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Genome-wide Identification and Characterization of Cytokinin Metabolic Gene Families in Chickpea (*Cicer arietinum*)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

In today's globe, when the population is growing every day, chickpeas are a crucial crop from an economic and nutritional standpoint. The regulation of cytokinin concentrations by the alteration of cytokinin metabolic genes offers a potential strategy for enhancing agricultural yield. These cytokinin metabolic genes are CKX, IPT, GLU, and ZOG. The IPT gene family produces cytokinins, CKX causes their degradation, and the zeatin O-glucosyltransferases (ZOG) gene family play roles in the reversible inactivation and β-glucosidases (GLU) is responsible for reactivation of cytokinins.

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These genes control the production of cytokinins in a controlled amount. Identification and characterization of the genes in these families is a crucial step in improving our comprehension of CK metabolism in chickpea. The sequences of every member of the chickpea cytokinin metabolic gene family were found and obtained for genome-wide analysis from the pulse database. These findings allowed for the identification of 7 CaIPT genes, 130 CaZOG genes, 32 CaGLU genes and 7 CaCKX genes in chickpea. The genes in each of these families all contain respective conserved domains. Since the soybean and Arabidopsis families of plants are dicots, just like the chickpea, all of the genes were renamed based on their actual orthologues. Using CDS and genome sequencing, the structural properties were made clear. These genes' promoter study revealed abundantly responsive elements for auxin, defense and stress, salicylic acid, abscisic acid, anaerobic induction, light adaptation, MeJA-R, and modulation of zein metabolism. All of the genes' expression profiles revealed that seeds consistently exhibited higher levels of expression at all phases of the plant's life cycle. The location of the cytokinin metabolic genes and the phenomena of gene duplication were revealed by localization and circos plot analysis respectively. Synteny studies showed that some cytokinin metabolic genes followed an evolutionary pathway that was mostly maintained across chickpea and the other three related species. Using a strings database, it was possible to predict how the proteins involved in cytokinin metabolism would interact with other proteins.

Keywords: Cytokinins; cytokinin dehydrogenase/oxidase; isopentenyl transferase; chickpea; gene expression.

1. INTRODUCTION

Chickpea is second only to the soybean as the most widely grown and consumed leguminous crop worldwide, giving it a significant economic impact. The protein content is thought to be superior to that of other pulses, and it is a healthy source of both carbohydrates and protein. Except for the amino acids that include Sulphur, which can be supplemented by including cereals in the diet, chickpea has large levels of all of the crucial amino acids. The main storage carbohydrate is starch, which is accompanied by dietary fiber, oligosaccharides, and simple sugars like glucose and sucrose [1]. In addition to being resistant to pests and diseases, chickpeas provide growers with a sustainable option because they require fewer chemical inputs [2].

Cytokinin metabolic genes are substantially related with plant yield, according to research in model plants [3]. Cytokinins are known as plant hormones that affect a variety of elements of a plant's physiology, growth and development, such as chloroplast differentiation, cell division, and the postponement of senescence [4]. By promoting cell division, cytokinins can help a plant produce more fruits and flowers, increase more biomass, and grow faster [5]. Although cytokinins are essential for the growth and developmental stages of plants, they can also have negative effects when present in too high or low amounts. High cytokinin levels can lead to abnormal growth, including the emergence of tiny or bushy plants. On the other hand, low cytokinin levels may result in slowed growth, decreased production, smaller height, and small leaves, as well as sluggish shoot growth [6]. Trans-Zeatin, cis-Zeatin, and dihydrozeatin-type CKs have additionally been discovered in plants, but their abundance depends on the species [7]. The majority of CKs are adenine derivatives with an isoprenoid or aromatic side chain connected to the N-6 of the purine ring [8]. Several gene families are involved in maintaining CK homeostasis, such as the isopentenyl transferases (IPTs) for the biosynthesis of cytokinins, the zeatin O-glucosyltransferases (ZOGs) are responsible for reversible inactivation, the -glucosidases (GLUs) responsible for reactivation, and the cytokinin oxidases/dehydrogenases (CKXs) responsible for destruction [9].

For the production of CK, IPTs are the relevant gene family. The degradation of transfer RNA (tRNA) and de novo synthesis are the two suggested routes for the production of CK [10]. The plant enzyme isopentenyltransferase (IPT) controls a rate-limiting step in the manufacture of the cytokinin (CTK) molecule. In both the abiotic and biotic stress responses, IPTs are known to play a significant role in controlling CTK homeostasis and plant hormone crosstalk. IPT gene family members, which can be divided into the adenylate ATP/ADP-IPT members or the tRNA-IPT members, control the first stages of plant CTK biosynthesis [11]. Studies of IPT-

driven alterations conducted in plants show that IPTs enhance stay-green properties, postpone senescence, lessen stress-induced oxidative injury, and safeguard the photosynthetic apparatus at a physiological level. These gains then frequently show up as increased or stabilized agricultural production, which is especially noticeable when the environment is stressed. These methods justify considering the IPTs as "master regulators" of fundamental cellular metabolic pathways, modifying plant adaptive responses to changing environmental challenges, and increasing yield potential. IPTs may have a significant role in determining seed production if their expression is sufficiently controlled in both space and time [12]. It is possible to enhance the development and growth of plants by using IPT genes under controlled expression [13].

Zeatin has been shown to be essential for controlling plant growth and development, with special importance for controlling shoot development. It also plays a function in defense and development in stressful environments [14]. Glycosylation is a mechanism in zeatin biology whereby sugar molecules are linked to hormone molecules. Zeatin O-glycosyltransferases are the specific enzymes responsible for carrying out this process. The addition of a sugar molecule, typically glucose or xylose, to the hydroxyl group at the O-6 position of the zeatin molecule is catalyzed by a set of enzymes called zeatin O-glycosyltransferases (ZOGs). As a result of this activity, the hormone's less active forms, zeatin-O-glucoside or zeatin-O-xyloside, are generated [15]. Zeatin-type CKs are naturally reversible to O-glucosylation. GLU serves as the catalytic agent for the deglycosylation of zeatin type CKs [16].

Glycoside hydrolase 1 family, which includes GLU genes, is important in controlling CK metabolism [9]. Cytokinins are de-glycosylated by β-glucosidase (GLU) genes. In Arabidopsis and rice, 37 and 47 GLUs, accordingly, have been found. Beta-glucosidase, the primary enzyme present in cellulase, completes the final step of hydrolysis of cellulose by converting cellobiose to glucose. Because glucose, the reaction's final product, inhibits it, the process is kept under control [17]. Given the sluggish rate of CK synthesis, it is probable that reversible CK activation and degradation play significant roles in preserving the overall CK pool in plants [18].

The decomposition of cytokinins is carried out by an enzyme known as CKX. The primary function of CKX is the irreversible degradation of cytokinin, which maintains the correct level of cytokinin required for plant activity [19]. It specifically transforms cytokinins into inactive forms that cannot stimulate plant development [20]. Two conserved domains, a FAD binding domain on the protein's N terminus as well as a CK binding domain on the C terminus, have been identified as playing a role in the catalytic functioning of CKXs [8]. Despite being present in all plant tissues, the CKX enzyme is most abundant in the leaves and roots. Controlling cytokinin levels is essential for healthy plant development and growth [21]. There are several factors that affect CKX activity. For example, external stresses like drought and intense heat can increase CKX activity, which reduces cytokinin levels and prevents plant development. In contrast, nutrients and light can increase cytokinin levels and decrease CKX activity [22]. The activity of CKX can be changed through genetic engineering. For instance, in order to reduce cytokinin levels and hasten plant senescence, researchers have been able to boost the expression of CKX in plants [23,24,25].

In conclusion, extensive functional research on the CK metabolic gene families have been conducted in model plants. But none of these gene family members have yet been identified or studied in chickpea, so there is extensive need to identify and characterize cytokinin gene family members in chickpea to develop an understanding of functioning of these genes in the respective crop.

2. MATERIALS AND METHODS

2.1 *In-silico* Extraction of Cytokinin Metabolic Genes

The homology search strategy was applied to find novel members in CK metabolic gene families. The pulse database (https://www.pulsedb.org/blast) was queried using the cDNA sequences and conserved domains of all already annotated genes from Arabidopsis, soybean, maize, and rice that were implicated in CK metabolism, including IPTs, CKXs, GLUs, and ZOGs. The corresponding sequences with the relevant were then downloaded as a result. HMMER scan (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) was used to confirm, with even more precision, the protein structure and conserved domains of these novel protein sequences.

2.2 Phylogenetic Analysis

MegAlign was used to verify the alignments of all the additional members of the cytokinin metabolic gene families using the conserved domain sequence. Then, independent multiple alignment was carried out using Clustal Utilizing the MEGA 11 tool, a neighborhood phylogenetic tree (bootstrap 1,000) was created for phylogenetic analysis utilizing the complete and conserved domain sequences of every member of the cytokinin metabolic gene family.

2.3 Chromosome Mapping

Chromosomes are physically mapped to demonstrate where a particular gene member is situated on each chromosome. The physical mapping of chromosomes is done using MG2C MapChart (http://mg2c.iask.in/mg2c_v2.1/) [26].

