

Genome-wide analysis and expression of the Plasma membrane intrinsic gene family in *Caragana korshinskii* Kom

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Research Article

Keywords: *Caragana korshinskii*, Plasma membrane intrinsic protein (PIP), Genome-wide analysis, Drought stress, Expression pattern

Posted Date: January 12th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8128395/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Plant Biology on July 28th, 2026. See the published version at <https://doi.org/10.1186/s12870-026-09485-8>.

Abstract

Plasma membrane intrinsic proteins (PIPs), as essential plant channel proteins located on the plasma membrane, play pivotal roles in the transfer of water, CO₂, and small uncharged solutes across the plasma membrane. Although the molecular mechanisms of PIPs have been extensively studied in model species, their characteristics and functions remain poorly characterized in *Caragana korshinskii*. Here, 13 *CkPIP* genes were identified through genome-wide analysis. *CkPIPs* were clustered into PIP1 (seven members) and PIP2 (six members) subfamilies, exhibiting conserved physicochemical properties, cis-acting elements that respond to abiotic stresses and phytohormones, and evidence of segmental duplication under purifying selection. Phylogenetic, structural, and expression analyses (RNA-seq and RT-qPCR) were performed to explore their evolutionary patterns and responses to drought stress. Under drought stress, *CkPIPs* exhibited tissue-specific expression dynamics: *CkPIP1;1*, *CkPIP1;2*, and *CkPIP2;2* was prominently upregulated, suggesting their key roles in drought adaptation. This study expands our understanding of *CkPIP* family characteristics and provides valuable molecular insights for drought-resistance research and forage improvement.

1 Introduction

Caragana korshinskii Kom, commonly known as the pea shrub, is a small leguminous shrub belonging to the family Fabaceae. This species exhibits remarkable traits such as tolerance to barrenness, resistance to salinity, cold hardiness, drought endurance, and high biomass yield [28]. Characterized by high nutritional value and palatability, it is widely distributed across sandy grasslands in the arid and semi-arid regions of northwestern China and Mongolia. Long-term adaptation to drought and saline environments has driven the evolution of specialized physiological mechanisms conferring exceptional drought resistance [29]. Its abundant flowering also makes it an important nectar-producing species. *C. korshinskii* plays a vital ecological role as a key species in windbreak, sand-fixation, and soil–water conservation forests. Its cultivation contributes to the restoration of degraded pastures, enhances the use of saline soils, and supports food security, sustainable animal husbandry, and ecological conservation [30].

Drought is among the most severe abiotic stresses limiting plant growth and productivity worldwide. To cope with water scarcity, plants have evolved diverse adaptive strategies at morphological, physiological, and molecular levels [1]. Among these mechanisms, maintaining water transport and cellular homeostasis is crucial for sustaining drought tolerance. Plasma membrane intrinsic proteins (PIPs), a subfamily of aquaporins, play an essential role in facilitating water transport across cell membranes [2]. PIPs help regulate water balance, stomatal movement, and osmotic responses, acting as key components in plant drought adaptation. These proteins are small intrinsic membrane proteins characterized by major intrinsic protein (MIP) domains located on the plasma membrane [3]. Their primary function involves forming selective pores that enable the regulated, single-file movement of water molecules across the membrane.

Plant PIPs are classified into two subfamilies, PIP1 and PIP2, based on sequence similarity [4,5]. Phylogenetic analyses have revealed the presence of PIPs in both bryophytes and gymnosperms, indicating that the divergence between the PIP1 and PIP2 subfamilies likely occurred before the evolutionary split between monocots and dicots [27]. Structurally, PIP1 and PIP2 differ primarily in the lengths of their N- and C-terminal regions, which correspond to distinct functional properties [6]. PIP2 proteins are highly efficient in facilitating water transport across the plasma membrane, whereas PIP1 proteins show lower water permeability and are often associated with the transport of small solutes. PIP1 proteins typically require co-expression with PIP2 proteins for proper membrane localization, highlighting their cooperative roles in regulating cellular water and solute dynamics [31]. Moreover, enhanced expression of PIP genes in transgenic plants has been linked to increased stomatal conductance, leaf expansion, and organ development [7].

Despite the recognized importance of PIPs in drought responses, most studies have focused on model organisms such as *Arabidopsis thaliana* and *Oryza sativa*. In *A. thaliana*, PIP1 and PIP2 proteins are actively involved in responses to abiotic stresses, including drought and salinity [8]. Empirical evidence indicates that their expression decreases under salt stress, leading to delayed recovery of hydraulic conductance and transpiration rates. Conversely, the overexpression of *OsPIP1-1* or *OsPIP2-2* enhances tolerance to both salt and drought stress [9]. However, extremophytic species such as *C. korshinskii*, known for their remarkable drought tolerance, have received limited attention. Investigating the role of PIPs in such species could provide novel insights into the molecular mechanisms underlying drought adaptation. Furthermore, the distinct evolutionary history and environmental adaptations of *C. korshinskii* make it an excellent model for exploring drought tolerance mechanisms in non-model plants.

A substantial gap remains in our understanding of the PIP gene family in *C. korshinskii*. While PIP genes have been extensively characterized in model plants, their diversity, evolutionary dynamics, and functional roles in forage plant remain poorly understood. Moreover, the expression patterns of PIP genes under drought stress and their potential contributions to drought tolerance in *C. korshinskii* have not been systematically investigated. Addressing these gaps is essential for advancing our understanding of plant drought tolerance and developing strategies to enhance crop resilience under water-limited conditions.

In this study, we performed a comprehensive genome-wide analysis of the PIP gene family in *C. korshinskii* to identify and characterize its members, elucidate their evolutionary relationships, and assess their expression patterns under drought stress. Our findings provide valuable insights into the roles of PIP genes in the drought tolerance mechanisms of *C. korshinskii* and establish a foundation for future research aimed at improving crop drought resilience and promoting ecological restoration in arid regions.

2. Materials and methods

2.1 Plant material

Seeds of *Caragana korshinskii* Kom. were collected in August 2022 by Jiaxing Song from Chaheze Township, Yulin City, Shaanxi Province, China (altitude: 1110.94 m; GPS coordinates: 109°59'82"E, 38°50'32"N). The collected seeds are currently preserved at the College of Grassland Science, Northwest A&F University. Genomic sequencing of the material has been conducted. Notably, no voucher specimen corresponding to the wild *C. korshinskii* seeds utilized in this study was prepared or deposited in any herbarium. Regarding genomic data, the raw and processed sequencing datasets remain unpublished at present. Seeds underwent three 3-min 75% ethanol sterilizations (rinsed with distilled water), then a sterile scalpel incision. Seeds were placed on moist filter paper (28°C dark, 24 h), stratified at 4°C for 3 d, then germinated seedlings were transferred to a growth chamber (16 h light/8 h dark, 22 ± 1°C) in nutrient soil:vermiculite:perlite (1:1:3). After 3 weeks, drought stress was induced by withholding irrigation. Leaf samples were collected at 0, 3, 6, 12, 24 d post-stress (3 seedlings/replicate, 3 biological replicates), frozen in liquid nitrogen, and stored at – 80°C for RNA extraction and RT-qPCR.

2.2 Genome-wide identification of the PIP gene family in *C. korshinskii*

To comprehensively identify *CkPIP* genes, genome and transcriptome data of *C. korshinskii* were analyzed. The hidden Markov model (HMM) profile of the conserved MIP domain (PF00230) was retrieved from the Pfam database using HMMER v3.0 implemented in TBtools. Candidate sequences were further confirmed using the NCBI Conserved Domain Database (CDD), and incomplete or redundant sequences were manually removed. Physicochemical properties, including amino acid length, molecular weight, isoelectric point, instability index, aliphatic index, GRAVY value, and subcellular localization, were predicted using ExPASy (http://web.expasy.org/compute_pi, accessed 1 November 2024) and Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plantmulti/>, accessed 1 November 2024). Chromosomal locations of *CkPIP* genes were mapped using MapInspect (<http://www.softsea.com/download/MapInspect.html>, accessed 1 November 2024) based on the genome annotation of *C. korshinskii*.

2.3 Multiple sequence alignment, phylogenetic analysis, and tertiary structure prediction

To explore the evolutionary relationships of PIP proteins, sequences from soybean (*Glycine max*) and *Arabidopsis thaliana* were retrieved from the CNCB (<https://ngdc.cncb.ac.cn/gwh/Assembly/84056/show>) and TAIR (<https://www.arabidopsis.org/>) databases, respectively. Full-length PIP protein sequences were aligned using MAFFT v7.4.1 (Katoh and Toh, 2008). An unrooted phylogenetic tree was generated using the Neighbor-Joining (NJ) method in MEGA v11, with 1,000 bootstrap replicates to assess node reliability. The resulting tree was visualized and annotated using Evolview v3 (<https://www.evolgenius.info/evolview/>). Conserved MIP domains of all *CkPIP* proteins were aligned and visualized using ggMSA with default parameters. The tertiary structure of a representative *CkPIP* protein was predicted using the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/>). Database (<https://alphafold.ebi.ac.uk/>).

