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ORIGINAL ARTICLE

Expansion and Diversification of the *14-3-3* Gene Family in *Camellia sinensis*

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Abstract

14-3-3 proteins are signal moderators in sensing various stresses and play essential functions in plant growth and development. Although, *14-3-3* gene families have been identified and characterized in many plant species, its evolution has not been studied systematically. In this study, the plant *14-3-3* family was comprehensively analyzed from green algae to angiosperm. Our result indicated that plant *14-3-3* originated during the early evolutionary history of green algae and expanded in terricolous plants. Twenty-six *14-3-3* genes were identified in the tea genome. RNA-seq analysis showed that tea *14-3-3* genes display different expression patterns in different organs. Moreover, the expression of most tea *14-3-3* genes displayed variable expression patterns under different abiotic and biotic stresses. In conclusion, our results elucidate the evolutionary origin of plant *14-3-3* genes, and beneficial for understanding their biological functions and improving tea agricultural traits in the future.

Keywords Evolutionary relationship · Phylogenetic tree · *14-3-3* gene · Abiotic stress · *Camellia sinensis*

Introduction

The sessile nature of plants requires the plants to continuously develop complex mechanism to adjust physiological processes response to changing environmental stress. 14-3-3 proteins are characterized as phosphothreonine /phosphoserine binding proteins, act as scaffolds to bind and regulate other proteins, thus to regulate complex environmental signaling pathways (Denison et al. 2011; Ferl et al. 2002). Therefore, 14-3-3 proteins play major functional roles in most processes, including signal transduction, primary metabolism, protein trafficking, and stress reactions.

Plant 14-3-3 proteins comprise multiple isoforms and they were named as GF14 or GRF for they are a part of protein/G-box complex (de Vetten and Ferl 1994; Rosenquist et al. 2001). With the completion of the genome sequence in a variety of plants, many members of 14-3-3 proteins have been annotated. 14-3-3 protein was originally identified in the model plant *Arabidopsis thaliana*. To date, many *14-3-3 s* have been identified in plant genomes, including Arabidopsis, rice, maize, soybean, grape, and so on (Chen et al. 2006; Cheng et al. 2018; Denison et al. 2011; Wang et al. 2019). Previous studies have demonstrated that plant 14-3-3 proteins have numerous roles in multiple physiological processes. For example, three barley *14-3-3* isoforms were expressed in embryo (Testerink et al. 1999). Most banana *14-3-3* genes displayed high expression during fruit development and postharvest ripening (Li et al. 2016). In Arabidopsis, reduction of *GRF10* and *GRF9* with antisense technology increased the leaf starch accumulation, indicating that 14-3-3 proteins regulate starch synthesis (Sehnke et al. 2001). Overexpression of cotton *14-3-3L* promoted fiber elongation and maturation, while downregulation of *14-3-3L* slowed fiber initiation and elongation (Zhou et al. 2015). Most Arabidopsis and rice *GRFs* displayed variable

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expression in response to heat, cold and salt stresses (Yashvardhini et al. 2018). Overexpressed *AtGRF6* in cotton improved tolerance to drought stress by regulate stomatal conductance (Yan et al. 2004). Due to their broad roles, the physiological functions of *14-3-3* genes are of great interest in plant science.

Tea is the world's most popular drink, rich in various helpful secondary metabolites (theanine, caffeine, flavonoids) and more than two billion cups are consumed every day (Brody 2019). As a type of evergreen woody plant, tea plant (*Camellia sinensis*) is primarily cultivated in tropical and subtropical regions. During growth, tea plant is often affected by various environment stresses (e.g., drought, heat, cold, salt, pest), which significantly constrain the yield and quality of tea products (Zhou et al. 2014). Therefore, analyzing the molecular mechanisms of tea plant response to environment stresses is of great significance, and many genes related to stress resistance have been discovered (Zhang et al. 2019). Tea plants underwent two whole-genome duplication (WGD) events by whole genome analysis (Wei et al. 2018; Xia et al. 2017). Many genes associated with secondary metabolisms (e.g., serine carboxypeptidase-like acyltransferase gene) and disease resistance (nucleotide-binding sites with leucine-rich repeats gene, pattern-recognition receptors gene) were significantly amplified in the tea plant genome (Wei et al. 2018; Xia et al. 2017). However, the *14-3-3* family in tea plant has not been characterized to date and the biological functions of this family remain unknown.

