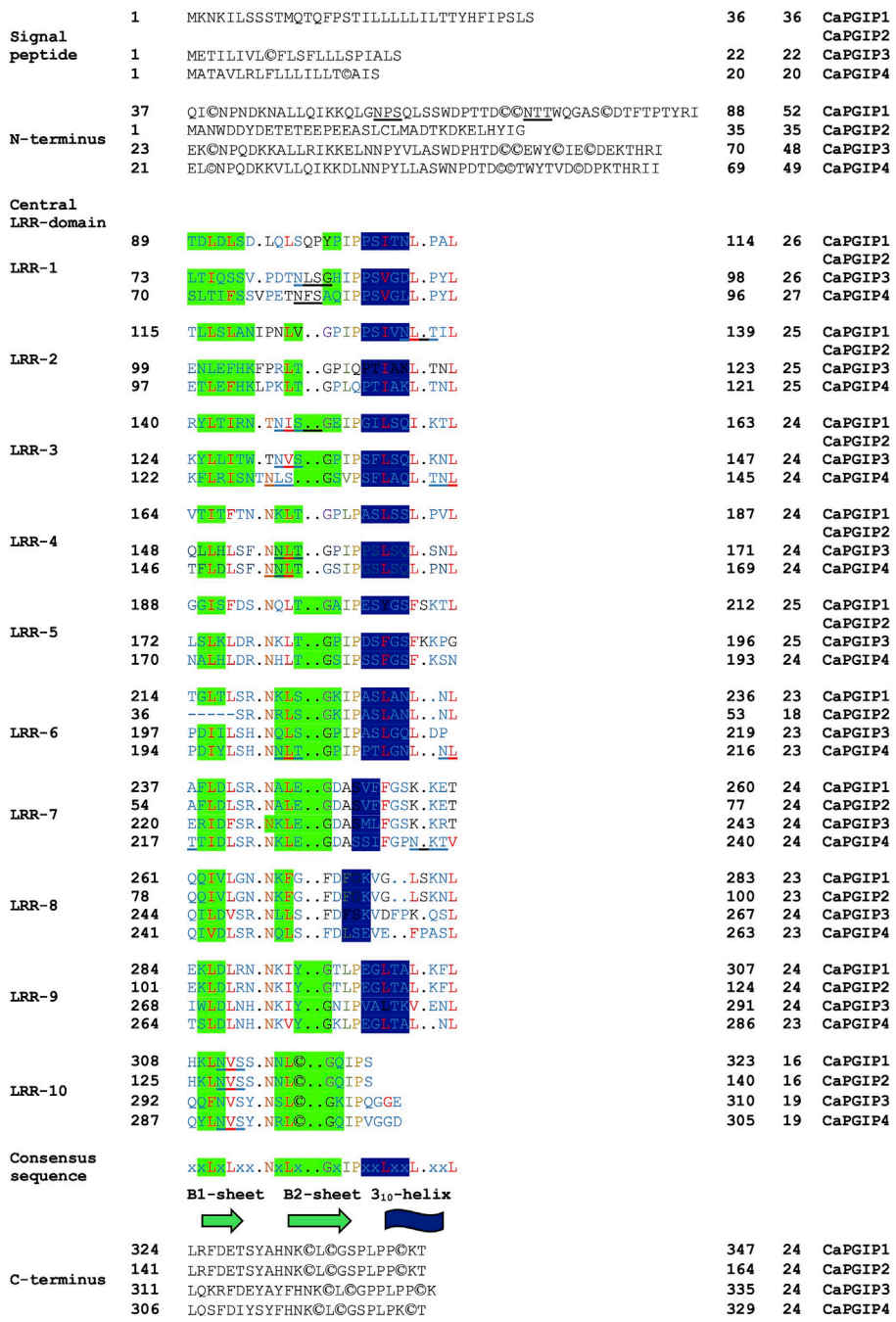

FIGURE 2
Translated structure of CaPGIP1,CaPGIP2,and CaPGIP4 based on PvPGIP2.A)Signal peptide,B)N-terminal domain,C)central LRK domain and D) C-terminal domains are indicated.Secondary structure elements (sheet B1,B2,and 310-helix) are highlighted.Five N-glycosylation sites (N-X-S/T) are underlined,cysteine residues are encircled.

by a fourth cluster, and the remaining PGIPs from other families, such as Actinidiaceae, Apocynaceae, Brassicaceae, Caricaceae, Cucurbitaceae, and Malvaceae, form the final cluster. CaPGIP1 and CaPGIP2 are members of the Leguminosae cluster and exhibit significant similarities to pea PGIP, PsPGIP1. CaPGIP3 and CaPGIP4 are outside the Leguminosae cluster, with CaPGIP3 sharing a high degree of similarity with its other legume counterparts, *V. radiata* PGIPs, VrPGIP1, and VrPGIP2. CaPGIP4 is in a separate cluster that includes PGIPs from various plant families.