2.4 Gene structure, conserved motif, and cis-acting element analysis

Conserved motifs within CkPIP proteins were identified using MEME v5.4.1 (<http://meme-suite.org/tools/meme>) with default parameters, except for the motif number, which was specified manually. Cis-acting regulatory elements were predicted in the 2-kb upstream promoter regions of each CkPIP gene using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

2.5 Chromosome localization and gene syntenic analysis

The chromosomal positions of *CkPIP* genes were determined from the *C. korshinskii* genome annotation (GFF3 format) and visualized using TBtools with Circos visualization mode. Gene duplication events among *CkPIP* genes were identified using MCScanX. The nonsynonymous substitution rate (K_a), synonymous substitution rate (K_s), and their ratio (K_a/K_s) were calculated for duplicated *CkPIP* gene pairs using Ka/Ks_Calculator v3.0. Genes with $K_a/K_s > 1$ were considered under positive selection, $K_a/K_s = 1$ under neutral evolution, and $K_a/K_s < 1$ under purifying selection.

2.6 Prediction of 3D Structures, Pocket Sites, and Interaction Networks of CkPIP Proteins

Three-dimensional (3D) structures of CkPIP proteins were predicted using the Phyre2 server (<http://www.sbg.bio.ic.ac.uk/phyre2>). Protein alignments were generated using HMM–HMM comparison in intensive mode to improve model accuracy. Model quality was assessed using Ramachandran plot analysis. Ligand-binding sites (pocket regions) were analyzed using the Phyre Investigator tool within the Phyre2 server. Predicted models with 100% confidence were retained, with amino acid residue coverage ranging from 78% to 98%.

A protein–protein interaction (PPI) network for 13 CkPIP proteins was constructed using homologous sequences from *Populus euphratica* and *Arabidopsis thaliana* via the STRING database (<https://string-db.org>, accessed 24 May 2024).

2.7 Transcription factor regulatory network analysis

Potential transcription factor (TF) binding sites within the 2-kb upstream promoter regions of *CkPIP* genes were predicted using the Plant Transcriptional Regulatory Map (PlantRegMap; <http://plantregmap.gao-lab.org/>), with *Arabidopsis thaliana* as the reference and a screening threshold of $P \leq 1 \times 10^{-5}$. Predicted TF regulatory networks were visualized using Cytoscape v3.9.1 (Shannon et al., 2003). A word cloud summarizing TF family frequencies was generated using the ggplot2 package in R. Additionally, PPI network analysis was performed using CkPIP protein homologs in *Ricinus communis* via the STRING database (<https://cn.string-db.org/>).

2.8 Heatmap analysis and RT-qPCR verification of the transcriptome data

To validate the expression response of *CkPIP* genes under drought stress, differentially expressed *CkPIPs* showing more than a twofold change in transcript levels were identified from the transcriptome data. Heatmaps were generated based on $\log_2$ -transformed FPKM values and visualized using TBtools. Expression patterns of selected genes were validated using RT-qPCR with FastReal qPCR PreMix (SYBR Green) (Tiangen, Shanghai) on a Roche LightCycler 480 system. Primer sequences are listed in Table S1. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with *CiEF1 α* (GenBank No.: KC679842) as the internal reference gene. Each RT-qPCR assay was performed with three technical replicates.

3. Results

3.1 Systematic identification of the PIP gene family in *C. korshinskii*

Characterization of the structural domain integrity of PIP genes using a hidden Markov model (HMM) resulted in the identification of 13 family members in *C. korshinskii*. Comprehensive analysis of these *CkPIP* proteins revealed considerable diversity in their physicochemical properties, providing insight into their potential functional and biological significance. Amino acid lengths ranged from 187 residues in *CkPIP2;5* to 289 residues in *CkPIP1;1* and *CkPIP1;2*. Such variation may influence protein conformation and function, with longer sequences potentially facilitating complex folding and interaction patterns, while shorter sequences such as *CkPIP2;5* may confer greater flexibility or higher turnover rates.

The molecular weights of *CkPIP* proteins ranged from 19.90 kDa (*CkPIP2;5*) to 31.13 kDa (*CkPIP1;5*), consistent with their amino acid composition and length. Isoelectric points (pI) ranged from 6.58 (*CkPIP2;3*) to 9.92 (*CkPIP1;5* and *CkPIP1;6*), suggesting distinct biochemical behaviors under physiological conditions. Proteins with higher pI values are likely to be positively charged at physiological pH, favoring interactions with negatively charged molecules such as membranes or nucleic acids, whereas those with lower pI values may preferentially associate with positively charged components [10].

All *CkPIP* proteins showed instability index values below 40, indicating overall structural stability. The observed hydrophobicity levels are consistent with their roles as membrane-associated proteins, where hydrophobic regions facilitate anchorage to the lipid bilayer. The aliphatic index ranged from 92.81 (*CkPIP2;3*) to 111.31 (*CkPIP1;6*), reflecting their potential thermostability. Higher aliphatic indices suggest a more compact and stable protein structure, which may enhance functional resilience under varying environmental conditions. Detailed physicochemical parameters are summarized in Supplementary Table S2.

3.2 Phylogenetic and classification analysis of PIP genes

To investigate the phylogenetic relationships of *C. korshinskii* PIPs, a neighbor-joining (NJ) phylogenetic tree was constructed. The tree included 13 *CkPIP* proteins, 23 soybean PIPs (*Glycine max*), and 13

Arabidopsis thaliana PIPs (Fig. 1). Phylogenetic analysis classified *CkPIPs* into two subfamilies: PIP1 and PIP2. The PIP1 subfamily consisted of seven members (*CkPIP1;1–CkPIP1;7*), and PIP2 included six members (*CkPIP2;1–CkPIP2;6*).

PIPs from the same subfamily clustered together regardless of species, indicating evolutionary conservation and suggesting that these genes have retained similar structural and functional characteristics over time. Furthermore, *CkPIPs* clustered closely with homologous soybean and *Arabidopsis* PIPs within each subfamily, suggesting that PIP1 and PIP2 subfamilies have undergone both divergent and convergent evolution. Divergent evolution is reflected in the separation of the two subfamilies, indicating distinct functional features, whereas convergent evolution is suggested by the close clustering of PIPs from different species within the same subfamily, implying that similar selective pressures have maintained analogous genetic traits.

3.3 Conserved motifs and gene structure of the *C. korshinskii* PIP gene family

To investigate the sequence characteristics of the PIP gene family, analyses of conserved motifs and gene structure were performed. Using the MEME suite, 10 conserved motifs were identified across 13 *CkPIP* members. Members of the same subfamily showed high consistency in motif type and number. Motifs 1, 2, 3, 4, 5, and 7 were frequently observed, with Motifs 3, 4, and 5 present in all members, highlighting their importance in maintaining PIP protein structural and functional integrity.

Subfamily-specific variations were evident; for example, all PIP2 members contained Motif 8, which was absent in PIP1 members, suggesting specialized functions for PIP2. Gene structure analysis revealed evolutionary patterns, with members of the same subfamily exhibiting similar exon–intron organization. Most PIP1 members exhibited consistent exon numbers and intron positions, aligning with their conserved motif profiles, indicating functional redundancy and evolutionary conservation. In contrast, inter-subfamily variation was observed, as some PIP2 members displayed distinct intron lengths or exon–splicing patterns, correlating with unique motifs such as Motif 8.

This interplay between gene structure and motif composition reflects PIP gene family evolution and suggests that conserved motifs within stable gene structures preserve essential protein functions. Conversely, subfamily-specific structural features and motifs may contribute to functional divergence (Fig. 2).

3.4 Three-Dimensional modeling prediction and network analysis of *CkPIP* proteins

To investigate the structural characteristics of the *C. korshinskii* PIP family, particularly in relation to abiotic stress responses, three-dimensional (3D) models were predicted for all 13 *CkPIP* proteins. Structural models were generated using the Phyre2 server with hidden Markov model-based alignment (HMM-HMM), achieving 100% confidence for all predictions. Residue coverage ranged from 78% to 98%

(Fig. 3), indicating high reliability of the predicted structures. All CkPIP models exhibited a conserved hourglass-like structure, which is likely essential for protein function, facilitating transmembrane transport relevant to abiotic stress tolerance.