In this study, we performed a genome-wide identification and analysis of 14-3-3 proteins in tea and other plant species. Phylogenetic analyses were performed to delineate the evolutionary history of the 14-3-3 family in major angiosperm lineages. The expression patterns in tissues and in response to abiotic and biotic stresses were characterized by examining publicly available RNA-seq data and qRT-PCR. The results explore the evolutionary relationship of *14-3-3* gene family in plant species and provide a theoretical basis for future studies of the biological functions of *14-3-3* gene family members in tea.

Materials and Methods

Genome-Wide Identification of Plant 14-3-3 Proteins

The protein sequences of *Arabidopsis thaliana*, *Coffea canephora*, *Theobroma cacao*, *Oryza sativa*, *Glycine max*, *Zea mays*, *Amborella trichopoda*, *Selaginella moellendorffii*, *Physcomitrella patens*, *Chlamydomonas reinhardtii* were downloaded from Phytozome (http://www.phytozome.net/) and TPIA (http://tpia.teaplant.org/index.html), respectively. HMMER search (E-value = 1e − 10) and NCBI Basic Local Alignment Search Tool algorithms (BLASTP, E-value = 1e − 10) were employed to search 14-3-3 proteins. All obtained protein sequences were examined for the presence of the 14-3-3 domain (PF00244) using the Hidden Markov Model (HMM) of Pfam (http://pfam.sanger.ac.uk/search) (Finn et al. 2016) and SMART (http://smart.emblheidelberg.de) (Letunic et al. 2015) (Table S1).

Multiple Sequence Alignment and Phylogenetic Analysis

Because the phylogenetic tree based on amino acid sequences was poor statistical support, we performed the Bayesian tree based on protein-coding nucleotide sequences (Table S2). Nucleotide sequences were aligned by MEGA and the hypervariable 5'and 3' ends were trimmed. The Bayesian tree was constructed with MrBayes 3.2.1 software with the fixed Whelan and Goldman model, four Markov chains and an average SD of 0.01 (Ronquist and Huelsenbeck 2003).

Gene Structure, Motif Analysis, Chromosomal Location and Synteny Analysis

Gene structure analysis was conducted using Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015). The motifs of tea 14-3-3 proteins were analyzed using the online MEME program (http://meme-suite.org/tools/meme) (Bailey et al. 2009). The motif distribution type was zero or one occurrence per sequence, maximum number of motifs:7, and only motifs with E-value > 0.05 were present. The location data of tea *14-3-3* genes were obtained from the genome annotation files and the chromosomal location was drawn and visualized using CIRCOS (Krzywinski et al. 2009). The gene duplication landscape was obtained using the MCScanX with the default parameters (Wang et al. 2012) and the syntonic map was generated using CIRCOS with the putative duplicated genes were linked by the connection lines.

Expression Analysis of Tea 14-3-3 Genes

To determine the expression patterns of tea *14-3-3* genes, the publicly available RNA-seq data were used as a resource (transcriptome data were downloaded from the TPIA database (http://tpia.teaplant.org/) (Xia et al. 2019). These transcriptome data include 8 tissues including apical bud, flower, fruit, young leaf, mature leaf, old leaf, root and stem. The fragments per kilobase per million reads (FPKM) value representing the expression level of *CsGRF* genes. The heatmap with k-means clustering was generated using R software.

RNA Extraction and qRT-PCR Analysis

The tea plants (*Camellia sinensis cv. Xinyanghong 10*) were grown in a green-house maintained at 23 ± 3 °C with 65 ± 5% room humidity and a 16/8 h (day/night) at Xinyang Normal University, Henan, China. The potted plants were watered and fertilized by the same standards. 2-year-old tea plants of uniform growth without signs of insects or disease were chosen for experiments.