2.4 Characterization of Physiochemical Characteristics

ExPASY was employed to ascertain the isoelectric point (PI), protein length (ML), and molecular weight (MW) of the novel members of CKX, IPT, GLU, and ZOG gene families. IPT, CKX, ZOG, and GLU gene subcellular localization was examined using the Softberry (http://www.softberry.com/cgi-bin/programs/proloc/protcomppl.pl) database. It provides details concerning subcellular localization and protein length of the CKX, IPT, GLU, and ZOG genes.

2.5 Prediction of Glycosylation Sites

In all of the cytokinin metabolic gene families, glycosylation sites are anticipated. The protein sequences of CaIPT, CaZOG, CaGLU, and CaCKX genes were submitted to the online tool NETNGlyc 1.0, which assisted in identifying N-glycosylation sites in each gene of these gene families [27].

2.6 Gene Structure, Conserved Domain and Motif Analysis

Using NCBI's CDD tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), conserved domains found in each sequence of the corresponding gene families were identified [28]. MEME (https://memesuite.org/meme/doc/install.html?man_type=web) was utilized for motif analysis [29]. By using their protein sequences, CKX, IPT, GLU, and ZOG MEME XML files were downloaded. TB tools (https://bio.tools/tbtools) then used these files to create graphs for the motif analysis for all of the gene families. All these files were substituted in TB tools to draw gene structure, conserved domain and motif graphs.

2.7 *In silico* Promotor Analysis

Promotor sequences for CKX, IPT, GLU, and ZOG were initially retrieved from the pulse database for *in silico* promotor analysis. PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was then used to carry out promotor analysis of these sequences. The data gathered from this test was entered into an excel spreadsheet. There are two approaches to create a graph from this data: either using Excel to create the graph or processing the data in TB Tools (https://bio.tools/tbtools).

2.8 Expression Analysis

The CKX, IPT, GLU, and ZOG gene family members' expression level data was acquired from publically accessible RNA-seq data, which shows the expression of all protein-coding genes and lncRNAs in various tissues and organs [30], for use in expression analysis. TB tools (https://bio.tools/tbtools) was used to process the data after that. With the use of TB tools, a heat map graph was created that depicts the expression level of all cytokinins that are regulating plant growth at various stages.

2.9 Gene Duplication Analysis

Circos of the cytokinin metabolic genes shows the duplication of the gene. Chromosome gene positions data is handled in TB tools (https://bio.tools/tbtools), which in turn generates a circos graph using advanced circos.

2.10 Synteny Analysis

Gene co-localization on chromosomes can be discovered via synteny analysis. Through processing their combined data in multiple synteny plots in Tb Tool (https://bio.tools/tbtools), chickpea was compared with pea, soya bean, and Arabidopsis to check orthologue genes.

2.11 Protein-Protein Interaction Analysis

Analysis of protein-protein interactions is carried out to learn more about the proteins nearby and

interacting with the protein in question. The strings database (https://string-db.org/) is used to analyze protein-protein interactions [31]. This tool can be used to study the relationship between these proteins, the pathways, and how they function.

3. RESULTS

3.1 *In-silico* Extraction and Phylogenetic Analysis of Cytokinin Metabolic Genes

The CKX, IPT, GLU, and ZOG genes that are already present in Arabidopsis, wheat, maize, rice, and soybean were employed to retrieve novel gene members in chickpea. Seven gene members of the CKX gene family, seven gene members of the IPT gene family, 32 gene members of the GLU gene family, and one hundred and thirty gene members of the ZOG gene family were found as a result. In terms of their physiochemical characteristics, each of these gene members is distinct from the others, and each gene member shares the conserved domains found in each gene family. To display all the new gene members, an unrooted phylogenetic tree is built for each gene family.

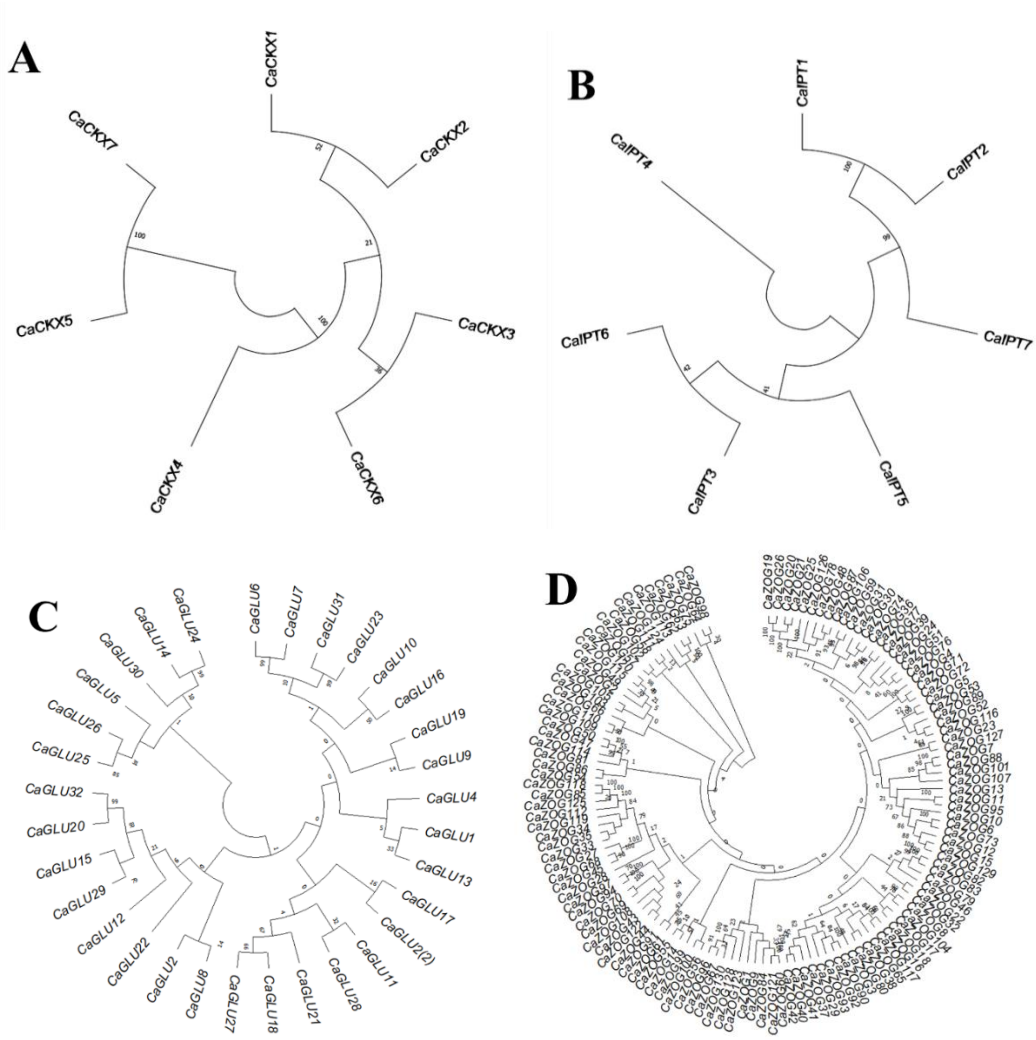


Fig. 1. Phylogenetic tree of cytokinin metabolic genes (A) Phylogenetic tree of 7 CaCKX genes, (B) phylogenetic tree of 7 CaIPT genes, (C) phylogenetic tree of 32 CaGLU genes and (D) Phylogenetic tree 130 CaZOG genes

The tree was created at the amino acid level employing the neighbor joining approach and bootstrapped with 1,000 replications.

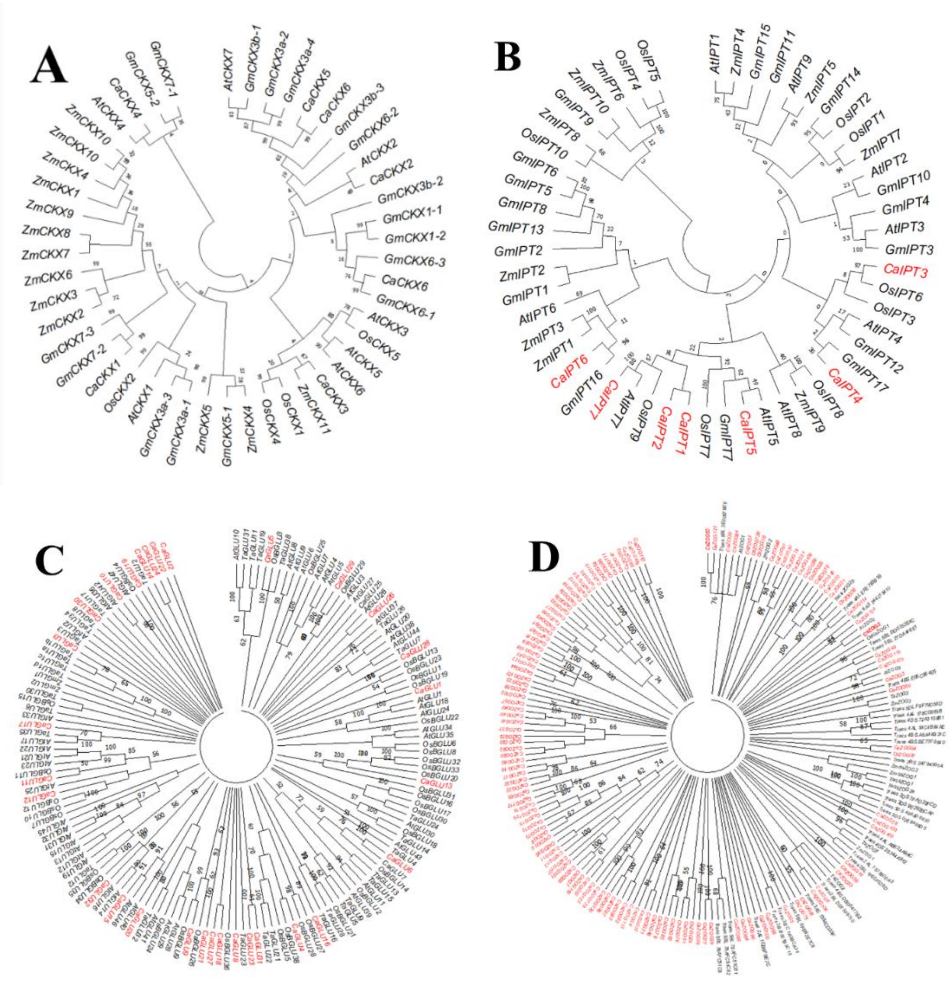


Fig. 2. Phylogenetic tree of (A) CKX, (B) IPT, (C) GLU and (D) ZOG genes from chickpea, Arabidopsis, rice, maize and soybean