Analysis of secondary structures (Supplementary Table S3) revealed notable variation among CkPIP proteins. The α -helix content ranged from 30.66% (*CkPIP1;7*) to 36.73% (*CkPIP1;5*), β -turns from 1.05% (*CkPIP1;7*) to 3.64% (*CkPIP1;6*), random coils from 43.27% (*CkPIP1;6*) to 52.60% (*CkPIP1;1*), and extended strands from 14.88% (*CkPIP1;1*) to 20.86% (*CkPIP2;5*). These variations in secondary structures may influence protein folding, stability, and functional roles, including substrate-binding, within the conserved 3D framework

3.5 Promoter cis-acting elements of the *C. korshinskii* PIP gene family

To explore the regulatory mechanisms of the *C. korshinskii* PIP gene family, cis-acting elements within the 2-kb upstream promoter regions of 13 *CkPIP* members were analyzed. The analysis identified elements associated with abiotic and biotic stress responses, growth and development, and phytohormone signaling. Stress-related elements included LTR (low-temperature response), ARE (anaerobic response), and MBS (drought-inducible MYB binding site). Specifically, *CkPIP1;1* contained eight LTR elements related to low-temperature adaptation, while *CkPIP2;4* harbored 20 ARE elements associated with anaerobic stress. Growth-related elements included the CAT-box (meristem expression) and circadian elements (rhythm regulation), with *CkPIP1;2* containing 73 growth-related elements, highlighting its potential role in meristem regulation. Phytohormone-responsive elements, such as TCA-element (salicylic acid response) and GARE-motif (gibberellin response), were identified, with *CkPIP2;4* containing 21 such elements. This diverse distribution of cis-acting elements among *CkPIP* members underscores their potential roles in stress resistance, growth regulation, and phytohormone signaling (Fig. 4).

3.6 Analysis of the chromosomal localization of PIP family genes in *C. korshinskii*

Chromosomal localization of the PIP family genes was examined using the *C. korshinskii* genome. The distribution of 13 *CkPIP* genes across chromosomes was analyzed (Fig. 5). The genes were unevenly distributed across six chromosomes (LG02–LG07), whereas LG01 lacked any *CkPIP* genes, suggesting possible reduced gene retention or evolutionary loss. LG03 and LG04 contained more than three genes each, representing major loci. This pattern may result from segmental or tandem duplication events, contributing to functional diversification. LG04, LG05, and LG07 contained two genes each, whereas LG02 harbored only one, reflecting variable evolutionary pressures on gene density. The absence of genes on LG01 may indicate regions with limited relevance to water transport or stress adaptation, or susceptibility to structural rearrangements. The uneven distribution suggests adaptive evolutionary strategies, with clustered genes potentially enabling coordinated regulation under environmental stimuli

such as drought or salinity, whereas singleton genes on other chromosomes may have evolved specialized roles in tissue-specific water homeostasis.

3.7 Gene duplication events and collinearity analysis of the *CkPIP* gene family

To explore the evolutionary trajectory of the *C. korshinskii* PIP gene family, genome-wide duplication events were analyzed using MCScanX, emphasizing the role of segmental duplication in gene family expansion. Eight gene pairs were identified as segmentally duplicated, forming collinear blocks across chromosomes (Fig. 6). Notably, *CkPIP2;2* (LG05) and *CkPIP2;4* (LG06) exhibited high sequence similarity and synteny, suggesting their origin from ancient segmental duplication events contributing to genetic diversity. These duplicated pairs may facilitate functional diversification, contributing to specialization in water transport and stress responses.

Cross-species collinearity analysis with *A. thaliana* and *G. max* revealed 15 and 44 collinear pairs, respectively, indicating conserved synteny across dicots and shared evolutionary origins. These results highlight the role of segmental duplication in *CkPIP* family expansion and the conservation of collinear blocks across distantly related species, providing a framework for understanding functional divergence and adaptive evolution (Fig. 7).

3.8 Analysis of the gene regulatory network of the *C. korshinskii* PIP gene family

To further investigate the functional characteristics of the *CkPIP* family, its gene regulatory network was analyzed. Thirteen *CkPIP* proteins were analyzed using the STRING database, with *G. max* protein sequences as a reference for comparative analysis. The resulting network (Fig. 7) comprised 15 nodes and 59 edges, representing proteins and their interactions. Five of the 10 *CkPIP* proteins exhibited interactions within the network.

Notably, GLYMA_20G170400 interacted with multiple *CkPIP* proteins, having the highest number of connections among all network members. This suggests that GLYMA_20G170400 may serve as a central hub within the PIP family (Fig. 8), contributing to its regulation and function. Its multiple interactions indicate a hub role, potentially modulating processes mediated by PIP proteins, including water transport, osmotic regulation, and abiotic stress responses (Supplementary Table S4).

3.9 Expression patterns of the *CkPIP* gene family under drought stress

RNA-seq data were used to analyze the expression patterns of the *CkPIP* gene family across multiple tissues and time points under drought stress (Fig. 9). The 13 *CkPIP* genes displayed distinct expression profiles across organs. Under drought, expression levels were dynamic, varying with tissue type and

stress duration. In stems, *CkPIP1;4*, *CkPIP2;1*, and *CkPIP2;2* was significantly upregulated after 7 and 14 days compared to day 0. Notably, *CkPIP1;4* exhibited a pronounced increase, suggesting a role in adaptive responses to prolonged water deficit, while *CkPIP2;1* and *CkPIP2;2* may be involved in water transport or stress tolerance. Across organs, *CkPIP1;1*, *CkPIP1;2*, and *CkPIP2;2* showed relatively higher expression under drought. In roots, *CkPIP1;1* and *CkPIP1;2* was moderately to highly expressed, indicating potential roles in root water uptake and growth regulation. In leaves, *CkPIP2;2* was highly expressed, likely contributing to leaf water status and photosynthetic efficiency under water-limited conditions. These differential expression patterns suggest a coordinated regulatory mechanism enabling adaptation to drought by modulating water transport and homeostasis across tissues and organs.

3.10 Response and expression pattern of *C. korshinskii* PIP family genes under drought stress

Expression patterns of 13 *CkPIP* genes under drought were further analyzed to elucidate their responses to abiotic stress. All genes showed varying degrees of drought responsiveness (Fig. 8). In leaves, all genes were upregulated after 6 days of drought. Specifically, *CkPIP1;2*, *CkPIP1;7*, and *CkPIP2;2* peaked at day 6, followed by a decline at day 24. Expression at day 6 was 3-, 2.3-, and 5-fold higher than baseline for *CkPIP1;2*, *CkPIP1;7*, and *CkPIP2;2*, respectively (Fig. 8). *CkPIP1;3*, *CkPIP2;5*, and *CkPIP2;6* peaked at day 12, then decreased by day 24, while *CkPIP1;1*, *CkPIP1;5*, and *CkPIP1;6* peaked at day 24. Cluster analysis revealed that genes from the same evolutionary branch exhibited similar drought responses, suggesting shared regulatory mechanisms.

These findings suggest that *CkPIP1;1* and *CkPIP2;2* may interact during drought stress. It is hypothesized that *CkPIP1;1* mediates early-stage responses, whereas *CkPIP2;2* sustains responses over extended periods. Their interaction may coordinate downstream regulation of genes encoding water transporters or antioxidant enzymes to mitigate drought-induced damage. Further studies are required to elucidate the precise molecular mechanisms underlying their interactions and functional roles in drought responses.

4. Discussion

C. korshinskii, native to the arid and semi-arid regions of Northwest China, exhibits notable drought resistance. Investigating its adaptive mechanisms provides a foundation for future advances in molecular breeding. Previous studies have shown that plasma membrane intrinsic proteins (PIPs) play critical roles in plant responses to drought stress. For example, *AtPIP1;1* regulates stomatal movement in *Arabidopsis thaliana* under drought conditions. Studies indicate that PIP expression decreases under salt stress, leading to delayed recovery of hydraulic conductance and transpiration rates. Overexpression of *OsPIP1-1* or *OsPIP2-2* enhances tolerance to salt and drought stress [32]. Specifically, *MaPIP1;1* improves salt and drought tolerance by modulating primary root elongation, water uptake, and membrane stability in *Arabidopsis* [11].

In this study, we analyzed genomic and transcriptomic data to assess the evolutionary status and structural features of 13 *CkPIP* genes. Our results indicate that several *CkPIP* genes respond to drought stress. GRAVY values, ranging from 0.249 (*CkPIP1;7*) to 0.618 (*CkPIP2;5*), provide insights into overall protein hydrophobicity and hydrophilicity. Furthermore, RNA-seq and expression pattern analyses revealed that *CkPIP* genes are associated with drought stress responses, highlighting the functional relevance of this gene family. These findings provide a molecular foundation for future functional studies of the *CkPIP* gene family. In summary, these results offer important insights into the genetic basis of stress tolerance in *C. korshinskii*.

PIPs play key regulatory roles in stress responses [4] and developmental processes [25]. With advances in genome sequencing, an increasing number of PIPs have been identified in legumes, including *Glycine max* [5], *Pisum sativum* (Schuurmans et al., 2003), and *Medicago sativa* [12]. In this study, we identified 13 *CkPIP* genes from the *C. korshinskii* genome. *CkPIP* nomenclature was based on homology with orthologs in *Arabidopsis thaliana* and *Glycine max* (Fig. 1). Our findings, together with the evolutionary phylogeny of the PIP family, indicate that all *CkPIP* genes can be classified into two subfamilies (PIP1 and PIP2), consistent with previously established PIP classifications [28]. This classification highlights genetic diversity across species and suggests that PIP gene duplication and functional differentiation occurred during plant evolution.

