A Genome-wide Analysis of the 14-3-3 Gene Family in Chinese Chestnut (*Castanea mollissima*)

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Abstract [**Objectives**] The 14-3-3 proteins are a class of highly conserved adapter proteins in eukaryotes that play a central role in signal transduction by recognizing phosphorylated target proteins and are crucial for plant growth and development. Chinese chestnut (*Castanea mollissima*) is an important woody grain crop in China. This study aimed to systematically identify the 14-3-3 gene family in chestnut and investigate their fundamental characteristics and functional clues, thereby laying a foundation for further elucidating the biological functions of this family in Chinese chestnut. [**Methods**] Using a combination of bioinformatics and molecular biology approaches, we conducted a comprehensive identification of the 14-3-3 family members in Chinese chestnut. Subsequently, we systematically analyzed their physicochemical properties, gene structures, conserved domains and motifs, gene duplication events, phylogenetic relationships, tissue-specific expression patterns, and codon usage bias. [**Results**] A total of nine 14-3-3 family members, designated CmGRF1 to CmGRF9, were identified and classified into two subgroups: epsilon (5 members) and non-epsilon (4 members). All CmGRF proteins were predicted to be hydrophilic. Phylogenetic analysis revealed a strong correlation between the clustering of CmGRFs and their respective gene structures, conserved domains, and motif compositions. Transcriptome data analysis indicated significant differences in the expression levels of different CmGRF members across various tissues and developmental stages. Codon preference analysis showed that CmGRFs tend to use codons ending with A/U, and their evolution is primarily driven by natural selection pressure. [**Conclusions**] This study provides the first genome-wide systematic analysis of the gene family in Chinese chestnut. The findings offer important theoretical insights and candidate genes for further research into the specific functions of these family members in chestnut growth, development, and stress responses.

Key words Chinese chestnut; 14-3-3 gene family; Phylogenetic analysis; Codon usage bias

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Many processes during the plant life cycle require specific signal transduction to accomplish essential biological functions. A common mechanism for relaying such information involves interactions between proteins undergoing reversible phosphorylation modifications[1]. However, phosphorylated proteins must bind to specific adapter proteins, known as phosphoserine/phosphothreonine-binding proteins, to execute their corresponding functions[2].

14-3-3 proteins are widely present in eukaryotes. They were first discovered in brain tissue and highly conserved, hydrophilic acidic proteins with a molecular weight of approximately 30 kDa and a length of about 250 amino acids. The discovery of 14-3-3 proteins in plants occurred approximately 20 years later than in animals. They were identified as part of the G-box complex and thus named GF14 (G-box factor homologue) or GRF (G-box regulating factor, or general regulating factor)[3]. As part of the G-box protein complex, which is ubiquitous in eukaryotes and highly conserved in structure, 14-3-3 proteins primarily exist as homodimers or heterodimers. Each momomer consists of nine anti-parallel α-helices and functions as dimeric phosphoserine-binding protein, capable of interacting simultaneously with two target proteins or with two domains of a single target protein. After binding to the phosphorylated serine/threonine residue within a short consensus motif on the target protein, 14-3-3 proteins interact with their targets mainly through three phospho-binding modes, ensuring their vital regulatory functions in vivo[4].

Members of the 14-3-3 gene family are classified into the epsilon (ε) and non-epsilon groups based on their gene structure and amino acid sequence similarity. Typically, genes in the epsilon group contain 6 - 7 exons and 4 - 6 introns, whereas those in the non-epsilon group generally possess only 4 exons and 3 introns[5]. By binding to the pSer/pThr residues of various target proteins (such as transcription factors and signaling proteins), 14-3-3 proteins modulate their activity, thereby regulating numerous biological processes including primary metabolism, stress responses, membrane transport, and signal transduction cascades[6].

The dimeric nature of 14-3-3 proteins forming either hetero- or homodimers, with each monomer comprising nine anti-parallel α-helices allows therm to act as scaffolds[7]. These structural features provide binding sites for interactions with 14-3-3 proteins themselves and their targets. The dimeric property enables a single 14-3-3 complex to bring two sites of the same protein proximity or to link two different proteins together within a single complex[8].

The repertoire of 14-3-3 target proteins is extensive. For instance, over 300 target proteins have been identified in

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Arabidopsis, interacting in either a phosphoserine-dependent or independent manner. Plants possess multiple isoforms of 14-3-3 genes. For example, the monocot rice has 8 isoforms[9], while the dicot *Arabidopsis* has 15 isoforms[10]. The presence of multiple isoforms in plants suggests that isofor-specific interactions with target proteins may be a key factor in regulating 14-3-3 function. These isoforms are encoded by multigene families with slight sequence variations.

In recent years, with the continuous release of plant genomes, genome-wide identification and analysis of 14-3-3 genes have been conducted in numerous species beyond model organisms like rice and *Arabidopsis*. For instance, 31, 14, 21, and 16 14-3-3 genes have been identified in cotton[11]. polar[12], rapeseed[13], and tomato[14], respectively. Studies have shown that 14-3-3 genes play important roles in plant growth, development, and stress responses[15]. For example, the *GsGF*14 gene in wild soybean is associated with stomatal and root hair growth[16]. Cotton Gh14-3-3L is involved in regulating fiber elongation and maturation[17]. Overexpression of *Arabidopsis AtGRF*14 in cotton enhanced drought resistance in transgenic plants[18], while overexpression of tomato *TFT*4 in *Arabidopsis* improved alkali tolerance[19]. Conversely, knocking out *AtGF*14 in *Arabidopsis* enhanced freezing tolerance[20].