3.3 Promoter analysis of *CaPGIPs*

To locate regulatory DNA elements, the 1,500 bp upstream sequence for all *CaPGIP* genes was analyzed. The TATA box and

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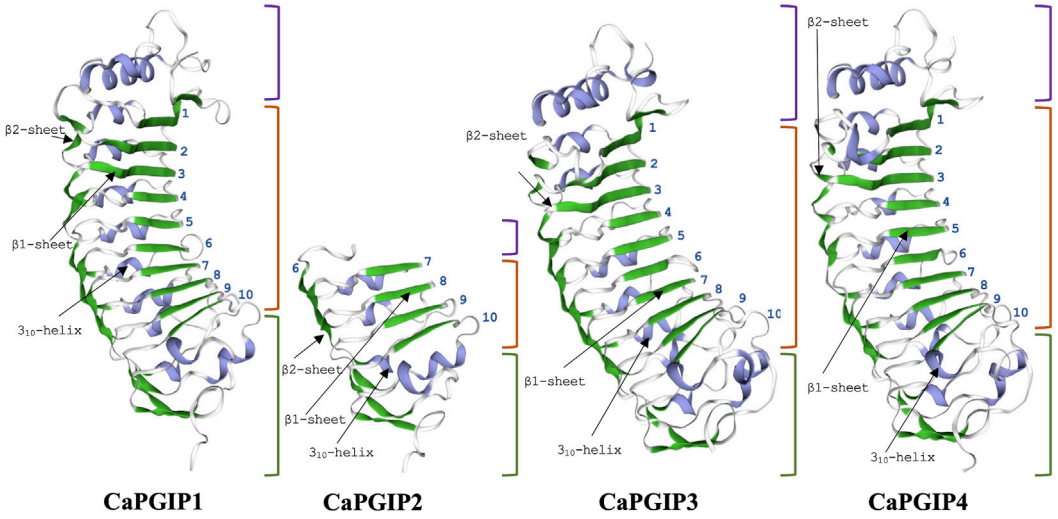

FIGURE 3
Homology 3D model of CaPGP1,CaPGIP2, CaPGIP3 and CaPGIP4 using PvPGIP2 as a template,b1, and b2 sheets are indicated by green color.310-helixes are indicated with purple color. purple-colored brackets indicate N-terminal domains,orange-colored brackets indicate central LRR domain with LRR numbers marked and gren-colored brackets indicate C-terminal domain.

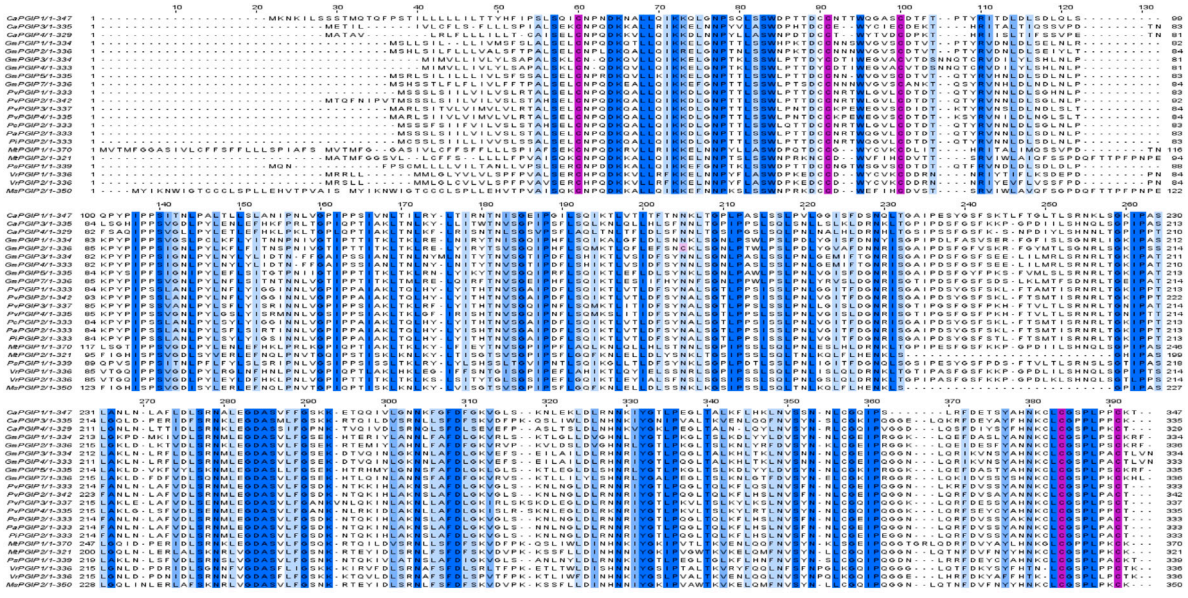

FIGURE 4
Multiple sequence alignment of chickpeas PGIPs[Capgip1(XP_004504732.1),Capgip3(XP_004493557.1),Capgip4(XP_012569258.1)], with other legume PGIPs such as soybean [GmPGIP1(XP_003524772.1),GmPGIP2(CAI99393.1), GmPGIP3 (NP_001304551.2), Gm PGIP4(CAI99395.1),GMPGIP5(XP_003524769.1),GmPGIP7(XP_003531070.1)], common bean [PvPGIP1(CAI11357.1),PvPGIP2(P58822.10,PvPGIP3(CAI11359.1),PvPGIP4(CAI11360.1)], runner bean [PcPGIP2(CAR92534.1)], tepary bean[PAPGIP2(CAR92533.1)], lima bean [PiPGIP2(CAR92537.1)], barrel clover [MtPGIP1(XP_003625218.1),MtPGIP2(XP_024626259.1)], peas [PsPGIP1(AJ749705.1)]mung bean[VrPGIP1(ATN23902.1),VrPGIP2(ATN23895.1), and alfalfa[MsPGIP2(ALX18673.1)]Blue color bar indicates conservation more than 50%, brighter the bar more the conservation, pink color bar indicates cysteine residues.