Multiple *CkPIP* candidates exhibited functional convergence with homologs in *Glycine max*; however, only a limited number of orthologous relationships were definitively identified. The observed divergence may result from both technical and biological factors. Firstly, the incomplete genome assembly of *C. korshinskii* relative to *G. max* likely impeded precise ortholog identification. Secondly, post-speciation evolutionary processes may involve neo-functionalization through gene family expansion in *G. max* or pseudogenization events in *C. korshinskii*. Importantly, phenotypic similarities may reflect conserved regulatory mechanisms rather than direct orthology.

Given the highly compartmentalized nature of plant cells, PIPs are distributed across various cellular and subcellular compartments [13]. The *CkPIP* gene family is divided into two subfamilies, each exhibiting distinct subcellular localizations, including the plasma membrane and vacuoles. This study identified that *CkPIP1;5* and *CkPIP1;6* localize to the vacuole, whereas other PIP subfamily members are predominantly localized to the plasma membrane. Additionally, diverse localization patterns were observed among the PIP1 subfamily members (Supplementary Table S2). Previous research has indicated that most plant cytoplasm is neutral or slightly alkaline, while vacuoles tend to be acidic. These findings suggest that differences in protein acidity and alkalinity may be closely associated with subcellular localization and biological function [14].

To assess the functional conservation of *CkPIPs*, we analyzed their gene structure, conserved motifs, and exon-intron distribution. Previous studies have shown distinct exon-intron organization of PIP genes between dicots and monocots: most *AtPIP* and *GmPIP* genes in dicots adopt a conserved "4 exons + 3 introns" structure [9, 15], with the three introns stably inserted between consecutive exons—an important

structural marker for the family's functional conservation in dicots. In contrast, *OsPIP* genes from monocot rice contain 0–4 introns, reflecting structural divergence between the two lineages, and their expression is tightly regulated by promoter activity [9, 15]. Consistent with findings in *Carica papaya* [16] and cucumber [17] that dicot PIP genes exhibit subfamily-specific exon-intron numbers, our results showed subfamily-specific distribution in *CkPIPs* (from dicot *C. korshinskii*; Fig. 2A), further supporting PIP functional conservation among dicots and within subfamilies. Motifs analysis revealed that certain motifs are unique to the PIP1 and PIP2 subfamilies, potentially contributing to functional specificity. Furthermore, ten out of thirteen sequences shared six identical motifs, indicating a high degree of conservation within subfamily motifs, consistent with studies on *CkWRKYs* [3]. While motifs were largely conserved across all *CkPIPs*, some were subfamily-specific (Fig. 2B).

Promoters containing abundant cis-acting elements are critical regulators of gene expression. In bananas, promoter activity of *MaPIP1;1* is associated with responses to drought and salt stress [18]. Analysis of the promoter cis-elements of *CkPIPs* identified drought-responsive elements, as well as elements associated with phytohormone regulation, including gibberellin (GA), abscisic acid (ABA), and auxin (Fig. 4). GA, a key phytohormone, mediates plant responses to osmotic stress via reactive oxygen species scavenging and signal transduction pathways. GA is hypothesized to modulate osmotic pressure by reducing leaf transpiration, with its synthesis and degradation during early osmotic stress influencing stomatal aperture and transpiration levels [19]. In response to osmotic stress, plants synthesize ABA, which regulates ion channels in the plasma membrane of guard cells, leading to solute efflux, guard cell shrinkage, and stomatal closure [20]. The salicylic acid (SA) response element (TCA-element) enhances tolerance by modulating gene expression under osmotic stress and drought. This study indicates that SA and GA₃ can either activate or inhibit *CkPIPs* (Fig. 4), suggesting that PIPs play a central role in the complex network of plant hormone signaling.

The presence of multiple co-collinear pairs with *Arabidopsis* and soybean PIP genes indicates the existence of genetic diversity among different species (Fig. 7) and supports the hypothesis that gene duplication and functional differentiation of PIP genes occurred during plant evolution [21].

Within the protein interaction regulatory network of the *CkPIP* gene family, plant glycerol-3-phosphate dehydrogenase (GPDH) catalyzes the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P), playing a pivotal role in glycerolipid metabolism and stress responses [22]. In *Glycine max*, G-3-P contributes to triacylglycerol (TAG) synthesis, enhancing both nutritional value and biofuel production from soybean oils [23]. Our findings indicate that several *CkPIPs* interact with GPDH (Fig. 8), suggesting that these proteins may exert their functions through this interaction. Previous research demonstrated that silencing *GhGPDH5* reduces drought tolerance in upland cotton (*Gossypium hirsutum* TM-1) and is potentially involved in stomatal closure movements [24]. Therefore, GPDH appears to play a central role in plant drought responses, and specific members of the *C. korshinskii* PIP family may interact with GPDH to modulate abiotic stress tolerance in forage plants.

PIP genes are known to participate in complex regulatory networks associated with abiotic stresses and phytohormone signaling [25]. In this study, *C. korshinskii* seedlings were subjected to drought treatment for 0, 7, and 14 days, with samples collected from roots, stems, and leaves for transcriptome sequencing. Differentially expressed *CkPIP* genes were identified by comparing transcriptome data before and after treatment, and their expression was validated via RT-qPCR. The analysis revealed distinct expression patterns of individual *CkPIPs* under varying treatments, providing a valuable resource for functional characterization.

Drought stress-responsive PIPs have been reported in multiple species. For instance, *AsPIPs* respond to drought conditions [34], *BrPIPs* respond to both salt and drought stress [33], and *CpPIPs* are also drought-responsive [16]. These findings underscore the importance of transcriptome sequencing in elucidating gene function. Integrating transcriptomic and genomic analyses further enhances functional predictions within gene subfamilies.

In the present study, RNA-seq data revealed that *CkPIP* genes display tissue- and time-specific expression under drought stress (Fig. 9). In stems, *CkPIP1;4*, *CkPIP2;1*, and *CkPIP2;2* showed significantly elevated expression after 7 and 14 days of drought stress compared to day 0, with *CkPIP1;4* exhibiting the most pronounced increase, suggesting its involvement in adaptive responses to prolonged water deficit. Additionally, *CkPIP1;1*, *CkPIP1;2*, and *CkPIP2;2* displayed higher overall expression under drought. Specifically, *CkPIP1;1* and *CkPIP1;2* in roots may facilitate water uptake and root growth regulation, whereas *CkPIP2;2* in leaves likely contributes to maintaining water status and enhancing photosynthetic efficiency.

Subsequent RT-qPCR analysis of all 13 *CkPIP* genes confirmed differential temporal expression under drought stress. Expression peaks were observed at 6 days for *CkPIP1;2*, *CkPIP1;7*, and *CkPIP2;2*; at 12 days for *CkPIP1;3*, *CkPIP2;5*, and *CkPIP2;6*; and at 24 days for *CkPIP1;1*, *CkPIP1;5*, and *CkPIP1;6* (Fig. 10). Cluster analysis indicated that genes from the same evolutionary lineage exhibited similar drought-responsive patterns, suggesting shared regulatory mechanisms. Notably, *CkPIP1;1* and *CkPIP2;2* may function cooperatively in drought response, with *CkPIP1;1* initiating early-stage responses and *CkPIP2;2* sustaining these responses over extended periods, potentially through coordinated regulation of downstream genes involved in water transport or antioxidant defense.

5. Conclusions

In this study, the PIP gene family in the genome of *C. korshinskii* was comprehensively analyzed, leading to the identification of 13 PIP genes. These genes were characterized through analyses of their evolutionary relationships, gene structure, gene duplication events, promoter cis-elements, and regulatory networks using bioinformatic approaches. Based on the classification of PIP families in *A. thaliana* and *G. max*, the *CkPIP* family was categorized into two subfamilies: PIP1 and PIP2. Genes within the same evolutionary branch exhibited similar genetic and structural characteristics, reflecting their conserved functions. Among the 13 *CkPIP* proteins, five demonstrated protein–protein interaction

relationships, with GLYMA_20G170400 exhibiting the highest number of interacting partners. Expression analysis under drought stress revealed that *CkPIP2;1*, *CkPIP2;2*, and *CkPIP1;1* was significantly upregulated, suggesting their potential as key candidates in the drought stress response.

Overall, this study provides a comprehensive analysis of the *CkPIP* protein family, elucidating their sequence characteristics, evolutionary relationships, and potential functional roles in drought adaptation. These findings offer a valuable reference and theoretical foundation for future research aimed at understanding the structural and functional properties of *CkPIPs* and improving drought stress resistance in *C. korshinskii*.