Chinese chestnut is an important woody grain crop in China and has recently gained increasing attention. The cloning and characterization of the chestnut 14-3-3 gene family have also attracted researcher's interest. These genes appear to play regulatory roles in responses to abiotic stresses (such as drought, salinity, alkalinity, and low phosphorus) and biotic stresses (such as *Xanthomonas* and *Euphorbia*)[21]. However, the functions of most members in the SITFT family remain unclear. With the rapid development of bioinformatics and modern molecular biology techniques, further analysis and refinement of the properties and functions of this gene family are necessary. In this study, a genome-wide identification was performed, and analyses were conducted on phylogeny, gene structure, conserved domains, motifs, phylogenetic relationships among different species, chromosomal distribution, and transcriptional profiles. The primary objective of this research is to provide comprehensive and novel insights into the CmGRF family, offering valuable references for studies on molecular and biological functions of these genes, as well as for genetic breeding research.

Materials and Methods

Identification of the Chinese chestnut 14-3-3 gene family

The publicly available protein sequences of 13 *A. thaliana* 14-3-3 proteins were downloaded from the TAIR website (http://www.arabidopsis.org/) to serve as seed sequences. The genomic data file and annotation file for the 'N11-1' chestnut cultivar were obtained from the China National GeneBank Database (https://ngdc.cncb.ac.cn/gwh). BlastP was employed to align the known 14-3-3 protein sequences against the chestnut protein database with an E-value threshold of 1e-5 to mine homologous sequences. The resulting sequences were further screened using the NCBI-CDD tool to identify candidate members of the chestnut 14-3-3 gene family. Concurrently, the integrity of the conserved domains in these candidate sequences was verified. The conserved domain for 14-3-3 (PF00244) was retrieved from the Pfam website (link provided above) and used for comparison analysis with HMMER 3.0 software to validate the candidate 14-3-3 members in the chestnut genome. Ultimately, nine members of the 14-3-3 family were identified. Based on their chromosomal locations, they were designated as CmGRF1 to CmGRF9.

Phylogenetic analysis of the chestnut 14-3-3 family

Reported 14-3-3 protein sequences from *A. thaliana* and *Oryza sativa* were downloaded for phylogenetic tree construction[22-23]. Multiple sequence alignment was performed between chestnut CmGRF1-CmGRF9 and *Arabidopsis* 14-3-3 protein sequences. A phylogenetic tree of the CmGRF family members was constructed using the Neighbor – Joining method in MEGA 7.0 software, with the bootstrap value set to 1 000 and other parameters kept as default.

Physicochemical properties, chromosomal localization and subcellular localization prediction of chestnut 14-3-3 family members

The physicochemical properties of chestnut *CmGRF*1-*CmGRF*9, including amino acid count, molecular weight, isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY), were analyzed using the ExPASy website (http://cn.expasy.org/tools). The subcellular localization of CmGRF1-CmGRF9 proteins was predicted using the BUSCA online tool (http://busca.biocomp.unibo.it/).

Chromosomal localization of chestnut 14-3-3 family members

The chromosomal positions of *CmGRF*1-*CmGRF*9 were extracted from the chestnut genome annotation file. The gene localization visualize (Advanced) function in TBtools software was used to map the chromosomal distribution of *CmGRF* gene family members and generate a chromosomal localization diagram[24].

Motif and gene structure analysis of the 14-3-3 family

The protein sequences of the 14-3-3 gene family were analyzed for conserved motifs using the MEME online tool (https://meme-suite.org/), resulting in the identification of 5 conserved motifs. These motifs were subsequently submitted to the InterProScan database (https://www.ebi.ac.uk/interpro/result/interprosca/) for functional annotation. Gene structure analysis of the 14-3-3 gene family members was performed using the Gene Structure View (Advanced) modules in TBtools[24]. Finally, the motifs, conserved domains and gene structures were visualized using the software's built-in program.

Analysis of *CmGRF* gene expression in different tissues of Chinese chestnut

To investigate the expression pattern of the *CmGRF* gene

family in different tissues, RNA-seq data from stems and buds of Chinese chestnut were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). Transcripts were assembled and evaluated using HISAT, String Tie, and Ballgown software to obtain gene expression levels across different tissues. The expression levels of *CmGRF*1-*CmGRF*9 were finally visualized using TBtools software.

Codon usage bias analysis of the 14-3-3 gene family

The relative synonymous codon usage (RSCU), effective number of codons (ENC), and GC1, GC2, and GC3 (GC content at the first, second, and third codon positions) of the 14-3-3 genes were calculated using CodonW1.4.2 (https://codonw.sourceforge.net/) and the online EMBOSS tool (https://www.bioinformatics.nl/emboss-explorer/). The RSCU value determines the usage frequency of synonymous codons for each amino acid within the 14-3-3 gene family. An RSCU value greater than 1 indicates a relatively high frequency of codon usage, while a value less than 1 suggests a relatively low frequency[25].