CAAT box motifs were discovered close to the start codons. CAAT and TATA box sequences were found at −40 and −229 upstream of *CaPGIP1*, respectively, and at −23 and −55 upstream of *CaPGIP3*. The TATA box was located at a −30 position in the *CaPGIP4* upstream sequence, whereas CAAT was at a −37 position. Elements/motifs associated with plant responses to hormones such as abscisic

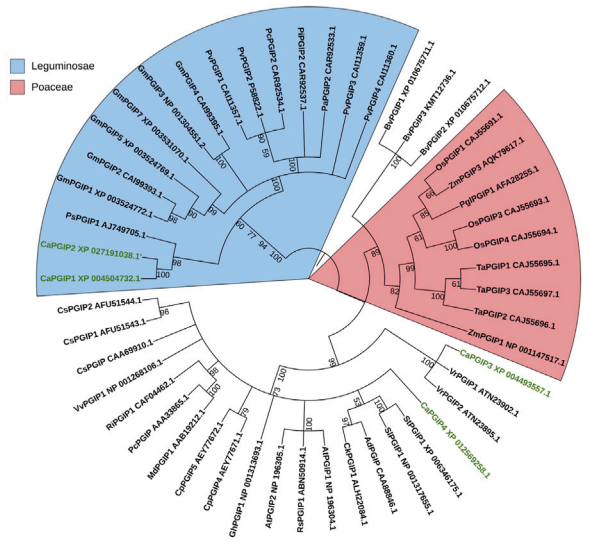

FIGURE 5
Phylogenetic tree illustrating the relationship between several PGIPs from various crop families and CaPGIPs.CaPGIPs are highlighted in green the values on the branches correspond to bootstrap values. Cucumis sativus PGIPs(CsPGIPs) are used as outgroup.

acid, gibberellic acid, jasmonic acid, and salicylic acid were also identified. Motifs for wounding response were identified as well. Crucially, numerous elements associated with pathogenicity responses were identified in the promoter regions of the *CaPGIPs*. Table 3 lists these putative cis-acting regulatory elements, as well as their locations and roles. Aside from the motifs mentioned above, additional cis-elements known to mediate tissue-specific activity and plant physiological processes were identified. Stress-related cis-acting regulatory elements associated with drought, dehydration, water, high light, and low-temperature stress, were also identified. All these elements are listed in Supplementary Table S3.

3.4 Cloning and characterization of *CaPGIPs*

CaPGIP genes were cloned and sequenced from the chickpea cultivar "Dwelley". *CaPGIP1* sequence matched GenBank sequence XM 004504675. However, a C was replaced by a T at the 720th nucleotide position, which was a synonymous substitution with no change in the coded amino acid. *CaPGIP3* sequence matched the GenBank sequence XM 004493500, and *CaPGIP4* sequence matched the GenBank sequence XM 012713804. Transcripts for *CaPGIP2* could not be amplified even with different sets of primers; hence, all subsequent investigations focused on *CaPGIP1*, *CaPGIP3*, and *CaPGIP4*.

3.5 Subcellular localization of *CaPGIPs*

DeepLoc-1.0 uses sequencing information to predict the subcellular localization of plant proteins. Based on the presence of signal peptides, it was inferred thatCaPGIP1, CaPGIP3, and CaPGIP4 were secretory and classified as extracellular proteins. CaPGIP2, on the other hand, was predicted to be found in the mitochondrion and cytoplasm. To validate those predictions, *Agrobacterium* cells carrying binary vectors of CaPGIPs and GFP fusions were infiltrated into *N. benthamiana* leaves for transient expression of encoded proteins in leaf mesophyll and epidermal cells. According to the excitation curves, the fluorescence of CaPGIPs-GFP fusion proteins was the same as GFP. CaPGIP1, CaPGIP3, and CaPGIP4 fluorescence was visible on the cell boundaries (Figure 6). As a result, they were most likely found on the cell wall or plasma membrane. CaPGIP2-GFP fluorescence was seen inside the cell in the cytoplasm and endoplasmic reticulum, and CaPGIP2 was most likely found in the cytoplasm and endoplasmic reticulum (Figure 6).

3.6 Absolute quantification of *CaPGIP's* transcripts

CaPGIP transcript levels were investigated at four growth stages using the indeterminate type of Kabuli chickpea variety Dwelley, which matures in 110–120 days. RT-qPCR was utilized to determine the absolute *CaPGIPs* expression levels (Table 1). Transcripts for *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* were ubiquitously detected in all the studied tissues. In the vegetative stages V1 and V6 stages were investigated (Figure 7). *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* transcript levels were higher in the V1 leaf compared to the root and stem. In V1 leaf *CaPGIP4* expressed significantly higher, followed by *CaPGIP3*, then *CaPGIP1*. For stem in the V1 stage, similar to leaf *CaPGIP4* expressed significantly higher, followed by *CaPGIP3*, then *CaPGIP1*. Roots in the V1 stage had a different expression pattern, where *CaPGIP3* expressed significantly higher, followed by *CaPGIP1*, then *CaPGIP4*. *CaPGIPs* expression was lower in the V6 vegetative stage compared to V1. In contrast to V1, the transcript levels of *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* were higher in the V6 stem compared to the leaf and root. In V6 stem *CaPGIP4* expressed significantly higher, followed by *CaPGIP3*, then *CaPGIP1*. For roots in the V6 stage, *CaPGIP4* and *CaPGIP3* expression levels were statistically similar and higher than *CaPGIP1*. For V6 leaves, *CaPGIP1* and *CaPGIP3* expression was statistically similar and lower than *CaPGIP4*.