Abbreviations

PIP, Plasma membrane Intrinsic Proteins; MIP, Major Intrinsic Protein; RT-qPCR, Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction; FPKM, Fragments per Kilobase of Transcript per Million Mapped Reads; Ka, Nonsynonymous Substitution Rate; Ks, Synonymous Substitution Rate; TF, Transcription Factor; PPI, Protein–Protein Interaction; HMM, Hidden Markov Model; CDD, Conserved Domain Database; CiEF1 α , Cyclophilin EF1 α ; GPDH, Glycerol-3-Phosphate Dehydrogenase; NCBI, National Center for Biotechnology Information; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; ATG, autophagy-related; DTT, dithiothreitol; ER, endoplasmic reticulum; HSP, heat shock protein; HSR, heat shock response; UPR, unfolded protein response.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

The dataset supporting the conclusions of this article is included within the manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We would like to express our gratitude to the College of Grassland Agriculture, Northwest A&F University for providing the experimental platform.

Authors' contributions

Wenyu Li led the conceptualization and methodology development of the study, in addition to conducting investigations, curating data, and drafting the original manuscript. Hatieti Madeti contributed to investigation efforts, collected experimental data, and performed statistical analysis. Xinyi Liu participated in investigations, curated key datasets, and assisted with statistical analysis. Jiahui Ren was involved in experimental execution, carried out investigation-related tasks, and organized research data. Yuze Wang contributed to investigation work, conducted statistical analysis, and verified the accuracy of the collected data. Zhilai Wang provided support for investigation activities, offered experimental assistance, and processed research data. Peizhi Yang oversaw the study, handled project administration, and facilitated funding acquisition. Xiaoxia Shen, as the corresponding author, provided overall supervision of the study, contributed to conceptualization, performed manuscript review and editing, and granted final approval for the publication of the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No. 32302478).

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Figures

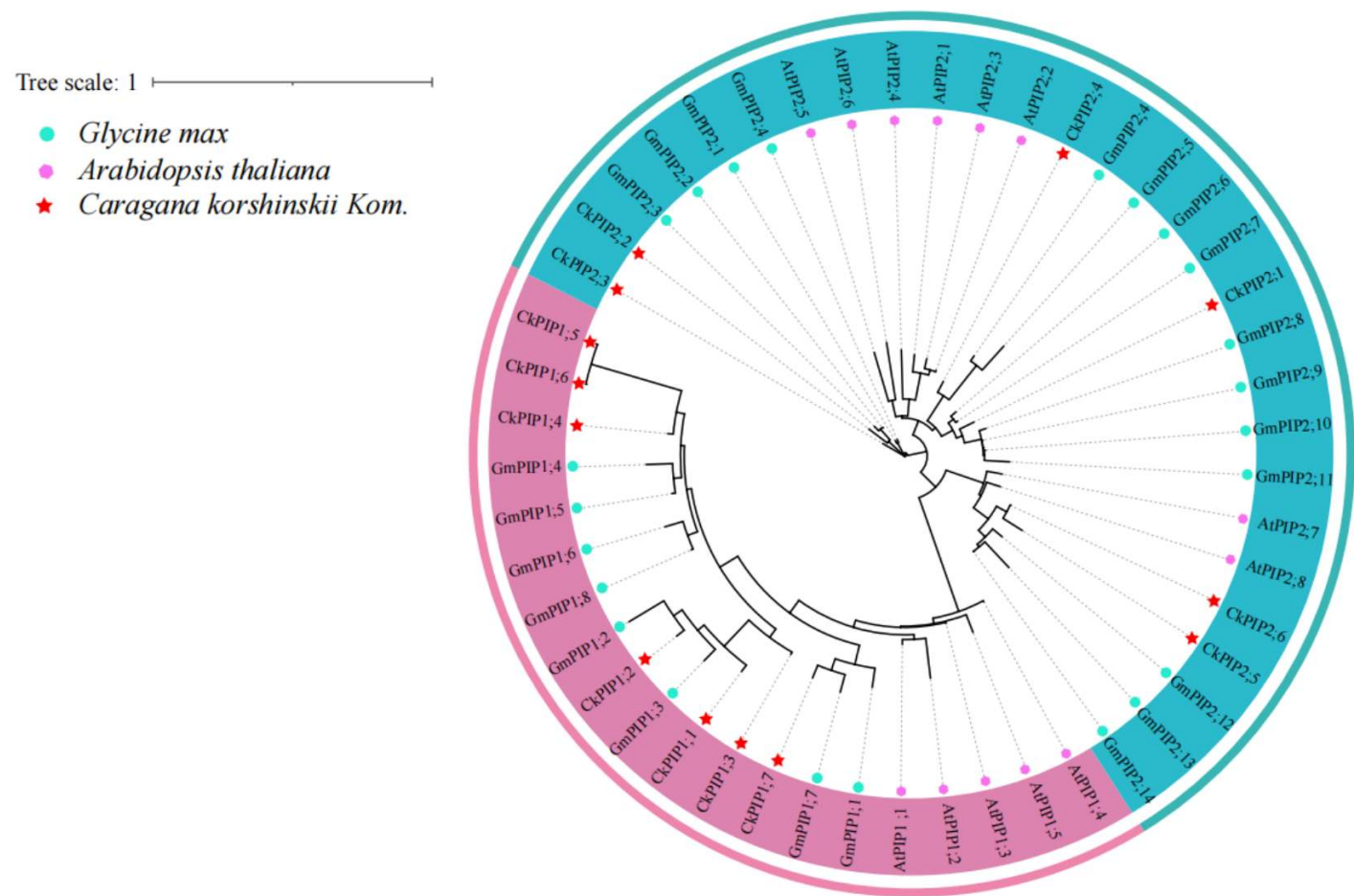


Fig. 1 Phylogenetic analysis of 49 PIPs proteins from *C. korshinskii*, *A. thaliana* and *G. max* constructed using the neighbor-joining (NJ) method. These PIPs were clustered into two subfamilies, designated as 1 and 2. CKPIPs, AtPIPs, and GmPIPs were marked with a red five-pointed star, pink circle, and blue circle, respectively.

Figure 1

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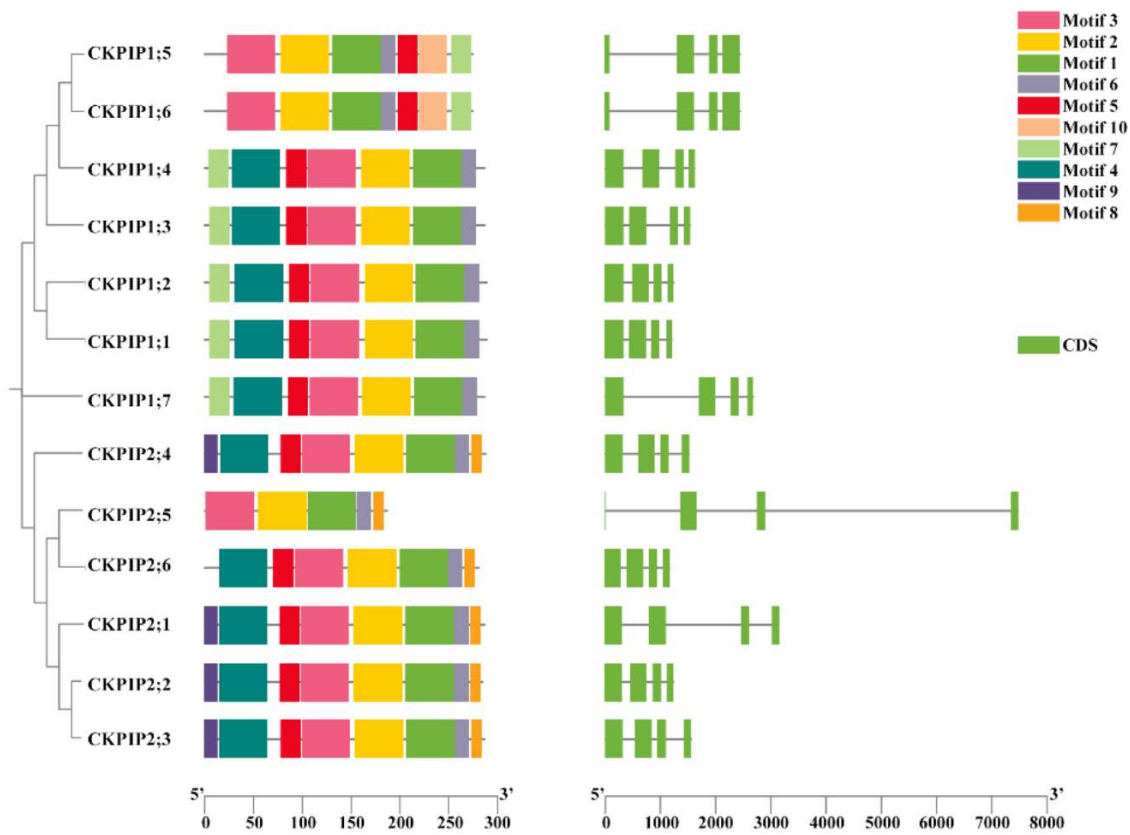


Fig. 2 Phylogenetic relationship, conserved motifs, and gene structure analysis of *CkPIP* family members of the *C. korshinski*.