The neutrality plot was generated with GC3 as the abscissa (X-axis) and GC12 (the average GC content of the first and second codon positions) as the ordinate (Y-axis). A scatter plot was created and fitted with linear regression line. A regression coefficient close to 1 indicates a strong correlation between GC12 and GC3, suggesting similar base variation patterns across all three codon positions. This implies that mutational pressure is the primary factor shaping codon usage bias in the 14-3-3 gene family members. Conversely, a lower regression on coefficient points to natural selection as a greater influencing force[26].

The theoretical ENC value (ENCexp) was calculated using the formula $ENCexp = 2 + GC3 + 29\ [GC3^2 + (1 - GC3)^2]$[27]. A standard curve was plotted with GC3 as the abscissa and ENCexp as the ordinate, generating an ENC-plot. An actual ENC value close to the ENCexp suggests that the codon usage bias of the gene is primarily influenced by mutation pressure. A significant deviation between the actual and expected values implies a greater impact from natural selection. Furthermore, an ENC value exceeding 35 indicates a weak codon usage bias[28].

Data analysis and the construction of the neutrality plot and ENC-plot were performed using Microsoft Excel 2023.

Results and Analysis

Phylogenetic analysis of 14-3-3 gene family

To elucidate the phylogenetic relationships among chestnut 14-3-3 proteins, a phylogenetic tree was constructed using the protein sequences of *C. mollissima* and *A. thaliana* (Fig. 1). The results indicate that the CmGRF proteins can be divided into two major groups: the epsilon (ε) group and the non-epsilon group. Specially, CmGRF3, CmGRF4, CmGRF5, CmGRF6, and CmGRF9 belong to the epsilon group, while CmGRF1, CmGRF2, CmGRF7, and CmGRF8 are classified as non-epsilon.

Identification and physicochemical characterization of 14-3-3 gene family members in Chinese chestnut

Through bioinformatic analysis, nine 14-3-3 family members were identified in the chestnut genome. Based on their respective subfamilies and chromosomal locations, they were designated as *CmGRF*1 to CmGRF9. ExPASy analysis revealed that the number of amino acids in these nine CmGRF proteins ranges from 246 to 266, with CmGRF2 being the largest (possessing 266 amino acids). Their molecular weights range from 279 09.50 to 300 62.79 Da, with an average of 288 73.02 Da. The isoelectric points vary between 4.73 and 6.09, averaging 4.98. The instability indices range from 37.48 to 49.87, and the aliphatic indices range from 81.75 to 92.22. All CmGRF1-CmGRF9 proteins exhibit strong hydrophilicity, with CmGRF8 showing the highest hydrophilicity. Subcellular localization predictions indicate that all nine CmGRF members are localized to the nucleus (Table 1).

Table 1 Analysis of physicochemical properties of the Chinese chestnut 14-3-3 gene family

Sequence ID	Name	Number of amino acid	Molecular weight	Theoretical pI	Instability index	Aliphatic index	Grand average of hydropathicity	Subcellular localization
EVM0003002.1	CmGRF1	262	29 761.59	4.85	49.23	86.07	−0.476	Nucleus
EVM0031859.1	CmGRF2	266	30 065.79	4.75	49.82	86.99	−0.511	Nucleus
EVM0022387.1	CmGRF3	246	27 909.50	4.94	49.87	81.75	−0.598	Nucleus
EVM0007139.1	CmGRF4	258	29 352.96	4.83	46.50	84.81	−0.533	Nucleus
EVM0008165.1	CmGRF5	252	27 942.89	6.09	37.48	88.17	−0.434	Nucleus
EVM0012667.1	CmGRF6	258	29 352.96	4.83	46.50	84.81	−0.533	Nucleus
EVM0000860.1	CmGRF7	248	28 197.94	4.90	48.21	89.35	−0.362	Nucleus
EVM0017167.1	CmGRF8	252	28 469.27	4.73	42.99	92.22	−0.287	Nucleus
EVM0011350.1	CmGRF9	253	28 804.25	4.86	49.48	84.55	−0.595	Nucleus

Chromosomal localization and collinearity analysis of the 14-3-3 gene family in Chinese chestnut

Based on the whole-genome annotation of Chinese chestnut, a chromosomal localization map of the *CmGRF* gene family was generated. The results (Fig. 2) showed that the chestnut 14-3-3 gene family members are distributed across chromosomes 1, 4, 5, 7, 9, and 12. The *CmGRF* genes were distributed on six chromosomes: *CmGRF*1 and *CmGRF*2 on chromosome 1, *CmGRF*3 on chromosome 4, *CmGRF*4, *CmGRF*5, and *CmGRF*6 on chromosome 5, *CmGRF*7, *CmGRF*8, and *CmGRF*9 on chromosomes 7, 9, and 12, respectively.