R1 and R4 were investigated in the reproductive stages, and the transcript levels were analyzed for flower, pod, and seed along with leaf, stem, and root (Figure 8). All *CaPGIPs* in leaves showed higher expression levels in the R1 stage compared to other tissues. In the R1 leaf, *CaPGIP3* expressed significantly higher, followed by *CaPGIP4*, then *CaPGIP1*. *CaPGIP1* and *CaPGIP4* expression levels were statistically similar and lower than *CaPGIP3 in* the R1 root. For stem and flowers in R1, *CaPGIP4* had a higher expression, followed by *CaPGIP3* and then *CaPGIP1*. Similar to the R1 stage, all *CaPGIPs* in R4 leaves expressed at higher levels than other tissues. In the R4 leaf, *CaPGIP4* expressed significantly higher, followed by *CaPGIP3*, then *CaPGIP1*. In R4 roots, *CaPGIP3* expressed significantly higher, followed by *CaPGIP1*, then *CaPGIP1*. For the R4 stem, *CaPGIP1* and *CaPGIP3* expression levels were statistically similar and lower than *CaPGIP4*. For

TABLE 3 Putative hormonal and pathogenesis related *cis*-acting regulatory elements identified in the promoter regions of *CaPGIPs*.

Cis - element	Position			Signal sequence	Function	References
	CaPGIP1	*CaPGIP3*	*CaPGIP4*			
ABRELATERD1	—	287(−),288(+),1415(−)	561(−),1132(−),1167(+)	ACGTG	Abscisic acid response	Nakashima et al., 2006
ABRERATCAL	—	286(−),287(+),1414(−)	560(−),1131(−),1166(+)	MACGYGB	Abscisic acid response	Kaplan et al., 2006
ACGTABRE	—	—	559(−)	ACGTGKC	Abscisic acid response	Hattori et al., 2002
ARFAT	—	—	857(+)	TGTCTC	Abscisic acid response	Nag et al., 2005
ARR1AT	320(−), 600(+),622(+), 637(−),969(−),990(−), 1008(−),1023(−),1054(−), 1223(−)	151(−),1293(−),1410(−), 1435(+),1488(−)	98(−),113(+),190(−),245(−), 355(+),371(−),872(+),896(−), 978(+),1106(+),1193(−), 1196(+),1275(−), 1360(+), 1446(−)	NGATT	Bacterial response	Ross et al., 2004
BIHD1OS	198(−),1337(−)	483(+),661(+),809(−), 1044(−)	1300(−),1369(+)	TGTCA	Pathogen response	Luo et al., 2005
GADOWNAT	—	—	559(−)	ACGTGTC	Abscisic acid response	Nakashima et al., 2006
GAREAT	123(+),144(+)	—	166(+),741(−)	TAACAAR	Gibberellic acid response	Ogawa et al., 2003
GT1CONSENSUS	209(+),236(+),283(+),356(+),402(−),422(−), 430(−),714(+),754(−), 843(+),1091(−),1092(−), 1130(−),1146(−), 1203(+),1369(−)	26(+),33(+),54(−), 73(+),87(+),391(−), 429(+),477(+),575(−), 612(+),668(−),712,(+)	53(+),66(−),86(−),106(−), 279(+),314(+),330(−),787(−), 799(−),907(+),908(+), 1021(−),1022(−),1031(−), 1046(−),1456(−),1470(−)	GRWAAW	Salicylic acid response	Buchel et al., 1999
		1102(−),1116(−),1136(−), 1310(−),1332(+),1465(−)				
GT1GMSCAM4	209(+),422(−),1091(−), 1146(−),1203(+),1369(−)	26 (+),87(+),391(−), 612(+),1136(−)	53(+),314(+)	GAAAAA	Pathogen response	Park et al., 2004
		1310(−),1332(+)	799(−),1470(−)			
SEBFCONSSTPR10A	—	660(+),783(−)	856(+)	YTGTCWC	Pathogen response	Boyle & Brisson, 2001
T/GBOXATPIN2	—	1415(−)	561(−),1132(−),1166(+)	AACGTG	Jasmonate response	Boter et al., 2004
WBOXNTERF3	—	—	412(−), 780 (−),1060(−), 1119 (+), 1293 (+), 1307 (+)	TGACY	Wound response	Nishiuchi et al., 2004