Figure 2

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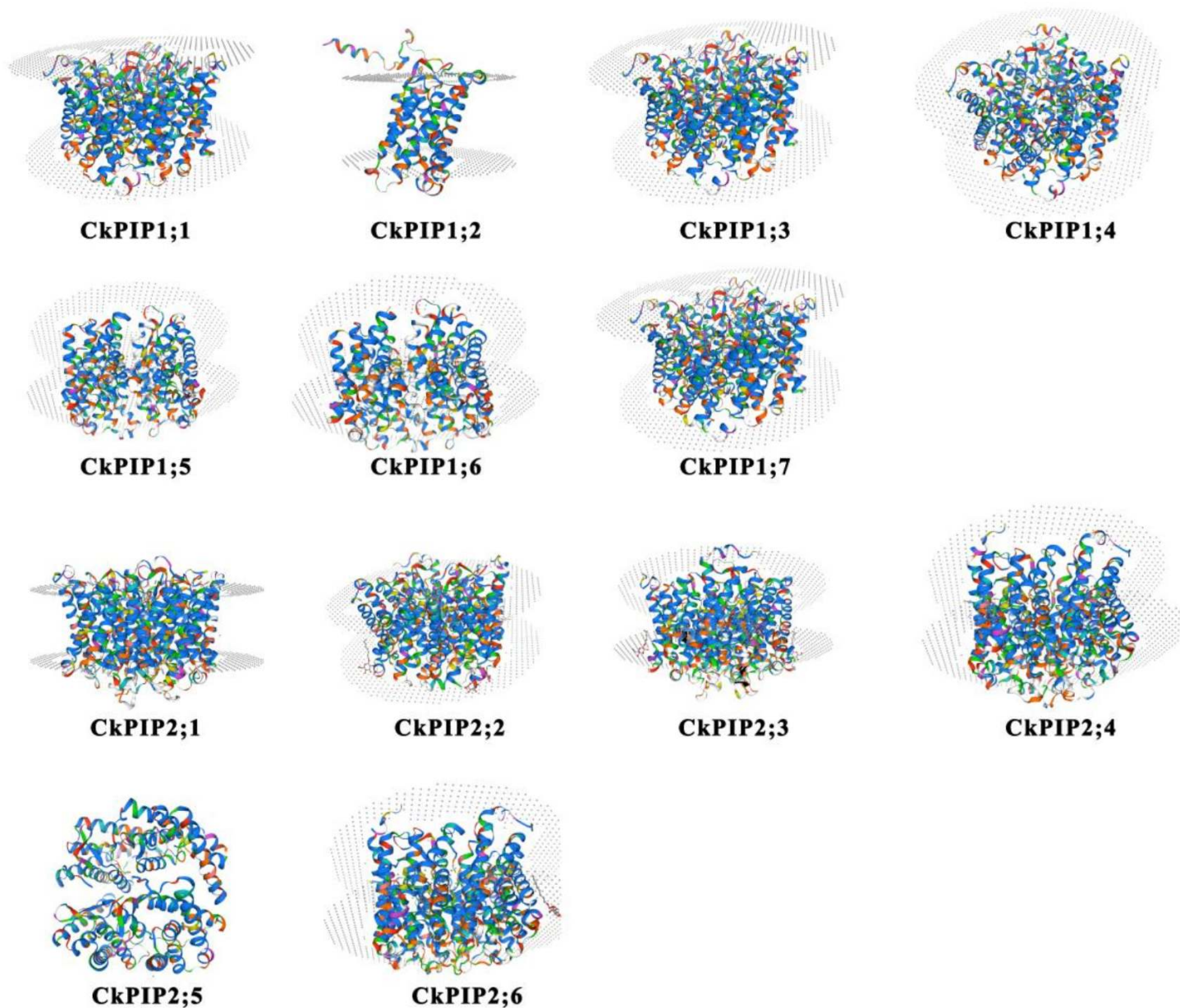


Fig. 3 Predicted tertiary structures of the selected 13 CkPIP protein using the AiphaFold 3. Modeled at a 100% confidence level by using Phyre2 server and the percentage residues ranged from 78 to 98%.

Figure 3

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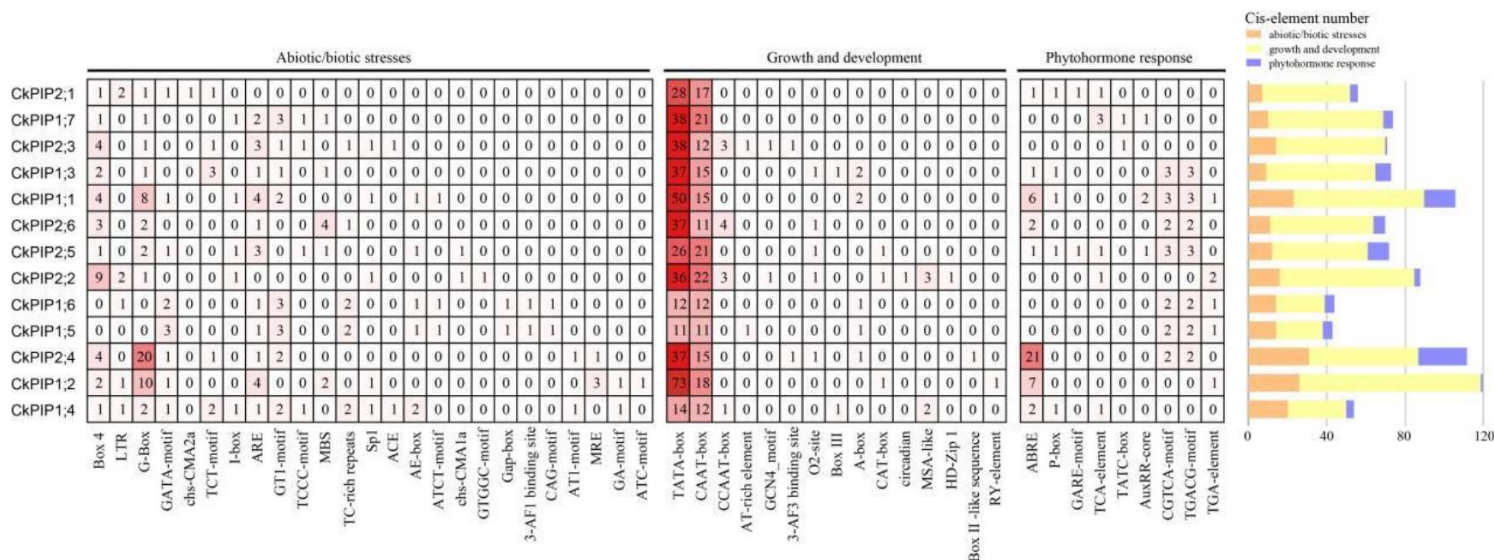


Fig. 4 *Cis*-acting elements analysis of *CkPIP* members. Three groups representing abiotic/biotic stresses, growth and development, and phytohormone response, respectively. Names of cis-acting elements are shown on the below.

Figure 4

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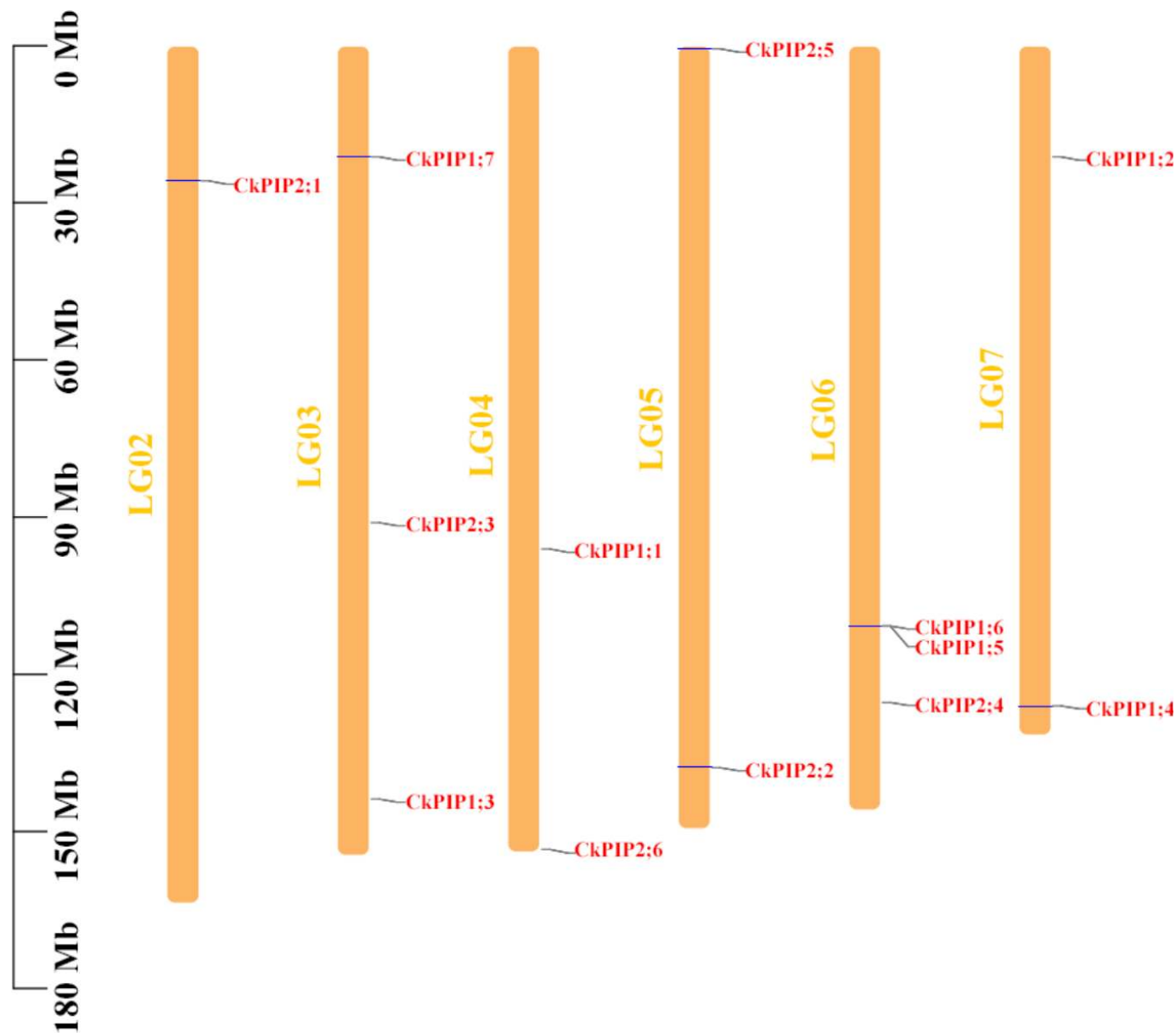