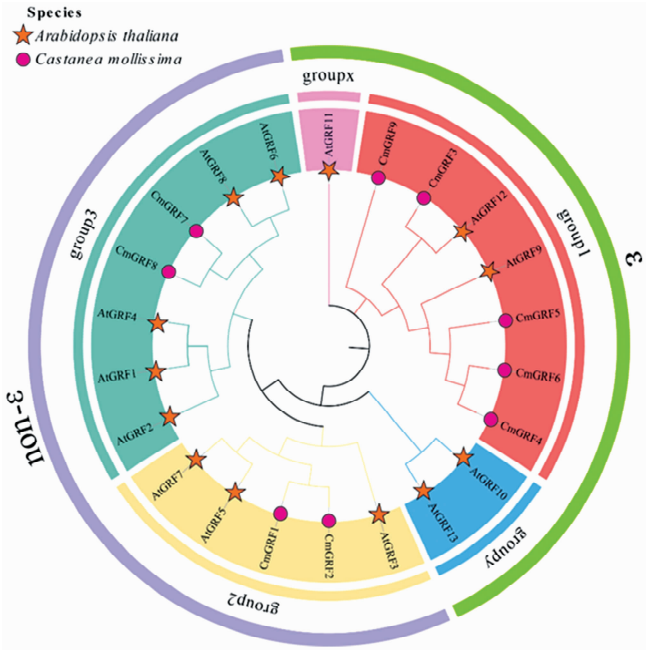

Fig. 1 Phylogenetic tree of the 14-3-3 gene families in Chinese chestnut (*Castanea mollissima*) and *A. thaliana*

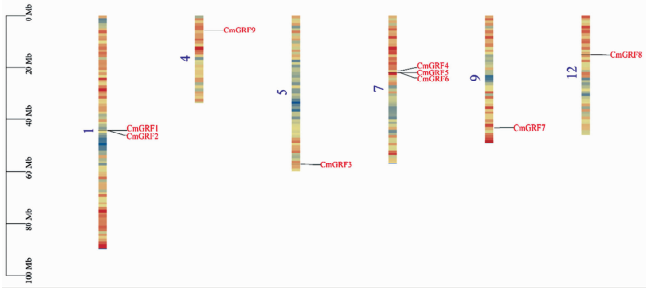

Fig. 2 Distribution of the 14-3-3 genes on chromosomes in Chinese chestnut

Analyses of collinearity within the *CmGRF* gene family (Fig. 3) revealed one pair of genes exhibiting a collinear relationship: *CmGRF*7 on chromosome 7 and *CmGRF*8 on chromosome 9. The Ka/Ks ratio for this pair was 0.053 3, indicating that it likely underwent strong purifying selection during evolution.

To further investigate the evolutionary relationships of the 14-3-3 gene family across different species, a collinearity analysis was performed among Chinese chestnut, *Arabidopsis*, and the monocot rice (Fig. 4). The results revealed a stronger association between Chinese chestnut and *Arabidopsis*, with eight collinear gene pairs identified. In contrast, no collinear pairs were found between Chinese chestnut and rice, indicating a closer phylogenetic relationship between Chinese chestnut and *Arabidopsis* compared to rice. Given that rice is a monocot, this suggests that the expansion of the 14-3-3 gene family may have occurred after the divergence of monocots and dicots. This observation is supported by previous research[29], which found no overlap between the 14-3-3 genes of monocots (rice, wheat, and maize) and dicots (tomato, watermelon, melon, potato, grape, polar, and *Arabidopsis*) across ten plant species, corroborating our hypothesis.

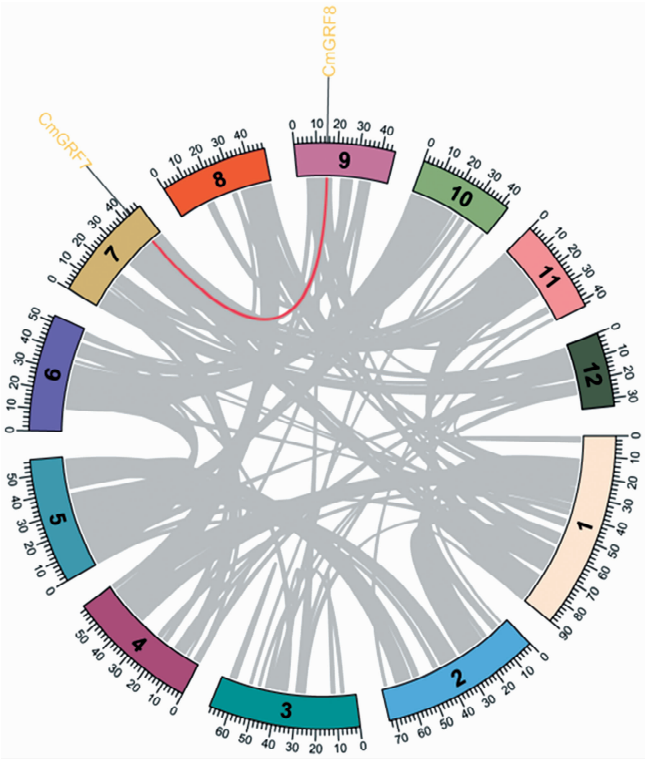

Fig. 3 Intra-genomic collinearity analysis of the 14-3-3 gene family in Chinese chestnut