For cold and salt stresses, plants were exposed to 4°C for 3 days and 100 mmol/L NaCl for 2 days. For drought stress, plants were exposed to drought by withholding water for 7 days, followed by a 24-h recovery period. For insect feeding treatment, fifteen larvae from 3rd or 4th instar *E. oblique* were placed on each of 3 totally unfed tea plants for obtaining geometrid-damaged tea plant. A group of plants that did not experience any stress were used as control (CK).

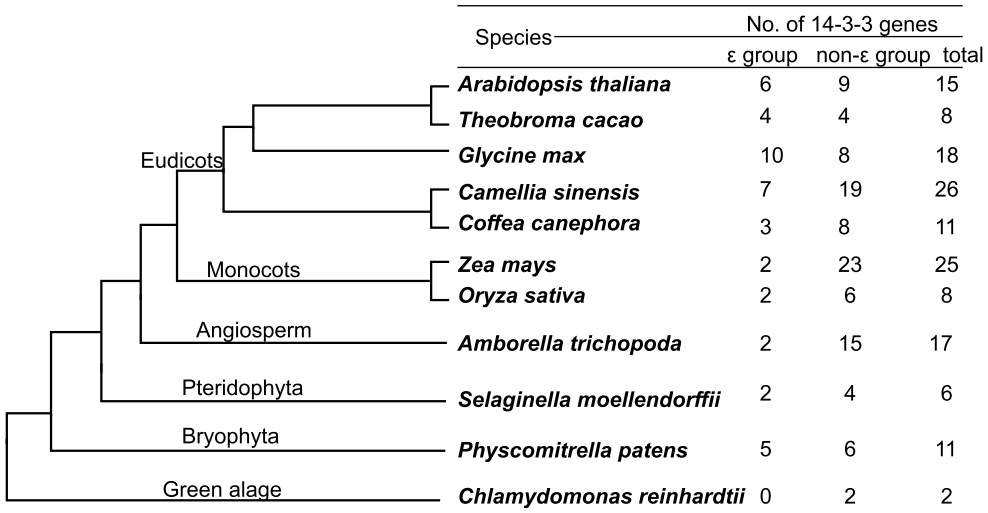
The Total RNA was isolated from the leaf for each treatment and RNA quality and quantity were determined using 1% agarose gel electrophoresis. One microgram of RNA was reverse transcribed to cDNA using the PrimeScript RT reagent Kit. Quantitative RT-PCR (qRT-PCR) was performed using SYBR Premix EX Taq on an ABI StepOnePlus machine. Relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. *CsPTB* was used as an internal control gene. The expression ratio was represented relative to the control value observed for the gene and fold change was calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt = (Ctgene—Ctptb) treatment − (Ctgene − Ctptb) control. The experiments were repeated in triplicate. Specific primers for *CsGRF* genes were designed using Primer 5 software (Table S3).

Results

Identification of GRF Genes in major Plant Lineages

The complete set of *GRF* genes were identified from tea and other major plant species based on Hidden Markov model (HMM) algorithm and BLASTP. 147 sequences, each containing a 14-3-3 domain, were identified from 11 different plant species including green algae *Chlamydomonas reinhardtii*, moss *Physcomitrella patens*, pteridophyta *Selaginella moellendorffii*, basal angiosperm *Amborella trichopoda*, monocots (*Oryza sativa*, *Zea mays*), and dicots (*Arabidopsis thaliana*, *Theobroma cacao*, *Glycine max*, *Camellia sinensis*, *Coffea canephora*) (Fig. 1). The tea and other 10 plants encode different numbers of GRF isoforms, ranging from two in the green algae *C.reinhardtii* to 11 in *P.patens*, 8 in *O.sativa*, 15 in *A.thaliana*, with the highest copy number being 26 in *C. sinensis*. These results indicate that GRFs were originated during the early evolutionary history of algae. Land plants contained higher copy numbers than algae, indicating that expansions of *GRF* genes occurred after land plants diverged from green algae (Fig. 1).

26 GRF proteins were identified in tea, with *CsGRF21* displayed the shortest coding sequence (363 bp), amino acid length (120 aa), and the smallest molecular weight (13.57 kDa), while *CsGRF14* showed the longest coding sequence (867 bp), amino acid length (288 aa), and the biggest molecular weight (32.85 kDa) (Table 1). Their isoelectric point (pI) values ranged from 4.67 (CsGRF16 and CsGRF18) to 8.78 (CsGRF21) (Table 1).