Fig. 5 Chromosomal distributions of *CkPIP* members in the *C. korshinski*.

Figure 5

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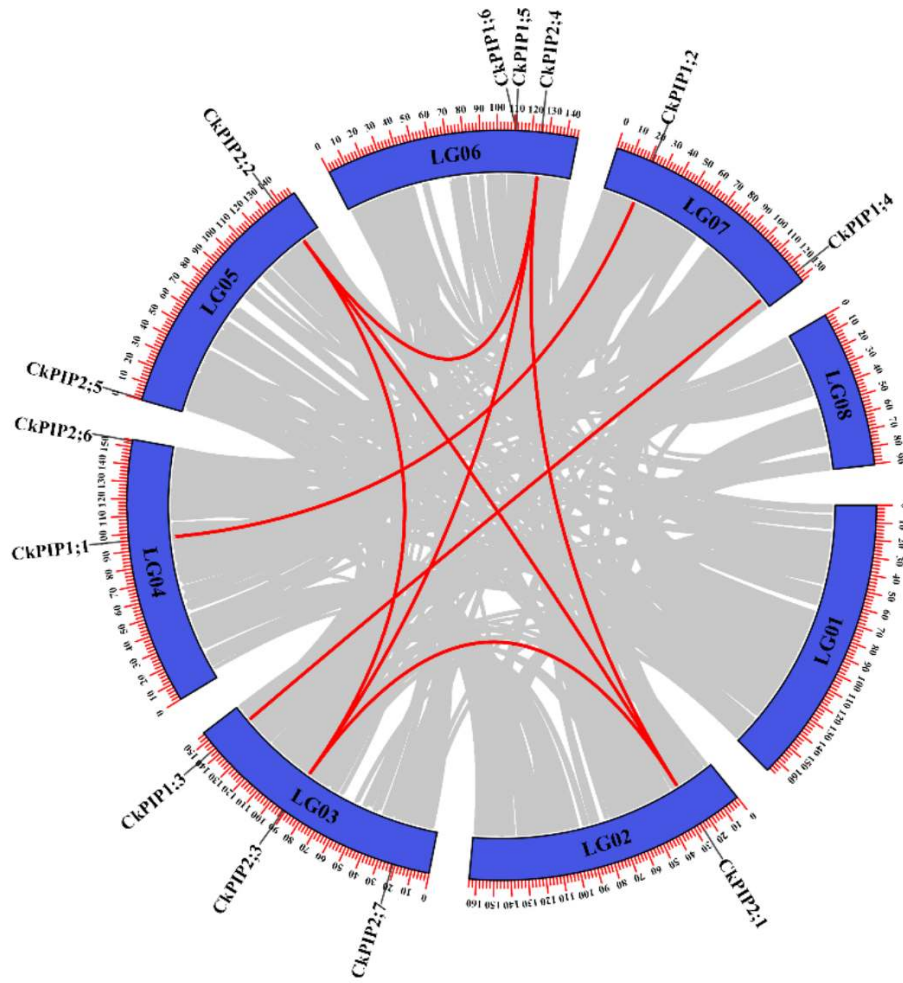


Fig. 6 Collinearity analysis of the CkPIP family members. The red lines represent gene pairs with collinearity relationships, the labels in the boxes denote chromosomes, and the scale ranging from 0 to 140 on the chromosomes indicates gene density.

Figure 6

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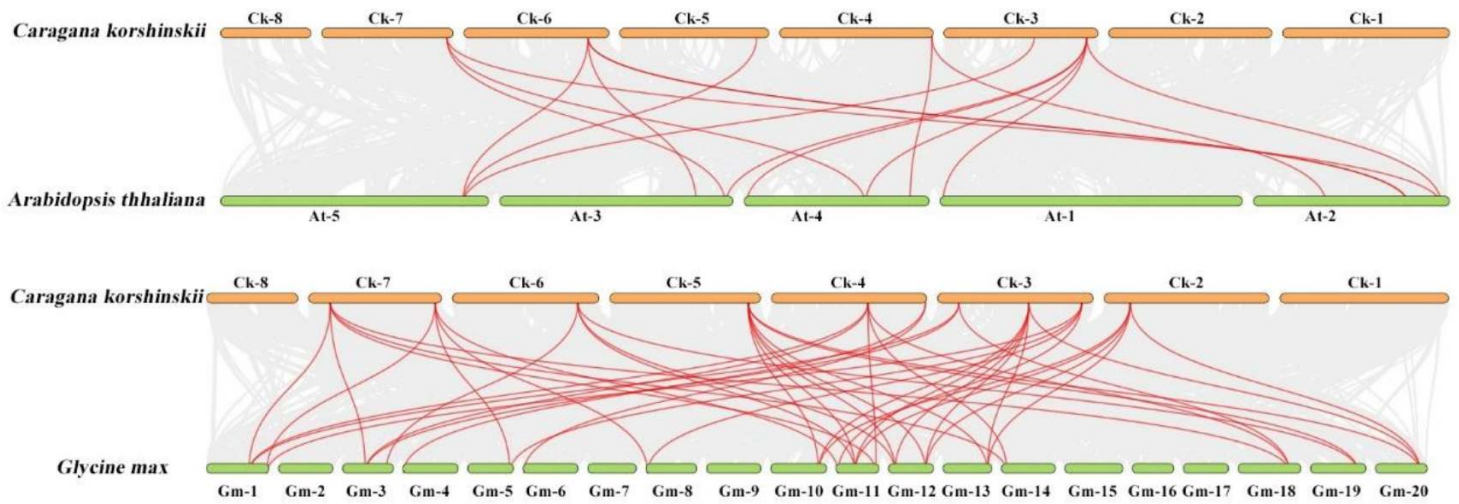


Fig. 7 Comparative genomics analysis of PIP gene collinearity between *C. korshinskii* and four model plants species (*A. thaliana* and *G. max*). The gray background lines indicate blocks of collinearity within *C. korshinskii* and the other designated plant species, while the red lines denote homozygous PIP genes.

Figure 7

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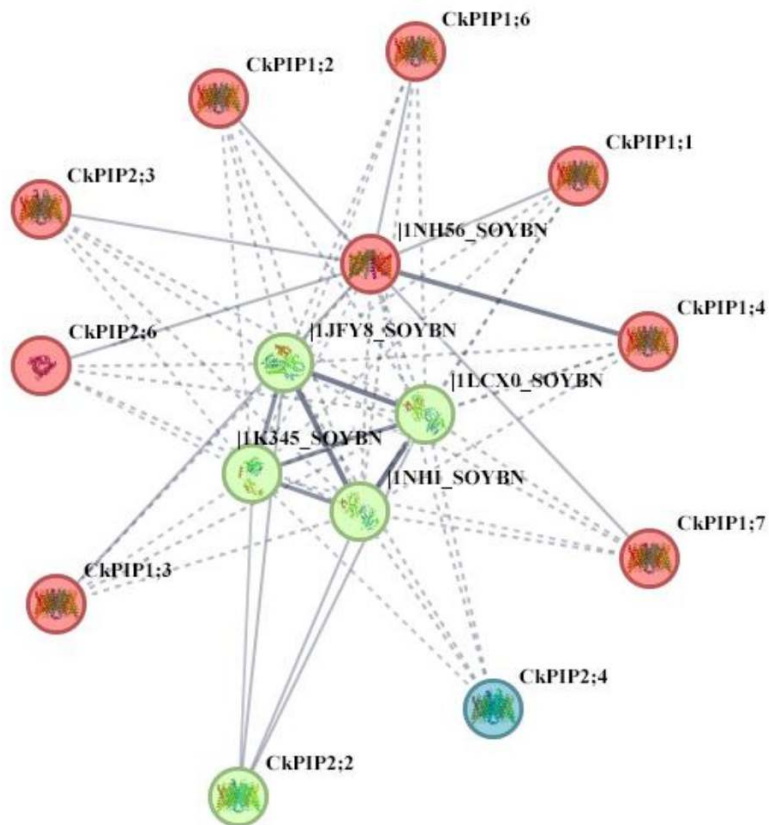


Fig. 8 Schematic depicts the protein interaction network of CkPIPs, with each node representing a protein and each edge denoting an interaction.