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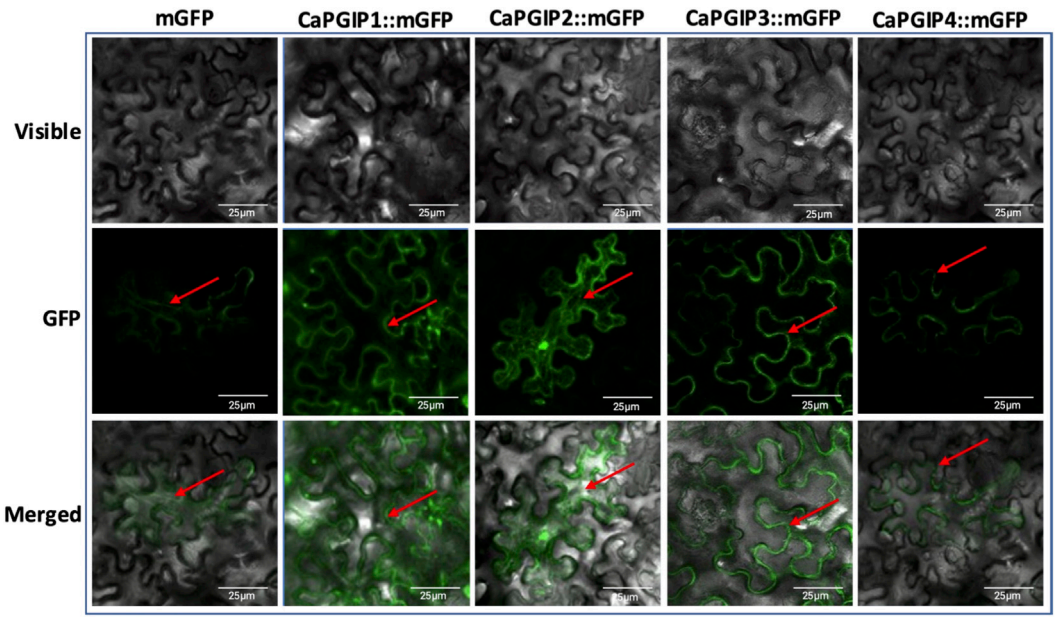

FIGURE 6
Subcellular localization of Capgips. Full-length Capgips fused with a green fluorescent protein (GFP) are transiently expressed in Nicotiana benthamiana leaves by agroinfiltration. The images show the fluorescence emitted by fusion proteins was captured 72 h after infiltartion using a laser scanning confocal microscope as mGFP fluorescence in green color, visible light in brightfield images, merged as merged images of mGFP and visible. Cells transformed with mGFP is the control. Two biological replicates along with two technical replicates per gene were infiltrated and imaged. Red arrows indicate localization.

pods and seeds in R4, *CaPGIP4* had a higher expression, followed by *CaPGIP3* and then *CaPGIP1*. Overall, the highest expression levels for all *CaPGIPs* were found in the leaves. Additionally, *CaPGIP4* showed the highest expression among the three genes, with levels about one and a half times higher than *CaPGIP3* and double the expression of *CaPGIP1*.

4 Discussion

Several plant species have PGIP-encoding small gene families; these multigene families encode proteins with similar LRR domains but different PG-inhibitory capabilities (D'Ovidio et al., 2006). This study demonstrated that the chickpea genome has a *PGIP* family of potentially three functional genes (*CaPGIP1*, *CaPGIP3*, and *CaPGIP4*) that are present on two chromosomes. Soybean is the only other legume where *PGIPs* are located on multiple chromosome (D'Ovidio et al., 2006). Like the genomic distribution of *CaPGIPs,* legume *PGIPs* are distributed across a broader genomic region, as observed in soybean, where *GmPGIP1*, *GmPGIP2*, and *GmPGIP5* are located on chromosome 5, and span a ~ 19.5 kbp region, while *GmPGIP3*, *GmPGIP4*, and *GmPGIP7*, present on chromosome 8, span a ~ 21 kbp region. In common bean, *PvPGIP1*, *PvPGIP2*, *PvPGIP3*, and *PvPGIP4* span an area of ~ 50 kbp on chromosome 2 (D'Ovidio et al., 2004; D'Ovidio et al., 2006).

So far, over eighteen legume PGIPs have been either computationally or biochemically characterized in 9 species. The reported sequence lengths of these legume PGIPs ranged from 321 amino acids for MtPGIP2 to 342 amino acids for MtPGIP1. All these PGIPs contain signal peptides, with GmPGIP4 having the shortest with 17 amino acids and PvPGIP2 having the longest with 29 amino acids. Protein molecular mass (kDa) ranged from 35.92 for MtPGIP2 to 38.17 for MtPGIP1. And the isoelectric point (pI) ranged from 6.79 for PsPGIP1 to 9.48 for PvPGIP4. Except for MsPGIP2 of alfalfa, all other legume PGIPs had 10 LRRs and all were intronless. Only MsPGIP2's genomic sequence contains 9 LRRs and a 154-bp intron. CaPGIP1, CaPGIP3, and CaPGIP4 exhibit very similar above-mentioned characteristics, with the exception that CaPGIP1 has the longest signal peptide among the known legume PGIPs so far, with 36 amino acids. Because of selection for transcription efficiency, conserved genes with relatively high levels of expression tend to lose introns (Zou et al., 2011). Intronless genes such as PGIP may play key roles in plant growth, development, or response to biotic or abiotic stresses (Liu et al., 2021).

Like many PGIPs, the central domain of CaPGIP1, CaPGIP3, and CaPGIP4 is comprised of 10 imperfect leucine-rich repeats (LRRs). Each is about 24 amino acids long and perfectly matches the extracellular LRR (eLRRs) consensus sequence xxLxLxx.NxLx. GxIPxxLxxL.xxL (Di Matteo et al., 2003). eLRRs are important in plant defense as they function as receptor-like proteins or receptor-like kinases to recognize diverse pathogen molecules, and plant hormones (van der Hoorn et al., 2005). The presence of xxLxLxxNxL core consensus in eLRRs is responsible for the *ß*–sheet structure formation (Zambounis et al., 2012). The LRRs are arranged tandemly in the PGIP's distinctive curved and elongated shape. The *ß*-sheets are parallelly organized on the