The tree was created at the amino acid level employing the neighbor joining approach and bootstrapped with 1,000 replications. The newly identified genes of chickpea are shown in red

At the amino acid level, 1000 bootstrap replications were used to build the trees using the neighbor-joining method. Using the entire amino acid sequence as well as the conserved domain sequence, the topology of all these phylogenetic trees was confirmed. According to the findings, the majority of the chickpea-derived genes had orthologues in other dicot species; for this reason, the names of the CK metabolic genes in chickpea were based on their similarity in closely related species.

3.2 Chromosomal Location

According to the findings, Ca_LG1 included four CaCKX genes, one each on Ca_LG4 and Ca_LG7. For CaIPT, there were three genes on Ca_LG1, two genes on Ca_LG4, one gene each on Ca_LG6, and Ca_LG7. On Ca_LG2, Ca_LG3, Ca_LG5, and Ca_LG8, no CaIPT genes were present. On each chromosome, the CaGLU and CaZOG genes were distributed differently.

3.3 Physiochemical Properties

The CKX, IPT, GLU, and ZOG genes in chickpea were found to have several physiochemical characteristics, including length, which is the number of amino acids in the gene, PI (isoelectric point), molecular weight, subcellular localization and glycosylation sites. These assays' results reveal that the amino acid length in case or CKX spans from 406 to 512 aa, the PIs ranges from 5.4 to 6.04, and the molecular weight varies from 44.838 to 57.650 kDa. Although they are primarily found in vacuole. In the case of IPT, the molecular weight ranges from 35.165 to 37.149 kDa, the amino acid

length ranges from 311 to 326 aa, and the PIs range from 7.75 to 8.80. Most of them are found in chloroplasts. Discussing GLU the site is usually chloroplast, and PIs from 5.05 to 5.4, the amino acid length ranges from 522 to 374 aa, and molecular weight from 60.147 to 42.745 kDa. ZOG genes are typically found in the extracellular region, despite the fact that the amino acid length of ZOG spans from 120 to 496, PIs varies from 4.52 to 7.64, and molecular weight spans from 19.425 to 180.452.

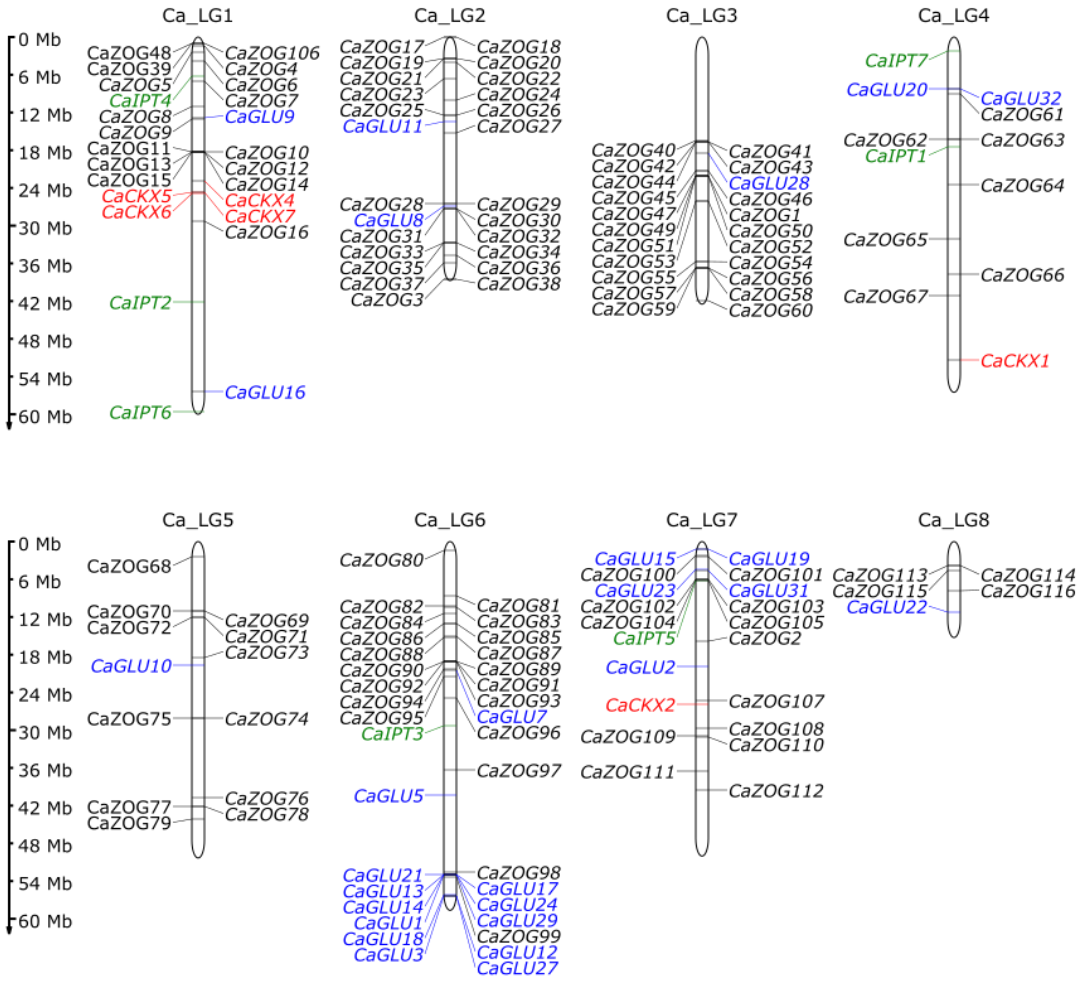


Fig. 3. Chromosomal location of cytokinin metabolic genes in chickpea

MG2C (http://mg2c.iask.in/mg2c_v2.1/) MapChart is used to physically map chromosomes. CaCKX genes are shown in red, CaIPT genes are shown in green, CaGLU genes are shown in blue and OG genes are shown in black

Table 1. Physiochemical characteristics of CaCKX genes

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation Sites
CaCKX1	406	5.4	44.838	Vacuolar	3
CaCKX2	330	6.27	36.612	Vacuolar	2
CaCKX3	301	6.31	33.621	Vacuolar	1
CaCKX4	529	6.52	59.640	Vacuolar	2
CaCKX5	512	5.50	57.283	Extracellular	2
CaCKX6	433	9.14	48.927	Extracellular	3
CaCKX7	512	6.04	57.650	Extracellular	2

Table 2. Physiochemical characteristics of CaIPT genes

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaIPT1	311	7.75	35.165	Chloroplast	1
CaIPT2	311	7.75	35.165	Chloroplast	1
CaIPT3	506	5.68	57.140	Cytoplasmic	2
CaIPT4	340	8.01	37.804	Chloroplast	2
CaIPT5	311	8.58	35.114	Chloroplast	1
CaIPT6	449	6.53	50.855	Cytoplasmic	0
CaIPT7	326	8.80	37.149	Chloroplast	1

Table 3. Physiochemical characteristics of CaGLU genes

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaGLU1	522	5.05	60.147	Chloroplast	3
CaGLU2	302	5.62	34.907	Chloroplast	2
CaGLU3	338	5.84	39.014	Chloroplast	3
CaGLU4	499	7.16	56.621	Chloroplast	2
CaGLU5	490	8.78	55.929	Chloroplast	0
CaGLU6	508	6.77	58.493	Chloroplast	3
CaGLU7	508	6.77	58.493	Chloroplast	3
CaGLU8	450	7.11	51.956	Chloroplast	4
CaGLU9	529	5.62	59.019	Chloroplast	5
CaGLU10	490	5.35	56.056	Chloroplast	2
CaGLU11	520	5.46	59.313	Chloroplast	0
CaGLU12	508	5.98	58.007	Chloroplast	5
CaGLU13	472	5.44	53.755	Chloroplast	3
CaGLU14	423	7.1	48.963	Chloroplast	1
CaGLU15	523	6.14	59.807	Vacuolar	4
CaGLU16	266	8.38	30.090	Cytoplasmic	2
CaGLU17	410	8.09	47.572	Chloroplast	1
CaGLU18	407	5.72	47.141	Chloroplast	2
CaGLU19	920	6.75	103.265	Chloroplast	4
CaGLU20	519	5.75	58.644	Vacuolar	6
CaGLU21	358	5.68	41.190	Chloroplast	1
CaGLU22	379	5.17	43.783	Chloroplast	1
CaGLU23	1092	5.74	123.685	Extracellular	6
CaGLU24	308	5.92	34.686	Chloroplast	1
CaGLU25	338	6.80	38.473	Vacuolar	2
CaGLU26	386	5.89	43.602	Vacuolar	1
CaGLU27	248	4.78	28.343	Chloroplast	2
CaGLU28	248	5.47	28.288	Chloroplast	1
CaGLU29	500	6.17	57.107	Chloroplast	1
CaGLU30	572	6.63	63.915	Extracellular	0
CaGLU31	1092	5.74	123.685	Extracellular	6
CaGLU32	374	5.4	42.745	Vacuolar	1