Figure 8

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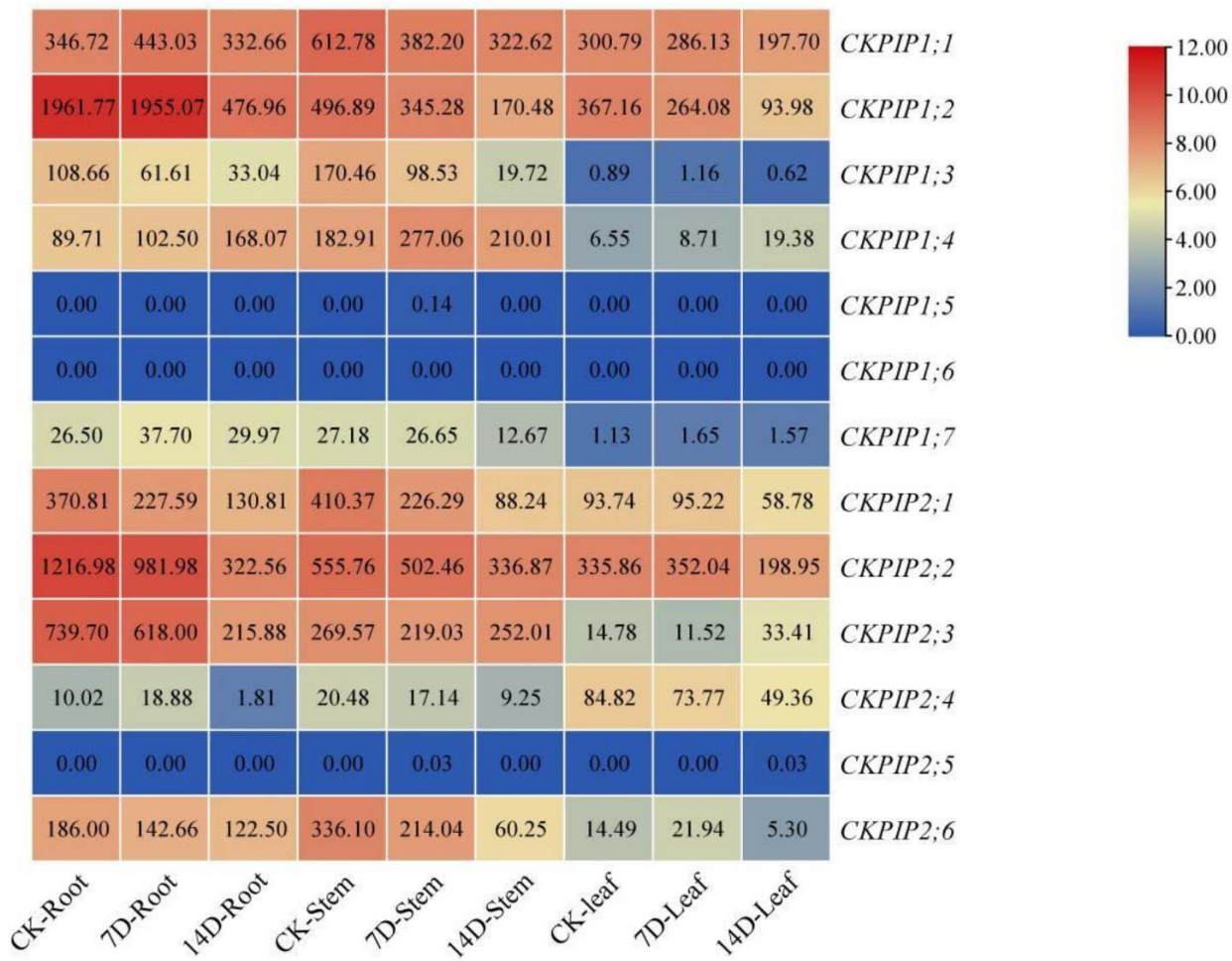


Fig. 9 The relative expression of *CkPIP* genes in roots, stems, and leaves following drought treatments at 0, 7, and 14 days from the RNA-seq analysis. The heatmap was generated based on triplicate FPKM values transformed using $\log_2$ transformation.

Figure 9

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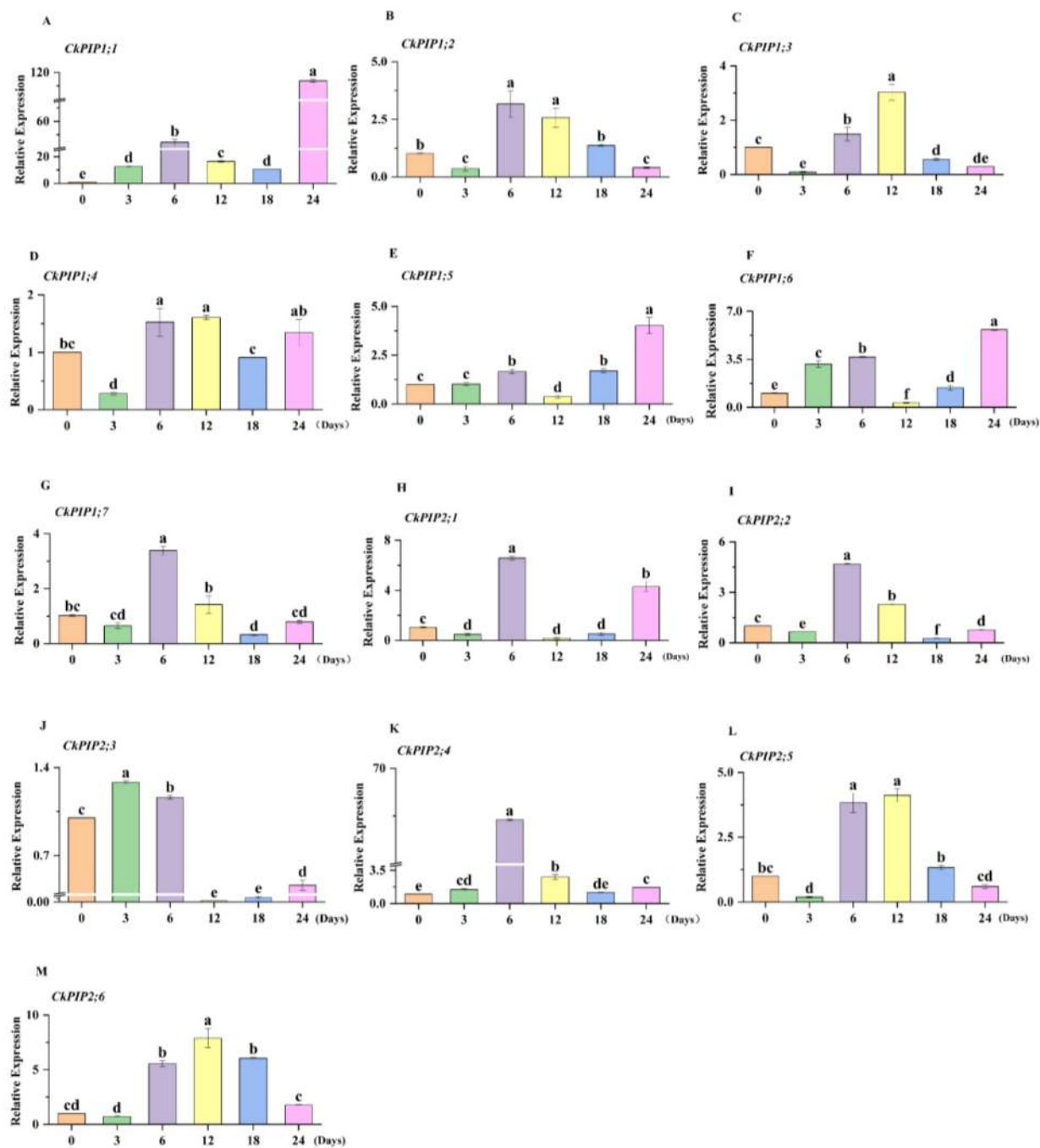


Fig. 10 The expression level of *CkPIP* genes in response to drought stress after 0, 3, 6, 12, 24 days.

Figure 10

See image above for figure legend

Supplementary Files

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