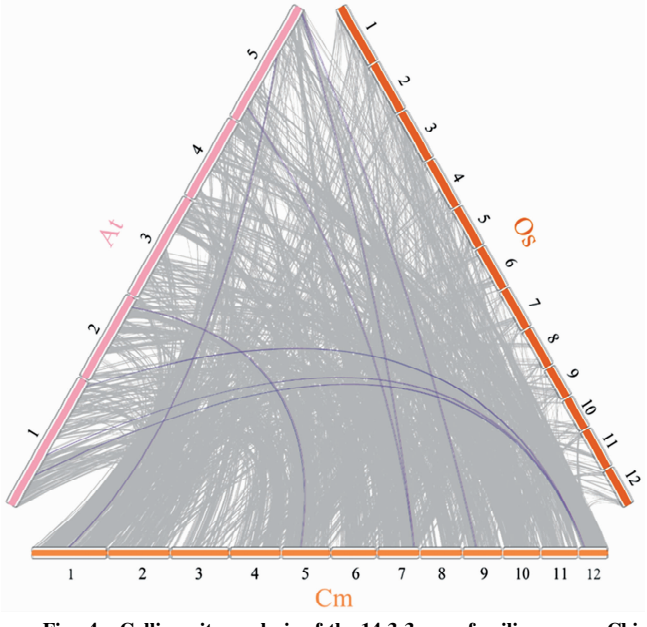

Fig. 4 Collinearity analysis of the 14-3-3 gene families among Chinese chestnut (Cm), *Arabidopsis* (At) and rice (Os)

Motifs and gene structure of 14-3-3 gene family in Chinese chestnut

The conserved motifs of 14-3-3 gene family analysis revealed that no significant difference in the motif composition between epsilon and non-epsilon 14-3-3 proteins (Fig. 5). All members

contain five motifs, except for CmGRF3 (which has four) and CmGRF5 (which has three). The number of exons varies among the chestnut 14-3-3 genes and is associated with their specific classifications. Among them, the non-epsilon members CmGRF1 and CmGRF2 contain four exons. CmGRF7 and CmGRF8, along with the epsilon member CmGRF5, possess three exons. The remaining epsilon-class members, excluding CmGRF5, all contain six exons.

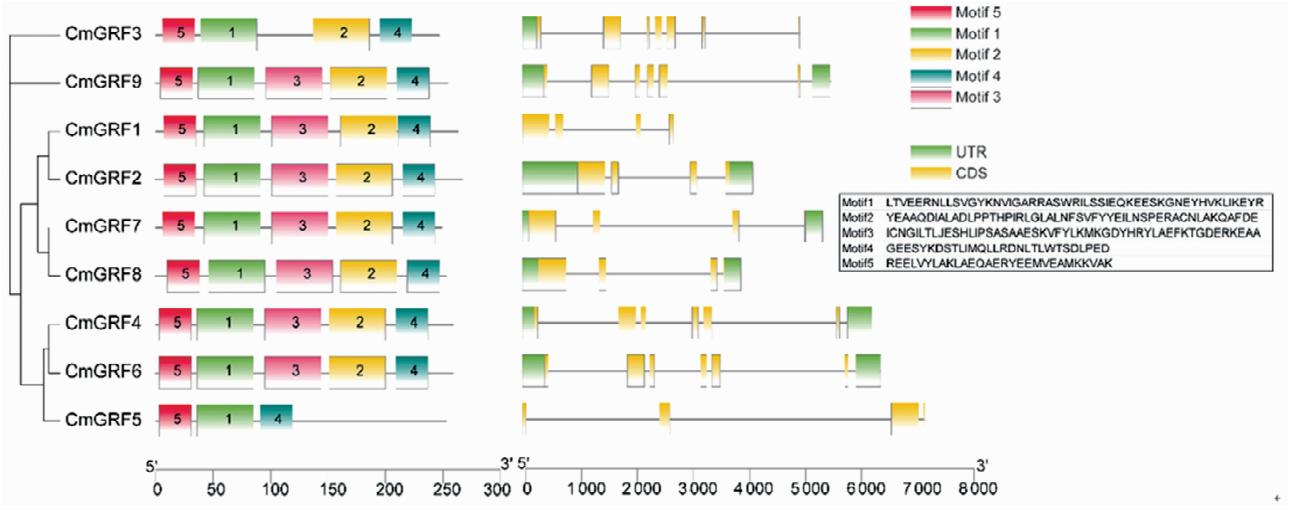

Fig. 5 Conserved motifs and gene structures of the 14-3-3 gene family in Chinese chestnut

Transcriptome analysis of the 14-3-3 gene family in buds and nuts

Fig. 6 Transcriptomic analysis of the 14-3-3 gene family in Chinese chestnut buds and nuts

To explore the potential functions of *CmGRFs*, we systematically analyzed the transcriptomic data of each member. The expression patterns of *CmGRFs* across various tissues were visualized using a clustered heatmap (Fig. 6). We found that *CmGRFs* were expressed in both fruits and buds at different development stages, with expression levels varying considerably between tissues and over time. The results indicated that *CmGRF*5 exhibited low expression in both buds and fruits, suggesting it might be a pseudogene. In contrast, *CmGRF*2 showed the highest expression levels in both tissues, implying it could be a major effector gene in the Chinese chestnut *CmGRFs*. During fruit development, the transcript levels of nearly all family members decreased over time. However, only a few genes showed a similar decreasing trend in buds. This may be because the demand for signal transduction decreases as fruits mature, whereas buds, even after maturation, still require active signaling.