Table 4. Physiochemical characteristics of CaZOG genes

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaZOG1	496	5.75	56.05920	Plasma membrane	3
CaZOG2	487	6.69	53.908	Extracellular	4
CaZOG3	443	7.07	50.424	Extracellular	1
CaZOG4	455	6.14	50.029	Extracellular	1

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaZOG5	1587	5.9	180.452	Extracellular	4
CaZOG6	458	6.81	51.378	Extracellular	0
CaZOG7	454	6.37	50.327	Extracellular	3
CaZOG8	455	4.81	50.906	Extracellular	8
CaZOG9	489	6.33	54.845	Extracellular	2
CaZOG10	458	5.99	51.496	Extracellular	2
CaZOG11	459	5.95	51.520	Extracellular	0
CaZOG12	457	6.22	50.946	Extracellular	1
CaZOG13	401	6.47	45.074	Extracellular	1
CaZOG14	448	6.07	50.057	Extracellular	1
CaZOG15	457	6.66	50.946	Extracellular	1
CaZOG16	474	5.00	53.432	Extracellular	3
CaZOG17	456	6.32	52.353	Plasma membrane	1
CaZOG18	456	5.99	51.981	Plasma membrane	1
CaZOG19	486	5.67	54.817	Extracellular	2
CaZOG20	484	5.47	54.382	Extracellular	1
CaZOG21	484	5.66	54.551	Extracellular	2
CaZOG22	443	5.11	50.112	Extracellular	3
CaZOG23	436	5.55	49.025	Extracellular	2
CaZOG24	476	5.76	53.562	Extracellular	1
CaZOG25	631	4.97	70.969	Extracellular	2
CaZOG26	486	5.85	54.626	Extracellular	3
CaZOG27	476	5.95	53.563	Extracellular	4
CaZOG28	466	5.93	52.020	Extracellular	1
CaZOG29	470	5.30	52.755	Extracellular	1
CaZOG30	459	5.13	52.071	Extracellular	5
CaZOG31	458	6.54	51.987	Extracellular	2
CaZOG32	455	5.81	51.938	Extracellular	4
CaZOG33	480	6.11	53.740	Extracellular	4
CaZOG34	479	6.22	53.370	Extracellular	4
CaZOG35	482	6.51	53.625	Extracellular	4
CaZOG36	494	6.23	55.437	Extracellular	3
CaZOG37	472	5.35	52.594	Extracellular	1
CaZOG38	466	5.07	51.948	Extracellular	3
CaZOG39	478	5.61	54.199	Plasma membrane	1
CaZOG40	468	5.66	51.887	Extracellular	1
CaZOG41	469	5.63	52.040	Extracellular	3
CaZOG42	468	5.41	51.950	Extracellular	0
CaZOG43	340	6.43	38.142	Extracellular	2
CaZOG44	475	5.94	52.469	Extracellular	2
CaZOG45	477	5.9	53.318	Extracellular	4
CaZOG46	335	5.05	37.596	Extracellular	1
CaZOG47	468	5.82	53.003	Plasma membrane	0
CaZOG48	485	5.48	54.796	Extracellular	4
CaZOG49	496	5.63	56.511	Plasma membrane	5
CaZOG50	469	5.73	53.044	Plasma membrane	3
CaZOG51	494	5.32	55.821	Plasma membrane	4
CaZOG52	430	5.38	48.318	Extracellular	2
CaZOG53	452	5.82	51.882	Extracellular	3
CaZOG54	664	6.67	74.142	Plasma membrane	2
CaZOG55	237	6.17	26.972	Plasma membrane	1
CaZOG56	315	7.11	35.853	Plasma membrane	2
CaZOG57	381	5.76	43.374	Plasma membrane	1
CaZOG58	297	5.66	33.617	Plasma membrane	3
CaZOG59	376	5.78	42.598	Plasma membrane	2

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaZOG60	480	5.74	53.274	Extracellular	2
CaZOG61	462	5.99	52.495	Extracellular	1
CaZOG62	479	5.81	53.043	Extracellular	4
CaZOG63	471	7.16	52.464	Extracellular	4
CaZOG64	189	4.86	21.371	Plasma membrane	1
CaZOG65	469	5.59	52.255	Extracellular	3
CaZOG66	465	6.31	52.885	Extracellular	0
CaZOG67	172	5.25	19.348	Extracellular	1
CaZOG68	476	5.66	53.040	Extracellular	2
CaZOG69	467	5.86	52.120	Extracellular	1
CaZOG70	468	5.5	52.294	Extracellular	3
CaZOG71	480	5.85	53.284	Extracellular	1
CaZOG72	480	5.75	53.081	Extracellular	1
CaZOG73	458	6.79	51.325	Extracellular	0
CaZOG74	457	5.62	51.237	Extracellular	2
CaZOG75	187	8.31	21.532	Plasma membrane	0
CaZOG76	488	6.23	54.750	Plasma membrane	2
CaZOG77	482	5.66	55.189	Extracellular	2
CaZOG78	483	5.79	55.285	Extracellular	2
CaZOG79	471	5.29	53.207	Extracellular	2
CaZOG80	466	5.67	52.398	Extracellular	1
CaZOG81	376	4.87	42.015	Extracellular	2
CaZOG82	471	5.24	52.813	Extracellular	3
CaZOG83	471	5.24	52.813	Extracellular	3
CaZOG84	734	8.14	82.533	Extracellular	4
CaZOG85	470	5.99	52.793	Extracellular	4
CaZOG86	300	5.07	33.754	Extracellular	3
CaZOG87	489	5.76	55.764	Extracellular	1
CaZOG88	454	6.21	50.468	Extracellular	3
CaZOG89	453	5.77	51.437	Plasma membrane	5
CaZOG90	476	6.15	53.362	Plasma membrane	1
CaZOG91	476	5.71	54.053	Plasma membrane	5
CaZOG92	476	6.15	53.362	Plasma membrane	1
CaZOG93	475	6.02	53.652	Plasma membrane	3
CaZOG94	465	5.63	51.419	Extracellular	2
CaZOG95	465	6.36	52.058	Extracellular	0
CaZOG96	172	4.94	19.290	Plasma membrane	1
CaZOG97	485	6.04	53.867	Plasma membrane	5
CaZOG98	155	4.99	17.445	Extracellular	0
CaZOG99	218	5.44	24.367	Plasma membrane	2
CaZOG100	492	6.11	54.546	Extracellular	3
CaZOG101	453	5.76	49.978	Extracellular	3
CaZOG102	473	6.31	53.059	Plasma membrane	2
CaZOG103	488	5.93	54.929	Plasma membrane	0
CaZOG104	476	5.74	53.696	Plasma membrane	3
CaZOG105	483	5.91	54.353	Plasma membrane	3
CaZOG106	471	6.13	53.613	Plasma membrane	3
CaZOG107	458	5.75	50.926	Extracellular	0
CaZOG108	473	6.34	52.244	Extracellular	3
CaZOG109	382	5.20	42.625	Extracellular	1
CaZOG110	387	5.76	43.179	Extracellular	5
CaZOG111	472	6.10	53.321	Plasma membrane	0
CaZOG112	479	6.76	54.144	Extracellular	4
CaZOG113	204	6.60	22.516	Plasma membrane	3
CaZOG114	466	5.34	53.284	Plasma membrane	1

Genes	Length (aa)	PI	Molecular weight (kDa)	Localization	Glycosylation sites
CaZOG115	484	5.45	53.598	Extracellular	1
CaZOG116	421	5.98	46.893	Extracellular	2
CaZOG117	470	5.58	53.071	Extracellular	3
CaZOG118	470	6.03	52.844	Extracellular	4
CaZOG119	470	5.67	52.735	Extracellular	4
CaZOG120	479	5.99	53.119	Extracellular	3
CaZOG121	476	5.83	52.504	Extracellular	1
CaZOG122	467	6.35	51.900	Extracellular	1
CaZOG123	369	7.60	41.434	Extracellular	2
CaZOG124	378	7.64	42.432	Extracellular	4
CaZOG125	508	6.95	56.901	Extracellular	4
CaZOG126	482	6.35	53.610	Extracellular	2
CaZOG127	449	5.72	49.784	Extracellular	0
CaZOG128	276	4.52	31.198	Extracellular	0
CaZOG129	457	6.66	50.946	Extracellular	1
CaZOG130	120	5.18	19.425	Plasma membrane	1