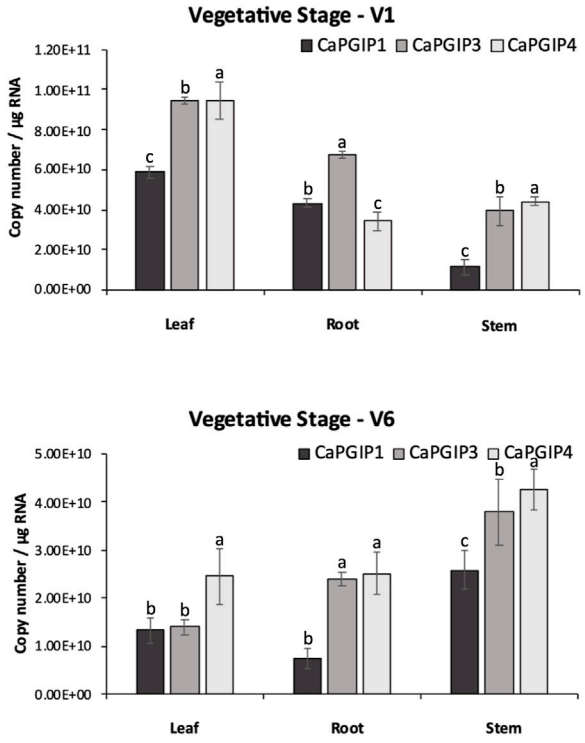

FIGURE 7
Absolute expression of Capgips. The number of transcript copies of Capgip1,Capgip3, Capgip4 was calculated in the leaf, stem, and root at vegetative growth stages V1 and V6. The abundance was normalized by the amount of internal control 18 SrRNA and 25 rRNA.The values are the means of three biological replicates each with three technical replicates. Error bars indicate standard deviation among the biological replicates (N = 3). Different letters indicate significant differences by ANOVA with Tukeys *ad hoc* test($p < 0.05$)

inner side of the protein to form the concave face, while the 3_{10}-helixes are parallelly organized on the outer side to make the convex face (Kobe and Deisenhofer, 1993). Solvent-exposed residues on the concave-sheet surface bind to pathogen molecules. CaPGIPs, have two types of β–sheets (β1 and β2) and 3_{10}-helixes. All LRRs of plant defense proteins have β1 sheet; however, the presence of β2 sheets is unique and seen only in PGIPs (Di Matteo et al., 2003). CaPGIPs are predicted to have several N-glycosylation on these *ß*-sheets which are vital for ligand binding for disease resistance, and the heterogeneity in *ß*-sheet residues or the glycosylation patterns contributes to the varying recognition specificities of LRR proteins (Ramanathan et al., 1997; van der Hoorn et al., 2005). The deduced CaPGIP proteins also contain conserved cysteine residues that form disulfide bridges crucial for the maintenance of secondary structures in PGIP (Veronico et al., 2011).

Previous research indicated that truncated variants of functioning NBS-LRR genes can be found within 100 kb of fully functional NBS-LRR genes. These truncated genes are often pseudogenes because of alternative splicing. These pseudogenes have large deletions due to various transposition events (Marone et al., 2013). CaPGIP2 is found within 30 kb of CaPGIP1. CaPGIP2's C-terminal end matches CaPGIP1 perfectly, implying that the majority of the central LRR domain and N-terminus might be deleted. Also, the presence of a nearly 10-kbp intron is atypical for PGIPs and most functional genes. Since it also lacks a fundamental signal peptide, it is classified as a non-secretory protein. For these reasons, CaPGIP2 was not subjected to investigation in this study beyond the subcellular localization analysis.

Alignment of CaPGIP amino acid sequences with amino acid sequences from various other legume PGIPs revealed that CaPGIPs are similar to those of other characterized PGIPs. Interestingly, alignment also indicated that CaPGIPs along with other legume PGIPs are highly conserved at the β2-sheet sites, which may be because β2-sheet are present only in PGIPs and are absent in other LRR proteins. Even though there is a higher level of similarity in the *ß* sheet regions, there are also many variable portions present in both *ß*-sheets. This variability is most likely responsible for the presence of multiple recognition specificities to target broader pathogen PGs (De Lorenzo et al., 2001; Matsushima and Miyashita, 2012). As per the phylogenetic analysis, CaPGIPs have a high degree of similarity with PGIPs from different plant sources. CaPGIP1 shared up to 70% similarity with *P. sativum* PsPGIP1 (AJI49705.1), and 69% with *G. max* GmPGIP3 (NP 001304551.2). It has been previously reported that PsPGIP1 and GmPGIP3 potentially inhibit several pathogens. For instance, PsPGIP1 has been identified as a possible defense factor against the pea-cyst nematode *Heterodera goettingiana* (Veronico et al., 2011). Encoded protein products of GmPGIP3 inhibited PGs from *Sclerotinia, Fusarium, and Botrytis* (D'Ovidio et al., 2006; Wang et al., 2015). Transgenic wheat expressing GmPGIP3 also showed enhanced resistance to *Gaeumannomyces graminis* var. *tritici*, and *Bipolaris sorokiniana* (D'Ovidio et al., 2006; Wang et al., 2015). Due to CaPGIP1's high sequence similarity to PsPGIP1 and GmPGIP3, it is likely to have similar functions and be engaged in nematode or fungal disease inhibition.