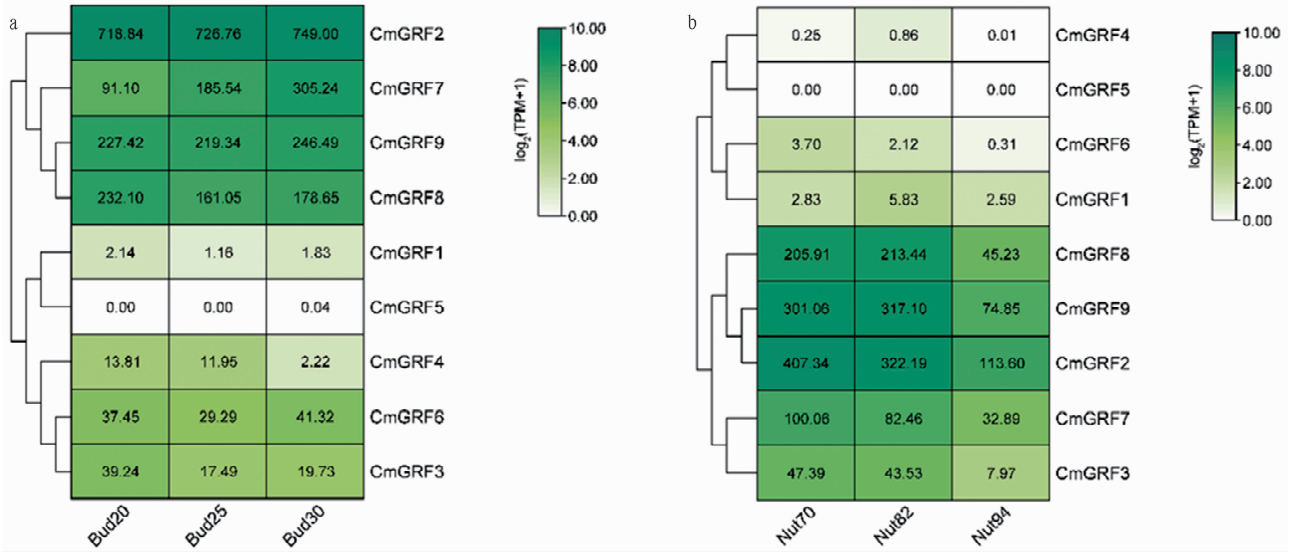

Fig. 6 Transcriptomic analysis of the 14 – 3 – 3 gene family in Chinese chestnut buds and nuts

Analysis of codon usage bias in the Chinese chestnut 14-3-3 gene family

The distribution range of GC3s reflects the selective pressure acting on a plant. A narrower GC3s distribution range indicates a stronger influence of natural selection on codon usage bias. Conversely, when the frequencies of the four nucleotide bases are equal, codon bias is influenced solely by mutational pressure. Additionally, plotting the theoretical standard curve of Effective

Number of Codons (ENC) can reveal the relative influence of mutation versus selection on codon usage. Codons plotting near the curve are primarily influenced by mutational pressure, while those deviating from the curve are subject to stronger natural selection[30].

Neutrality plot analysis of GC12 versus GC3 for the chestnut 14-3-3 gene family (Fig. 7) revealed a significant positive correlation ($r = 0.110\ 1$). The GC3 content ranged from 40% to 70%, while GC12 was confined to a narrower range of 42% –46%. With most data points not distributed along the regression line, the result suggests that natural selection, rather than mutation pressure, was the dominant force shaping the codon usage bias in these genes, as evidenced by the lack of substantial difference in base composition among the three codon positions.

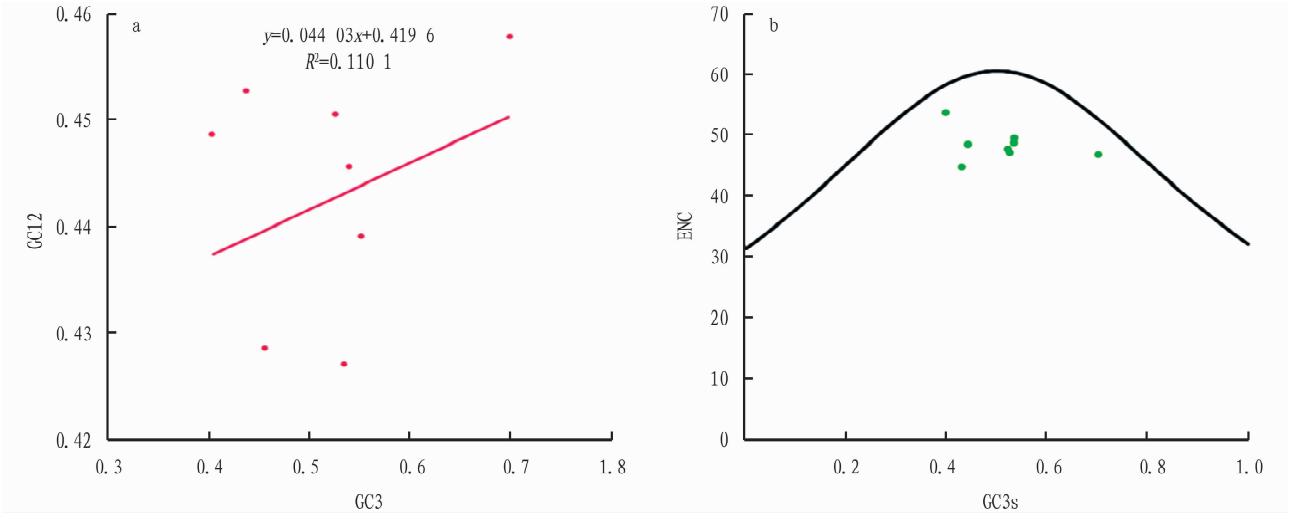

Fig. 7 Codon usage bias analysis of the Chinese chestnut 14-3-3 gene family (a. Neutrality plot analysis; b. ENC-plot analysis)

An ENC-plot analysis of the 14-3-3 gene family revealed that all loci of the gene family lie away from the expected curve. The distribution trend does not align with that of the standard curve, and the points are situated relatively far from the expected curve. This further indicates that codon usage bias in the 14-3-3 genes is strongly influenced by natural selection, which is consistent with the results from the neutrality plot analysis.