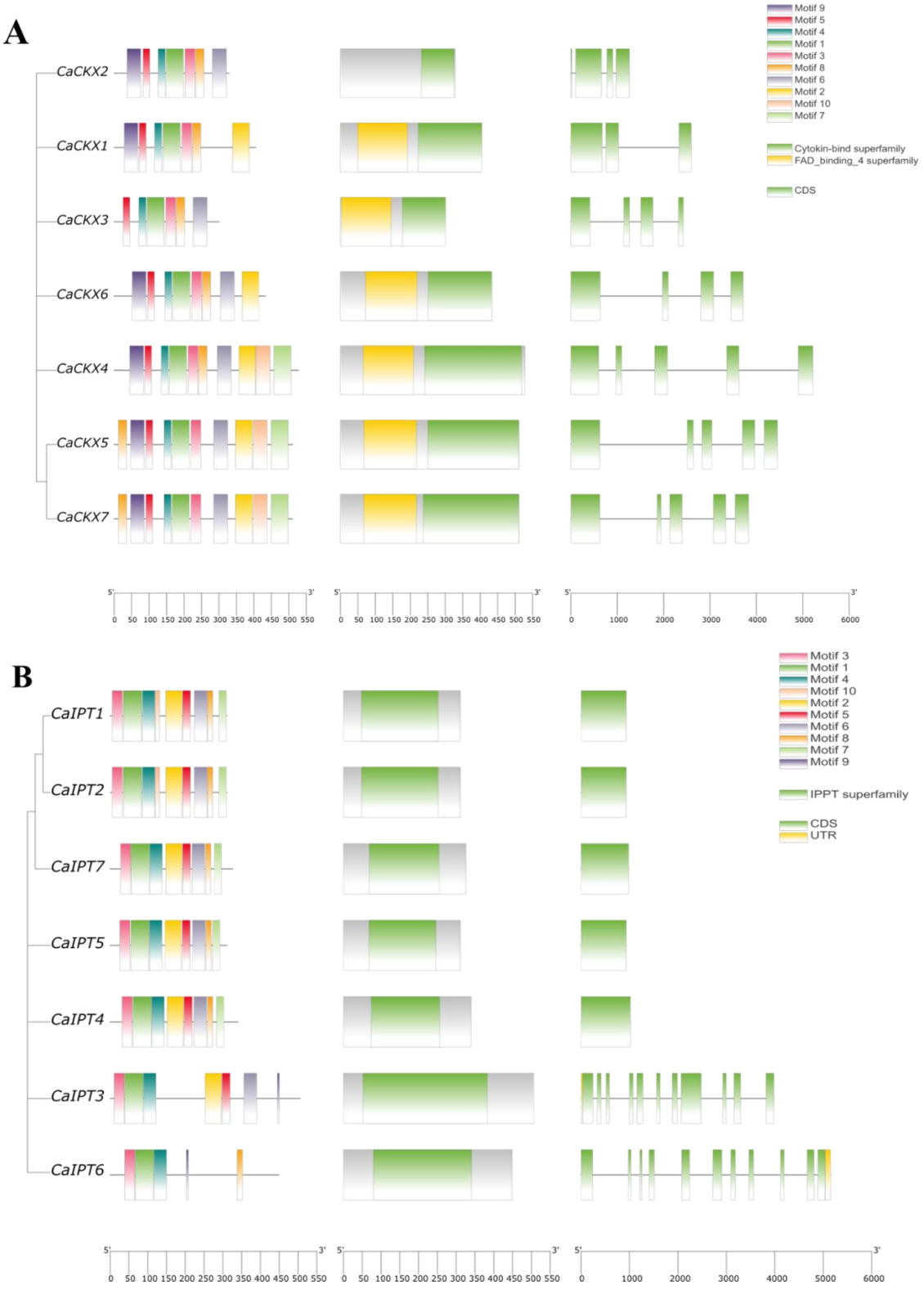
3.4 Gene Architecture and Protein Motif Analysis

Exons are shown as colored boxes, while introns are shown as single lines. The 5' and 3' ends' untranslated regions (UTRs) are shown as blue colour lines. The full-length gDNA and cDNA of chickpea were used to predict the gene structure of cytokinin metabolic genes. Minimum of one intron was present in *CaCKX3* while other genes showed more than one intron in there structure. *CaIPT1, CaIPT2, CaIPT4, CaIPT5* and *CaIPT7* had no introns at all. Introns are present only in *CaIPT3* and *CaIPT6*. CaGLU23 and CaGLU31 both seemed to be related in phylogenetic analysis, hence there gene structure also confirms it as both have same number of introns and exons with same gene length. Gene structure of CaGLU genes varies differently as some have no introns at all while others show introns. Two conserved domains, one of them from the FAD binding-4 superfamily and the other from the cytokinin binding superfamily, were found in the CaCKX family according to the examination of the domain architecture. The CaIPT gene family's domain architecture investigation revealed the existence of a single IPPT superfamily with a conserved domain. Similar to this, CaGLU's domain architecture research revealed the existence of a single Glycohydrolytic superfamily with a conserved domain. When CaZOG family's domain architecture was examined, the conserved domain it revealed belonged to the superfamily of Glycosyltransferase. The results show that a total of 10 motifs were selected to be examined, and various motifs could be seen in the members of the CKX, IPT, GLU, and ZOG genes. The placement of all motifs in the direction of the phylogenetic tree of the cytokinin metabolic gene family members demonstrates that every member of the specific family belongs to the same clade and has shared domains, proving that these metabolic genes have gone through the same evolutionary process. In the CKX gene family, each gene has a unique motif, however in the IPT gene family, CaIPT1, CaIPT2, CaIPT5, and CaIPT7 all have the same motif while the others have different motifs. Nearly all of the genes in the GLU gene family have unique molecular motifs. The motifs for the ZOG gene family are the same.

3.5 Analysis of cis Regulating Elements

According to the findings of this investigation, cis elements responsible for methyl jasmonic acid responsiveness in the CaCKX family are primarily present, with cis elements responsible for abscisic acid responsiveness being most prevalent. Similar to methyl jasmonic acid, the IPT gene family also has cis elements that control light and abscisic acid response, with light responsiveness being the most prevalent. The GLU gene family contains the cis elements necessary for auxin responsiveness, defense and stress, abscisic acid and salicylic acid responsiveness, anaerobic induction, zein metabolism regulation, and light responsiveness. However, this family has an abundance of the cis elements necessary for light responsiveness. Talking about the ZOG gene family auxin-responsive element, ATBP-1, abscisic acid responsiveness, defense and stress responsiveness, anaerobic induction, low-temperature responsiveness, MeJA-R, salicylic

acid responsiveness, circadian control, palisade mesophyll cells, light adaptability, control of zein metabolism, and expression of the meristem and endosperm. In contrast, light responsiveness is also the most prevalent cis element in the ZOG family.



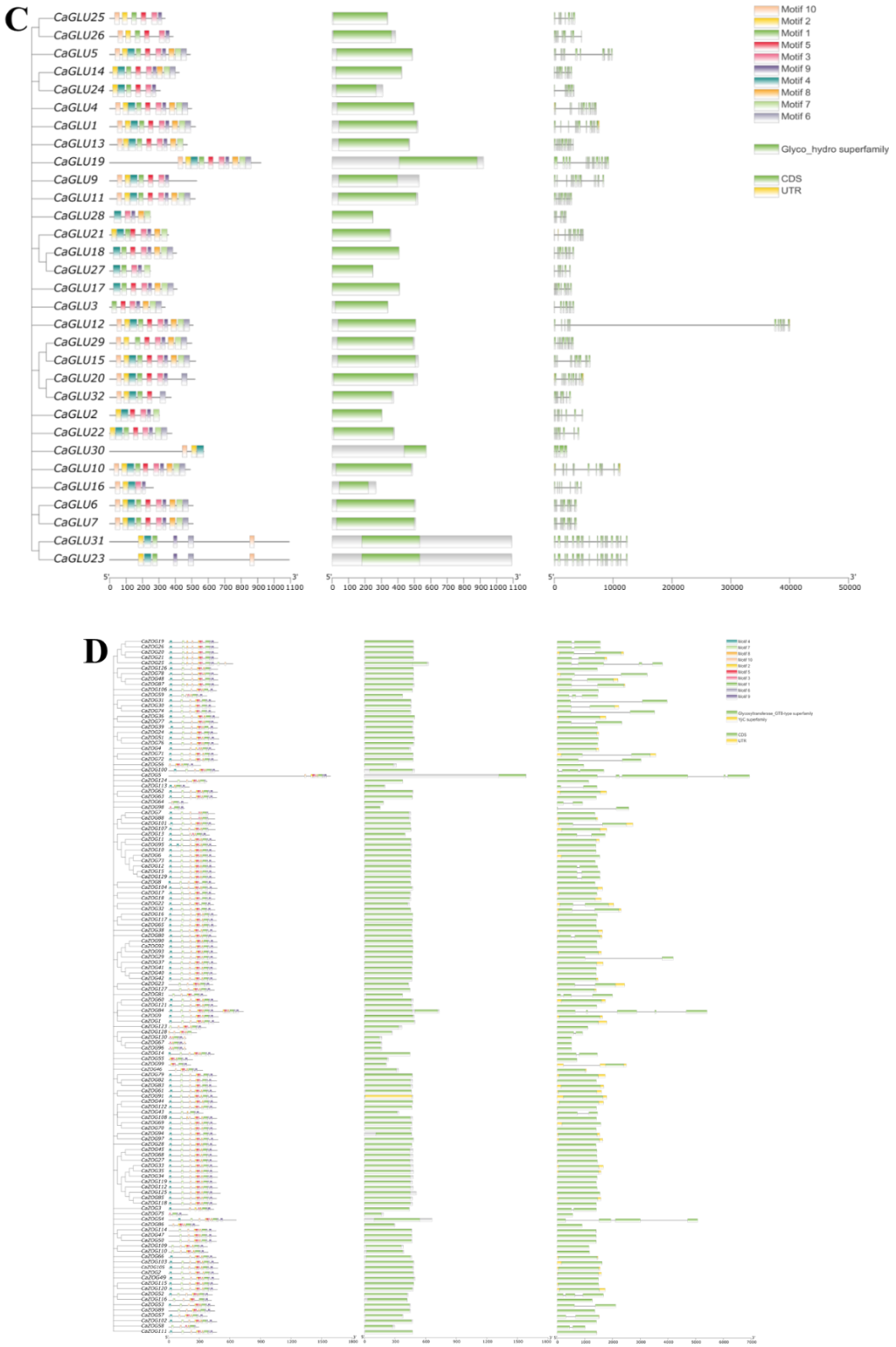


Fig. 4. Gene architecture and protein motifs of (A) CaCKX genes, (B) CaIPT genes, (C) CaGLU genes and (D) CaZOG genes