CaPGIP3 is more similar to the two tightly linked PGIPs of *V. radiata*, with 64% and 69% similarity to VrPGIP1 and VrPGIP2, respectively. Both VrPGIPs are known to provide resistance to bruchids (*Callosobruchus spp*) (Kaewwongwal et al., 2017), and CaPGIP3 may play a similar role in chickpeas against bruchids. CaPGIP4 had higher similarity to tree fruit PGIPs, with 72% similarity to *Malus domestica* MdPGIP1 (AAB19212.1) and 71% similarity to *Pyrus communis* PcPGIP (AAA33865.1). MdPGIP1 protein inhibited PG production in *Colletotrichum lupini* and *Aspergillus niger* (Oelofse et al., 2006). Unlike CaPGIP1 and CaPGIP3, CaPGIP4 may only be effective against pathogenic fungi.

The TATA box and CAAT box motifs are widely found in functional gene's promoter and enhancer regions (Xue, 2002; Svensson et al., 2006). TATA boxes function as a motif for recruiting transcription initiation machinery and RNA polymerase II, while CAAT boxes improve protein binding (Joubert et al., 2013; Liao et al., 2015). TATA and CAAT boxes are conserved eukaryotic cis-elements that are found in many plant gene promoters, including *PGIPs*. *CaPGIP1*, *CaPGIP3*, and *CaPGIP4* all have several TATA and CAAT boxes upstream of the start codon ATG, indicating that they are functioning genes. Plant *PGIPs* are typically expressed after pathogen infection and wounding response (Kalunke et al., 2015), hence the presence of multiple pathogenicity-related and wounding motifs in the *CaPGI1, CaPGIP3,* and *CaPGIP4* promoter region. Apart from pathogens, *PGIPs* are triggered by phytohormone treatment in several plant species (Stotz et al., 1993; Yao et al., 1999;

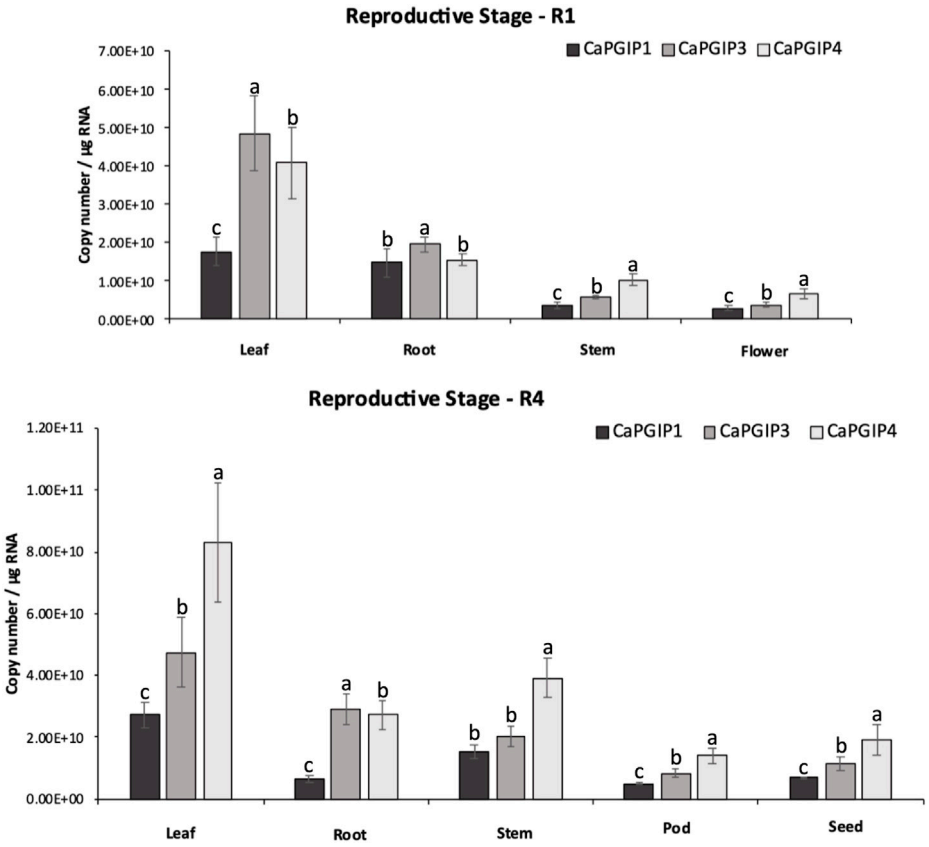

FIGURE 8
Absolute expression of Capgips. The numbers of transcript copies of Capgip1,Capgip3, and Capgip4 were calculated in the leaf, stem, root and flower at reproductive growth stage R1, and leaf, stem,roor, pod and seed at reproductive growth stage R4. The abundance was normalized by the amount of internal control 18 SrRNA and 25 rRNA. The value are the means of three biological replicates each with three technical replicates. Error bars indicate standard deviation among the biological replicates ($N = 3$) Different letters indicate significant differences by ANOVA with Tukeys *ad hoc* test ($p < 0.005$).