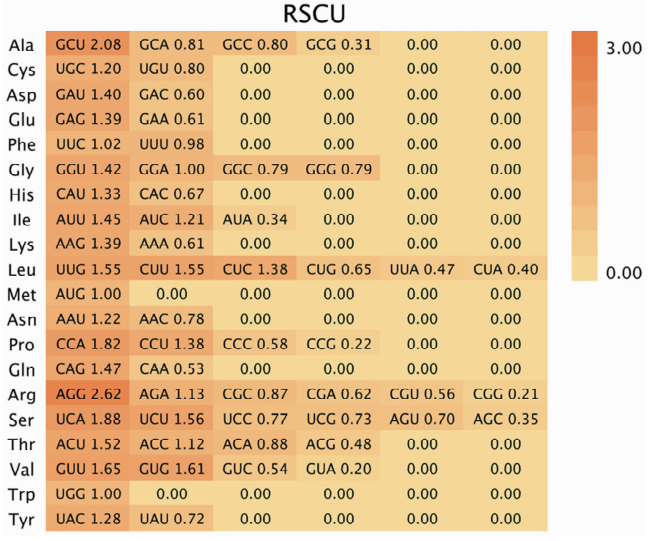

Fig. 8 RSCU values of codons in members of the Chinese chestnut 14-3-3 gene family

Analysis of the RSCU values for the Chinese chestnut 14-3-3 gene family (Fig. 8) revealed that AUG (Met) and UGG (Trp) were excluded from codon preference analysis as they encode methionine and tryptophan, respectively, with no codon usage bias. The stop codons (UAA, UAG, UGA) were also excluded. The results showed 28 codons with an RSCU value greater than 1. Among these, 4 codons terminated with A, 11 with U, 6 with G, and 7 with C. This distribution is consistent with the pattern where monocotyledonous plants more frequently use codons ending with C and G, while dicotyledonous plants prefer codons ending with A and U (T)[31]. Furthermore, the data indicate a clear preference for codons ending with U in the Chinese chestnut 14-3-3 gene family.

Discussion

The 14-3-3 proteins are a widely expressed protein family in eukaryotes, known to play distinct regulatory roles in various physiological processes and tissues of plants. Therefore, investigating the functions of plant 14-3-3 proteins is of significant importance. Against the backdrop of environmental degradation, the impact of abiotic stress on plant physiology and crop yield is increasingly severe. Research into the mechanisms of plant abiotic stress response is crucial for enhancing stress tolerance. Given that 14-3-3 proteins play a key role in abiotic stress responses, studies focusing on them represent a critical avenue for addressing stress-related challenges.

In this study, we identified nine CmGRFs from the Chinese chestnut genome. The majority of these CmGRFs were found to be unstable proteins, with an average molecular weight (MW) of 288 73.02 Da. They were all acidic and hydeophilic, which is largely consistent with previously published findings[32]. Subcellular localization predictions indicated that all nine members are nuclear

proteins, suggesting that their functions are primarily carried out in the nucleus. Motif analysis revealed the presence of five conserved motifs in the Chinese chestnut 14-3-3 gene family. Among these, motif 1, motif 2, and motif 5 were identified as characteristic motifs. Furthermore, the genes contain varying numbers of exons, indicating potential divergence in their gene structures. This structural variation suggests that different members of the 14-3-3 gene family may play distinct functional roles in the organism.

A phylogenetic analysis of 14-3-3 proteins from various species was conducted to elucidate the evolutionary relationships of the 14-3-3 family between Chinese chestnut and other species. The clustering of members from different species within the same group implies a shared origin and close evolutionary relationship. The 14-3-3 members analyzed included those from *A. thaliana*, *Branchipodium distachyon*, and *Manihot esculenta*. It is hypothesized that the origin of the 14-3-3 family predates the divergence of monocots and dicots. Our evolutionary analysis also revealed that certain sub-branches within different groups may have undergone more frequent gene loss and amplification events. This suggests that the structure and function of 14-3-3 proteins are prone to divergence, indicating functional diversity in gene expression and protein function among family members[33].

Of particular note, we observed that all nine CmGRFs exhibited a non-random distribution pattern across the Chinese chestnut chromosomes. They were located on only 6 of the 12 chromosomes. Specifically, except for two members on chromosome 1 and three members on chromosome 5, the remaining four chromosomes each harbored only a single member. At least two large-scale duplication events have occurred during chestnut evolution. The first was a chromosomal block duplication after the monocot-dicot divergence (approximately 170 – 235 million years ago, MYA)[34], followed by a polyploidization event after the Arabidopsis-chestnut split (approximately 90 MYA)[35]. This second duplication event corresponds to the differentiation of the 14-3-3 genes in *Arabidopsis* and chestnut.