The domains are predicted using the NCBI CDD program (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Motif analysis is carried out using MEME (https://memesuite.org/meme/doc/install.html?man_type=web). Different motifs are shown with different colors. In gene structure exons are depicted filled green boxes, UTR are shown by yellow boxes and introns are depicted as single lines

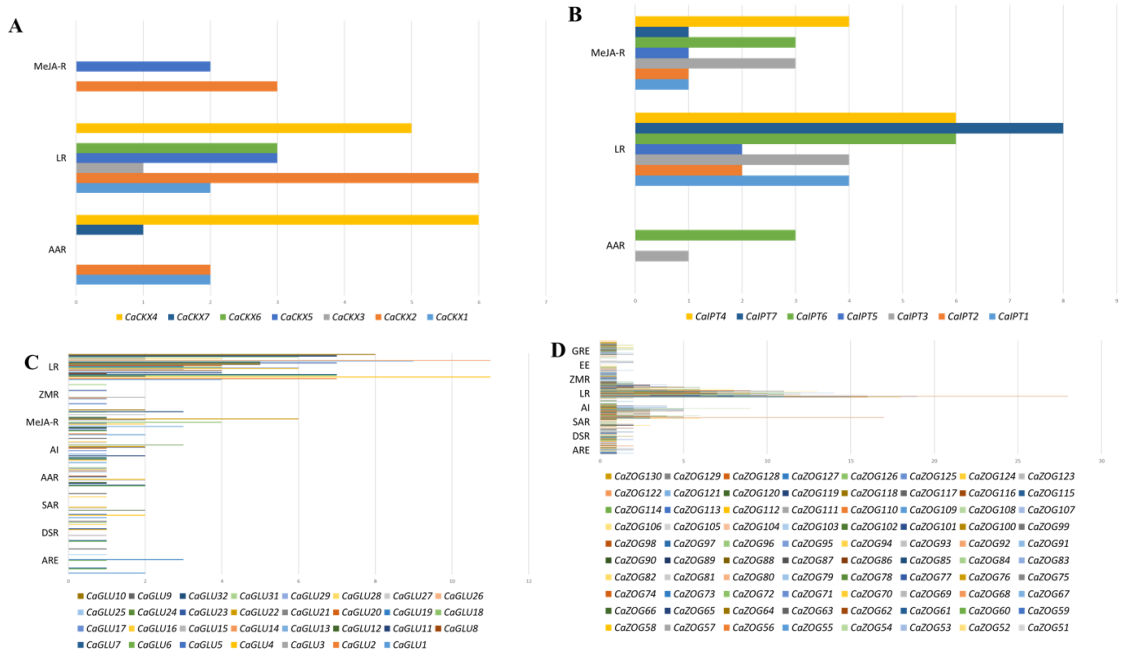


Fig. 5. Cis element analysis of (A) CaCKX genes, (B) CaIPT genes, (C) CaGLU genes and (D) CaZOG genes

Auxin-responsive elements, ATBP-1, defense and stress responsiveness, salicylic acid responsiveness, low-temperature responsiveness, anaerobic induction, abscisic acid responsiveness, light adaptability, circadian control, control of zein metabolism, MeJA-R, expression of the meristem as well as endosperm, and responsive elements for palisade mesophyll cells are abundantly seen in the promoter analysis of cytokinin metabolic genes. Data for promotor analysis is retrieved using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/)

3.6 Expression Analysis

According to the heat map's data, it is clear that the *CaCKX1* gene is expressed at a relatively high level in almost all phases of plant growth for the CaCKX gene family. When compared to *CaCKX1, CaCKX5* exhibits a similar tendency, however it is less pronounced. Additionally, seeds had the maximum expression of *CaCKX5* and *CaCKX1* 20 days following pollination. Ten days post pollination, the mature leaf, the mature seed coat, and the corolla, the seed shows a relatively high expression of *CaCKX1*. When compared to the other 4 genes, the expression of the *CaIPT1*, *CaIPT2*, and *CaIPT4* genes is somewhat higher. Nearly all plant growth stages exhibit high levels of expression, however seeds exhibit the highest levels post 20 days. *CaIPT3* expression is minimal at all developmental stages, but it is marginally elevated in seeds 20 days post pollination. When compared to the CaCKX and CaIPT gene families, the expression trend for the CaGLU gene family is noticeably different. *CaGLU4, CaGLU5*, and *CaGLU19* have the highest levels of expression in seeds 30 days post pollination out of the 32 CaGLU genes. The root tips are where *CaGLU3* and *CaGLU27* are most expressed, with their expression also being slightly higher in the roots, root hair, and sprouting seedling. According to the CaZOG gene family's expression profiling, *CaZOG79* and *CaZOG98* express themselves more than the other CaZOG genes during almost all phases of plant growth and development. Twenty days after pollination, the expression of and *CaZOG39*, *CaZOG60*, *CaZOG75, CaZOG79, CaZOG97, CaZOG98, CaZOG100*, and *CaZOG101* is elevated in seeds. Androecium has strong *CaZOG100, CaZOG101*, and *CaZOG88* expression. It is also possible to witness expression in developing seedlings, root hair, mature leaves, bracteol, and calyx.

3.7 Gene Duplication Analysis

The CaIPT genes circos exhibits duplication in *CaIPT4* and *CaIPT5* at chromosomes 7 and 1, respectively. Other than these two genes, no other IPT gene family member in chickpea shown duplication. Using a circos plot, gene duplication in CaGLU genes was also

discovered. On chromosomes 2 and 6, as well as *CaGLU9* and *CaGLU4*, there is a duplication of genes between *CaGLU11* and *CaGLU7*, as is evident from the Circos plot. There is also duplication of genes between *CaGLU11* and *CaGLU7.*