Ferrari et al., 2003). *PGIP* expression in rice, alfalfa, and pepper is induced by abscisic treatment. *PGIPs* from rapeseed, rice, barrel clover, and pepper are triggered by jasmonic acid and salicylic acid (Song and Nam, 2005; Janni et al., 2006; Hegedus et al., 2008; Lu et al., 2012; Wang et al., 2013). Rice *PGIPs* are induced by gibberellic acid treatment (Janni et al., 2006; Lu et al., 2012). The presence of multiple cis-acting elements in *CaPGIP1, CaPGIP3,* and *CaPGIP4* that regulate abscisic acid, gibberellic acid, jasmonic acid, and salicylic acid pathways suggests that they may play comparable roles to those seen in many plant *PGIPs*. Like other families of defense-related genes, *PGIPs* demonstrate tissue-specific activity. The grapevine *VvPGIP1* gene is only expressed in roots and ripening berries, and its expression is developmentally regulated (Joubert et al., 2006). Several cis-acting elements that influence tissue-specific responses, particularly root-specific responses, were found in all *CaPGIPs,* indicating a role in plant development or resistance to pathogens that enter the plant system through the roots. *Brassica juncea PGIPs* are associated with high temperature and drought stresses (Bhardwaj et al., 2015), while Arabidopsis *AtPGIP1*, and apple's *MdPGIP1*, are induced in response to cold stress (Kalunke et al., 2015). The presence of regulatory elements associated with drought, dehydration, water, high light, and low-temperature stress in the *CaPGIP*promoter suggests that they may play a role in plant stress.

Bioinformatic analysis and subcellular localization confirmed thatCaPGIP1, CaPGIP3, and CaPGIP4 are secretory proteins, and they are located in the plasma membrane or the cell wall. DeepLoc-1.0 analysis indicated CaPGIP2 might be found in the mitochondrion or the cytoplasm, and localization experiments revealed *CaPGIP2* was found in the cytoplasm and endoplasmic reticulum. The locations of CaPGIPs within the plasma membrane/cell wall were consistent with the locations of other legume PGIPs. Localization on the plasma membrane is crucial as these proteins play a role in defense responses as cell surface receptors to detect pathogen PGs in the apoplast (Rodriguez-Palenzuela et al., 1991). CaPGIP2 localization to the cytoplasm and endoplasmic reticulum might be because of the lack of signal peptide in CaPGIP2 and suggests it might not be involved with the PG interaction in the apoplast.

Several studies investigated*PGIP* gene expression in response to external stimuli. On the other hand, analyzing gene expression in the absence of external stimuli or treatment enables the correlation of the expression of different *PGIP* genes within a crop. Furthermore, pathogens can infect plants at any stage of their life cycle, and *PGIP* gene families, like other defense-related gene families, have been demonstrated to exhibit variable expression patterns (Kalunke et al.,

2015). Because *PGIP* genes exhibit functional redundancy and sub-functionalization at the protein level (De Lorenzo et al., 2001; Federici et al., 2006), tissue-specific expression of *PGIP* genes is feasible, allowing them to respond more effectively to a variety of environmental stimuli (D'Ovidio et al., 2004). In terms of pathogen PG specificity, plant *PGIP* genes can express at higher levels in distinct growth stages and tissues that correspond to pathogen infection (Cantu et al., 2008). Absolute expression analysis showed *CaPGIP* genes has higher expression in leaf tissue and the least in pod tissues. *B. vulgaris's BvPGIP* genes were reported to be highly expressed in roots in comparison to leaf tissue during normal growth and development (Li and Smigocki, 2016). *Carica papaya's CpPGIP4* and *CpPGIP6* genes were shown to be ubiquitously expressed in root, stem, leaf, seed fruit pulp and peel. However, the *CpPGIP* gene transcripts were most abundant in fruit pulp and peel and decreased during ripening (Broetto et al., 2015). Tissue-specific differences have been reported in apples, where higher transcript abundance was in leaves and fruit, least in the stem (Zhang et al., 2010). In blackberry *PGIP* gene expression was more abundant in young leaves and fruit compared to old leaves and ripe fruit (Hu et al., 2012). In raspberries, *PGIP* transcripts were detected in fruit but not in flowers (Johnston et al., 1993). The varying expression levels of *CaPGIP* genes in different tissues at different growth stages indicates that *CaPGIP* genes might respond to different external stimuli.

5 Conclusion

In conclusion, this study is the first to characterize chickpea PGIPs. Two additional PGIPs on chromosome 3, CaPGIP3 and CaPGIP4 were identified in addition to the previously reported CaPGIP1 and CaPGIP2 on chromosome 6 and this necessitated modifying the genomic organization of CaPGIPs. CaPGIP1, CaPGIP3, and CaPGIP4 displayed a typical PGIP sequence identity with an N-terminal domain, a central LRR domain with ten imperfect LRRs, and a C-terminal domain. Multiple sequence alignment shows that CaPGIP amino acid sequences are highly similar to those of other described legumes. The phylogenetic study of CaPGIPs indicated that CaPGIP1 and CaPGIP3 are similar to legume PGIPs, and CaPGIP4 falls outside the legume PGIP cluster. *CaPGIP*'s promoter sequences harbor cis-elements that regulate response to various external stimuli. CaPGIP1, CaPGIP3, and CaPGIP4 are localized to the cell wall or plasm membrane. Absolute quantification of the *CaPGIP* transcript levels under untreated conditions demonstrates that *CaPGIPs* have tissue-specific expression. Interestingly, CaPGIP2 lacked most of the characteristics typical of a PGIP and warrants further investigations.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

VE and WC conceived the study and designed the experiments. VE performed the experiments. VE analyzed the data. VE wrote the manuscript with contributions from all co-authors. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2023.1189329/full#supplementary-material

SUPPLEMENTARY TABLE S1
Genes identified through a homology database search in Cicer arietinum genome that exhibited PGIP features.

SUPPLEMENTARY TABLE S2
Primers used in this study to isolate, clone, and for the RT-qPCR expression analysis of the Capgips.

SUPPLEMENTARY TABLE S3
Other putative *cis*-acting regulatory elements identified in the promoter regions of *Capgips*.

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