In Chinese chestnut, we found that *CmGRFs* are expressed in buds and nuts, with expression levels varying significantly across different tissues and developmental stages. This tissue-specific expression pattern during specific growth and developmental processes is generally consistent with previously reported expression patterns of 14-3-3 family members in cotton[36] and grape[37]. In grape, the expression levels of most *VviGRFs* differ substantially among various tissues and organs. For instance, *VviGRF*12 generally shows low expression in most tissues but is undetectable in floral organs such as stamens, bus, flowers, and pollen. Conversely, *VviGRF*15 is highly expressed in most tissues and organs except leaves, petals, and pollen, suggesting that *VviGRFs* may be involved in regulating grape development and fruit ripening[37]. In cotton, 31 *GhGRFs* also exhibit distinct expression levels across 15 examined tissues. Nine *GhGRFs* show relatively low expression in all tissues, while another 15 are highly expressed across all tested tissues, indicating their potential involvement throughout cotton growth and development. Furthermore, *GhGRF*8-*A*, *GhGRF*6-*A*, and *GhGRF*6-*D* show expression levels in 0 – and 1 – day post-anthesis ovules similar to those 15 highly expressed genes, suggesting they may function as important tissue-specific regulators[36]. These collective findings strongly corroborate our results.

The 14-3-3 proteins are extensively involved in various plant cellular processes and growth developmental stages, such as cell elongation, seed germination, vegetative growth, and reproductive development. The activation of plasma membrane H (+) – ATPase via C-terminal binding with 14-3-3 proteins facilitates cell elongation[38]. For instance, during the rapid elongation phase of cotton fiber development, Gh14-3-3 genes are induced[39]. In barley seed germination, 14-3-3 3A/B/C are induced, displaying distinct spatial expression patterns[40], while Jerusalem artichoke exhibits high 14-3-3 gene expression abundance during sprouting.

These proteins also regulate vegetative growth, including root growth, hypocotyl elongation, photomorphogenesis, and photoperiod responses. Mutations in *A. thaliana* 14-3-3μ/*υ* lead to shortened roots and altered chloroplast developmeng, which are modulated by light intensity and wavelength[41]. Additionally, 14-3-3λ/k interacts with PIF3 to mediate light signaling in Arabidopsis[42]. In rice, 14-3-3 proteins interact with root hair development-related factors to regulate root growth[43]. Overexpression of bamboo (*Phyllostachys edulis*) Pe14-3-3b in Arabidopsis results in longer root, thicker stems, earlier bolting, and accelerated growth[44].

By mediating phytohormone signal transduction, 14-3-3 proteins influence growth, development, and stress responses. In *Arabidopsis*, the regulate auxin-dependent phototropism by modulating NPH3 localization[45] and control gibberellin GA biosynthesis through interaction with RSG[46]. They may also interact with the brassinosteroid BR receptor kinase BRI1 to regulate leaf development[47]. In rice, qGL3 induces phosphorylation of *OsGRF*14*b*, modulating nuclear-cytoplasmic shuttling and transcriptional activity of *OsBZR*1, ultimately negatively regulating BR signaling and grain length[48].

Flowering represents a critical transition from vegetative to reproductive growth. In chrysanthemum, *Cm*14-3-3μ cooperates with the CCT transcription factor *CmNRRa* to suppress flowering[49], whereas overexpression of rapeseed BnGF14-2c promotes flowering independent of vernalization[50]. In *Arabidopsis*, 14-3-3 acts as a scaffold protein, forming a florigen activation complex (FAC) with Florigen FT and the bZIP transcription factor FD. This complex activates *APETALA*1 expression, initiating floral organ formation[51]. Similarly, in rice, Hd3a first complexes with 14-3-3 protein GF14c in the cytoplasm, then translocates into the nucleus to form FAC with FD1, inducing MADS15 transcription and flowering[52]. Structurally, 14-3-3 serves as an adaptor mediating indirect interaction between Hd3a and FD1, forming a W-shaped dimer where two Hd3a monomers bind the C-terminus, while phosphorylated C-termini of two FD1 proteins attach to the positively charged pocket within the W-structure[53]. Conversely, OsCEN2 competes with Hd3a to form a florigen repression complex (OsCEN2-GF14f-OsFD2), with GF14f negatively regulating grain development and filling[54]. In potato, St14f forms FAC with StSP6A and StFDL1 to promote tuberization, where flexibility in helix of St14f is crucial for StFDL1 recognition[54]. Upregulation of 14-3-3 proteins is also observed during tuber formation in chayote[55]. During cassava root expansion, three 14-3-3 proteins are induced,

with MeGRF9/12/15 upregulated and MeGRF5/7 downregulated[56]. In tomato, TFT1 and TFT10 likely regulate sugar metabolism during fruit development by modulating sucrose phosphate synthase (SPS) activity[57], 14-3-3 gene expression increases during fruit development but declines during ripening[58].

Given that 14-3-3 genes exhibit distinct expression patterns across tissue and developmental stages in many plants[56], their differential expression in Chinese chestnut tissues and organs, particularly the higher expression levels of CmGRF1, 8, and 9, suggests these isoforms may play significant roles in regulating diverse biological processes in chestnut.

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