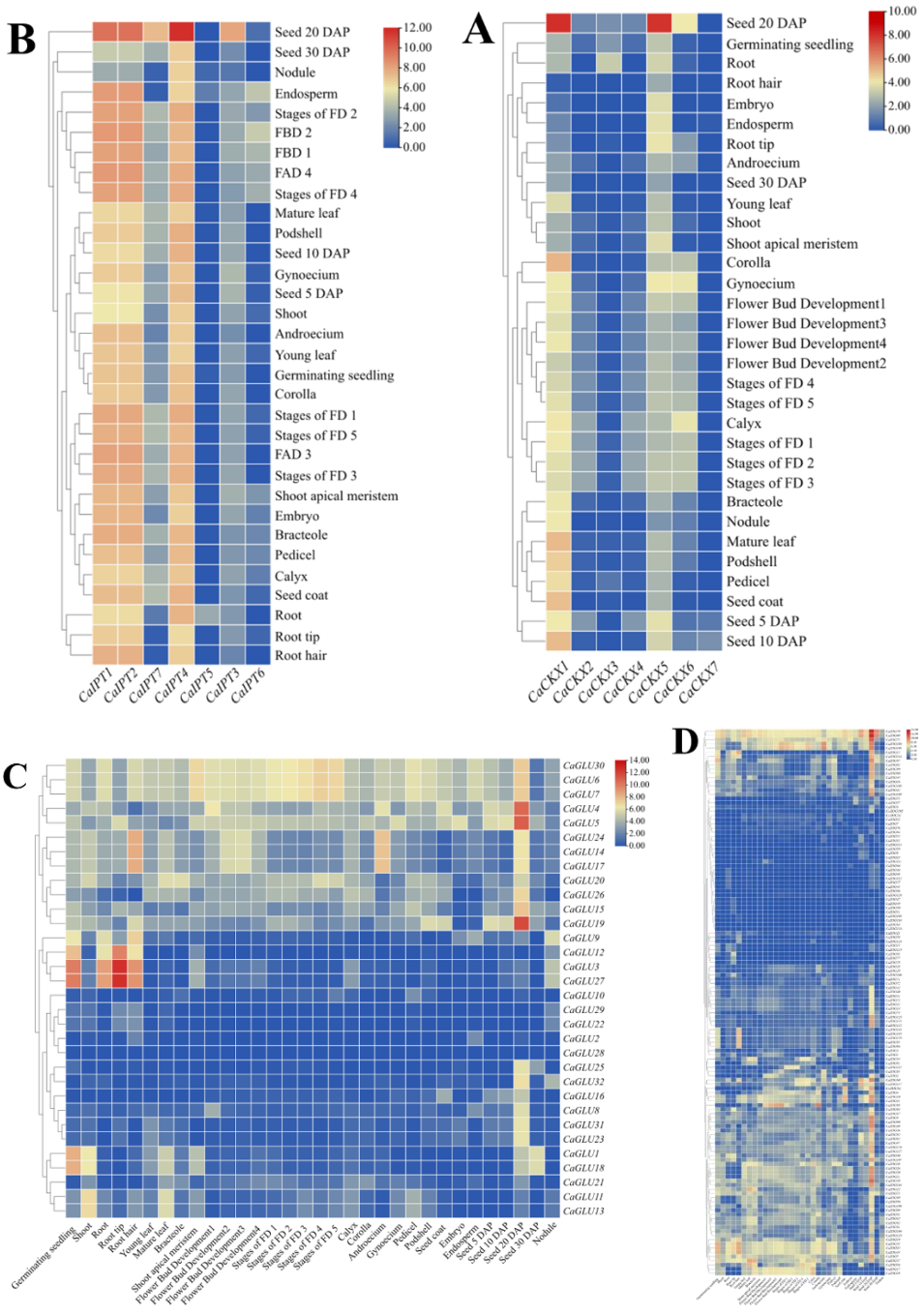


Fig. 6. Expression analysis of (A) CaCKX genes, (B) CaIPT genes, (C) CaGLU genes and (D) CaZOG genes

Publicly available data from openly accessible RNA-seq data, which shows the levels of gene expression coding for proteins and lncRNAs in diverse tissues/organs, was used to analyze the spatial and temporal activity of cytokinin metabolic gene members. Datasets were collected from plants at different stages of growth for distinct tissues. Based on the differential transcriptome frequency of cytokinin metabolic genes, a heat map was produced using the software TBtools. The scale bar displays the expressiveness level from lowest (blue) to highest (red)

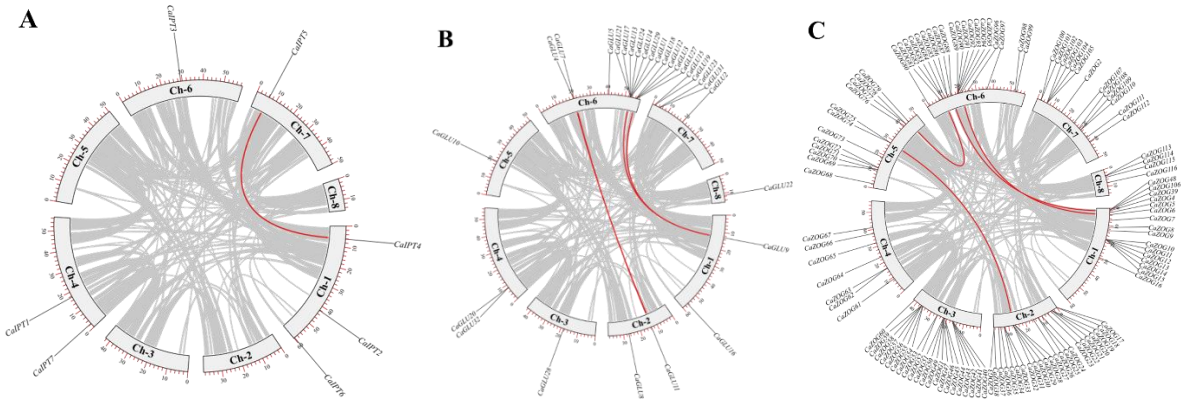
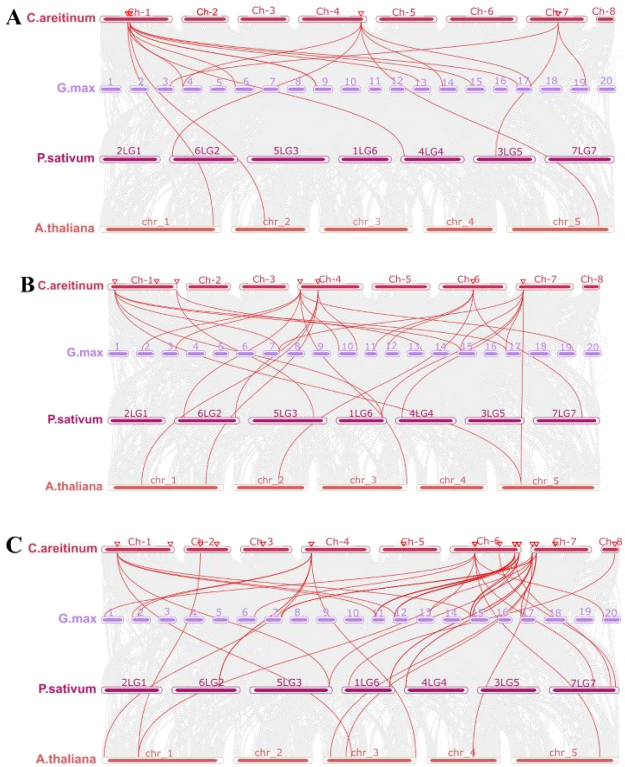


Fig. 7. Circos plot of (A) CaIPT genes, (B) CaGLU genes and (D) CaZOG gens.
Red lines indicate gene duplication on several chromosomes. Tb Tool (https://bio.tools/tbtools) advanced circos is used to create circos plots.

Similar to this, the CaZOG genes circos plot demonstrates the duplication of the genes for *CaZOG4* and *CaZOG88, CaZOG6* and *CaZOG95, CaZOG30* and *CaZOG74*, and *CaZOG79* and *CaZOG83*.

3.8 Synteny Analysis

The co-localization of cytokinin metabolic genes in chickpea is demonstrated in this analysis along with three other crops, including pea, soya bean, and Arabidopsis. These tree species were chosen to study the orthologue genes because like chickpea these species are also dicots and are related to chickpea. Distinct colors have been used to depict the chromosomes of the four distinct crops. The grey lines in the background denote the collinear blocks in the genomes of chickpea and the other three species. The collinear blocks found in chickpea chromosomes are indicated by the red lines.



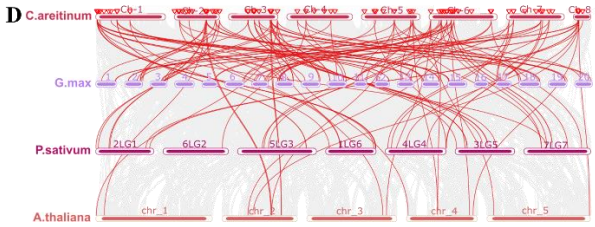


Fig. 8. Synteny analysis of (A) CaCKX genes, (B) CaIPT genes, (C) CaGLU genes and (D) CaZOG genes

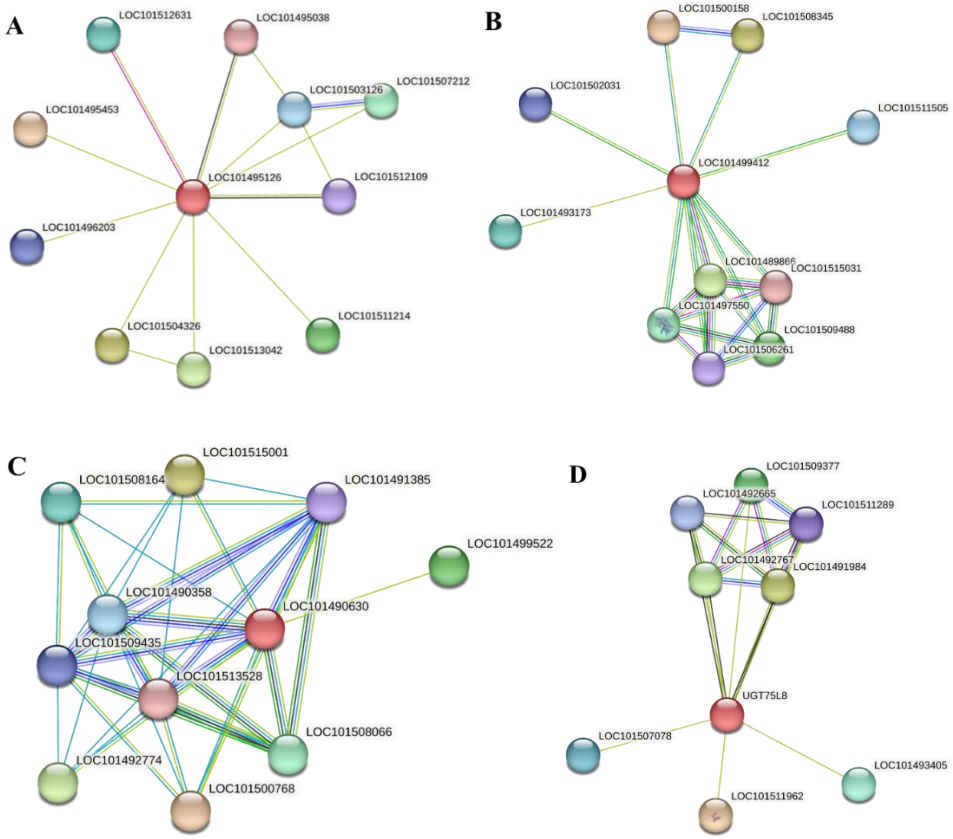


Fig. 9. Protein networking shown by (A) CaCKX gene family, (B) CaIPT gene family, (C) CaGLU gene family and (D) CaZOG gene family

Each node in the network represents a protein that has undergone post-translational modifications or had splice isoforms collapsed. Colored nodes represent search proteins and the preliminary shell of interactors, while white nodes represent the second shell. Empty nodes represent proteins that lack a known 3D structure, while filled nodes represent proteins that do. As a result, proteins cooperate to complete an action without necessarily physically sticking to one another, as connections are designed to be exact and meaningful. Protein-protein analysis is done using the Strings database (https://string-db.org/).

The multiple synteny plot between Arabidopsis and the legumes chickpea, pea, soybean, and was created using TB tools (https://bio.tools/tbtools). Chromosomes of various species have varied colors. Collinear blocks that are found in chickpea chromosomes are shown by the red lines.

3.9 Protein-protein Interaction

CaCKX's protein networking reveals that a number of additional proteins are located nearby (LOC101495126). The protein-protein interaction of CaCKX demonstrates that it interacts with a variety of other proteins, including tRNA dimethylallyltransferase 9-like (LOC101503126),

tRNA dimethylallyltransferase 2-like (LOC10150 7212), chloroplastic; Upstream in-frame stop codon (LOC101511214), Histidine-containing phosphotransferase protein 6-like (LOC101512109 and LOC101495038), Adenylate dimethylallyl transferase (cytokinin synthase); Adenylate isopentenyl transferase 3, Histidine-containing phosphotransferase protein 6-like (LOC101512109 and LOC101495038), an uncharacterized protein LOC101513042, Histone acetyltransferase type B catalytic subunit-like (LOC101504326), adenylate isopentenyl transferase-like; The sequence of the model RefSeq protein was modified relative to its source genomic sequence to represent the inferred complete CDS: substituted 1 base at 1 genomic stop codon (LOC101496203), ATP synthase delta chain, chloroplast-like (LOC101512631) and F-box/LRR-repeat protein 15-like (LOC101495453).

Protein networking of CaIPT (LOC101499412) reveals that the proteins present in close proximity to it include Cytokinin trans-hydroxylase (LOC101500158 and LOC101508345), isoform X3, Pyridoxine/pyridoxamine 5'-phosphate oxidase (LOC101502031), Uncharacterized protein (LOC101511505), Farnesyl pyrophosphate synthase 1-like (LOC101497550), Cytokinin dehydrogenase 1-like LOC101493173), chloroplast-like (LOC101489866) and Heterodimeric geranylgeranyl pyrophosphate synthase small subunit, chloroplast-like (LOC1015150310, LOC101506261 and LOC101506261) and 4-hydroxy-3-methylbut-2-enyl diphosphate reductase. Uncharacterized protein loc101499522, (R)-mandelonitrile lyase-like (LOC101508164), Endoglucanase 17-like (LOC101515001, LOC101492774 and LOC101500768), Alpha-glucosidase-like isoform X1 that belongs to the glycosyl hydrolase 31 family (LOC101508066) and Beta-glucosidase 44-like that belongs to the glycosyl hydrolase 1 family (LOC101490358, LOC101513528, LOC101491385 and LOC101509435) show networking with CaGLU gene family. CaZOG's protein-protein networking demonstrates its networking with cell division cycle 5-like protein (LOC101511962), transcription factor MYB24-like (LOC101507078), Myb-related protein 315-like (LOC101493405), leucoanthocyanidin dioxygenase-like; (LOC101511289), bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase (LOC101492767 and LOC10149 1984), Flavanol synthase/flavanone 3-hydroxylase-like (LOC101509377) and Glutathione S-transferase F11-like (LOC101492665).

4. DISCUSSION

Cytokinins (CKs), a class of phytohormones, are essential for managing plant growth. They have established functions in cell development, nutrition signaling, and leaf withering [32]. According to certain theories, many gene families maintain CKs in equilibrium for robust plant growth. Model plant CK metabolism-controlling genes have already been identified and well characterized. The IPT, GLU, ZOG, and CKX metabolic (CKs metabolic) genes of Arabidopsis, rice, and maize were used to examine the chickpea local genomic database using a comparative genomics technique. Novel genes included seven CaCKX, seven CaIPT, thirty-two CaGLU, and one hundred thirty CaZOG. The systematic naming of newly found genes was done following their orthologues in closely related species [33]. The names of the novel genes were chosen in a methodical manner. The nomenclature was genuinely based on similarities between newly discovered gene members in chickpea and previously discovered genes in other species, including Arabidopsis, soybean, maize, rice etc. It was very clear from the phylogenetic tree that, in comparison to other species, the majority of the chickpea members were discovered to be closely linked to Arabidopsis. This relationship is due to the fact that both Arabidopsis and chickpea are dicots, whereas the other species being studied are monocots. Phylogenetic analysis employing sequence similarity is a potent technique for foretelling orthologue genes that are interesting and their roles in key crop species [9]. Softberry and NetNGlyc servers, respectively, have been used to predict subcellular localization and glycosylation locations. The subcellular localization of genes may vary and presence or absence of glycosylation sites in each family member predicts their distinct activities and substrate subtleties [34]. The gene structure analysis done via Gene Structure Display server (http://gsds.cbi.pku.edu.cn) also confirms homology between genes that are show similarity in phylogenetic analysis as their gene structure is also same. By affecting the efficacy of gene promoters, cis-regulatory elements perform a crucial part in modulating gene expression. Knowing the intended cis-elements is useful when designing complex functional research. The bulk of the CaCKX, CaIPT, CaGLU, and CaZOG genes' putative promoters were

predicted to have ABA-, sulphur-, auxin-, drought-, light-, and SA- responsive cis regulatory elements. Due to the diverse regulatory elements that predict their expression in various plant tissues, these gene families might be able to stabilize CK content across an array of environmental stresses. The CaCKX1 gene is evidently expressed at an elevated level in practically every stage of plant growth, as shown by the data from the heat map. Comparing *CaCKX5* to *CaCKX1*, a similar tendency is there, but it is not as strong. Maximum CaCKX1 and CaCKX5 seed expression occurs 20 days after pollination. The mature corolla, mature seed coat, and mature leaf appear ten days post pollination, and the seed has a comparatively significant expression of *CaCKX1*. The expression of the CaIPT1, CaIPT2, and CaIPT4 genes is a little higher than that of the other 4 genes. High levels of expression are present in nearly all plant development stages, however seeds show most elevated levels after 20 days. At every stage of development, *CaIPT3* expression is very low, however seeds 20 days after pollination have a slight increase. The expression pattern for the CaGLU gene family differs substantially from that of the CaCKX and CaIPT gene families. Out of the 30 CaGLU genes, CaGLU4, CaGLU5, and CaGLU19 are expressed at the greatest levels in seeds 30 days after pollination. In roots, root hair, and seedlings that are just starting to sprout*, CaGLU3* and *CaGLU27* are slightly more expressed. CaZOG79 gene and CaZOG98 gene expression is more than each of the other CaZOG genes throughout practically all stages of plant development and growth, according to the expression profile of the CaZOG gene family. 20 days after pollination, the expression of *CaZOG79* gene, *CaZOG98* gene, *CaZOG75* gene, *CaZOG100* gene, *CaZOG101* gene, and *CaZOG97* gene is higher in seeds. *CaZOG100, CaZOG101*, and *CaZOG88* are substantially expressed in Androecium. They also exhibit a higher level of expression in growing seedlings, mature leaves, root hair, calyx, and bracteol. The cytokinin hormone family of plants is hypothesized to affect both the qualitative and quantitative aspects of yield. The increased yield of wheat is influenced by an elevated level of cytokinins [35]. Additionally, the CaCKX, CaIPT, CaGLU, and CaZOG genes—which have previously been linked to yield—were discovered to be associated with abscisic acid, light responsiveness, drought, zeatin, and MeJA while studying the cis regulatory elements in chickpea, confirming the link between cytokinin metabolic genes and yield. Some of the genes in the CaIPT, CAGLU, and CaZOG families have demonstrated the gene duplication phenomenon. Similar to this, synteny analysis also demonstrates gene duplication and conservation of homologous genes between chickpea and other three dicot species (pea, Arabidopsis and soybean).

5. CONCLUSION

Chickpea is a valuable leguminous crop and it provides wholesome sustenance for a growing global population. Genomic and CDS sequences were retrieved from an online service to do genome-wide characterization of the cytokinin metabolic genes full-length protein. Presence of cytokinin metabolic gene families were confirmed using their protein sequences. With the help of this data 7 CaCKX genes, 7 CaIPT genes, 32 CaGLU genes and 130 CaZOG genes were identified in chickpea. Analysis of the exon/intron structure showed that cytokinin metabolic genes are more conserved. Phylogenetic analysis showed that cytokinin metabolic genes families in chickpea are more evolutionary related to dicot crops as chickpea itself is dicot. Spatial and temporal expression profiling of the cytokinin metabolic genes showed that genes are more expressive in seeds. Demonstrating that these genes may be involved in response to light, and cis-element analysis supported this by demonstrating the presence of several light responsive elements in the promoter regions of cytokinin metabolic genes. Gene duplication was observed in gene members of CaIPT, CaGLU and CaZOG gene families.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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