Species	No. of 14-3-3 genes		
	ε group	non-ε group	total
Arabidopsis thaliana	6	9	15
Theobroma cacao	4	4	8
Glycine max	10	8	18
Camellia sinensis	7	19	26
Coffea canephora	3	8	11
Zea mays	2	23	25
Oryza sativa	2	6	8
Amborella trichopoda	2	15	17
Selaginella moellendorffii	2	4	6
Physcomitrella patens	5	6	11
Chlamydomonas reinhardtii	0	2	2

Fig. 1 Summary of the *GRF* gene among 11 species

Table 1 List of all *CsGRF* genes identified in the tea genome

Gene name	Gene locus	Length (aa)	pI	Molecular weight (Da)	Group
CsGRF1	CSS0013665	251	5.33	29,468.48	Non-ε
CsGRF2	CSS0025248	256	4.89	28,912.03	Non-ε
CsGRF3	CSS0039114	252	5.91	28,357.38	Non-ε
CsGRF4	CSS0042487	251	5.30	28,398.45	Non-ε
CsGRF5	CSS0032859	263	4.76	29,681.27	Non-ε
CsGRF6	CSS0014184	263	4.76	29,681.27	Non-ε
CsGRF7	CSS0042088	261	4.76	29,435.09	Non-ε
CsGRF8	CSS0004258	253	4.77	28,551.35	Non-ε
CsGRF9	CSS0009214	260	4.73	29,282.74	Non-ε
CsGRF10	CSS0023510	259	4.92	29,646.27	ε
CsGRF11	CSS0024947	259	4.76	29,450.08	ε
CsGRF12	CSS0008011	261	4.95	29,737.39	ε
CsGRF13	CSS0017401	245	5.02	27,838.32	ε
CsGRF14	CSS0001272	288	5.08	32,852.02	ε
CsGRF15	CSS0002272	261	4.70	29,412.99	Non-ε
CsGRF16	CSS0000996	253	4.67	28,613.45	Non-ε
CsGRF17	CSS0013188	257	4.86	29,509.27	ε
CsGRF18	CSS0014576	253	4.67	28,594.41	Non-ε
CsGRF19	CSS0042610	233	4.77	26,460.00	Non-ε
CsGRF20	CSS0034316	253	4.71	28,593.42	Non-ε
CsGRF21	CSS0034623	120	8.78	13,569.34	Non-ε
CsGRF22	CSS0027025	278	4.8	31,742.74	Non-ε
CsGRF23	CSS0013218	127	7.78	14,549.64	Non-ε
CsGRF24	CSS0036348	260	4.71	29,543.96	Non-ε
CsGRF25	CSS0018642	253	4.97	28,896.43	ε
CsGRF26	CSS0001833	127	8.50	14,486.54	Non-ε

Phylogenetic Classification of GRF Genes into Two Groups

The GRF protein sequence can be divided into three sections: the conserved core region, and a more divergent amino and carboxyl terminus, respectively. The core region contains nine antiparallel a-helices which are function in protein dimerization. The amino acid sequences of tea GRF were highly conserved in the core region with nine a-helices were identified, while its N-terminal and C-terminal regions were more divergent (Fig. 2). This characterization was same as the GRF proteins in other plants, such as Arabidopsis, rice and soybean (Chen et al. 2006; Wang et al. 2019; Wu et al. 1997).

Although many plant GRF proteins have been identified, the evolutionary origin was still not clear. In previous studies, many neighbour-joining (NJ) phylogenetic trees were constructed based on GRF amino acid sequences, however, these NJ trees were not good in statistical support. To explore the evolutionary origin of plant *GRF* gene family members, we used the core protein-coding nucleotide sequences and phylogenetic tree was conducted with Bayesian method (Fig. 3). Based on the phylogenetic analyses, plant *GRF* genes can be divided into two major groups (ε group and non-ε group) with non-ε group contains more isoforms (Fig. 3), consistent with the former classification in other plants, including Arabidopsis (Ferl et al. 2002), rice (Yashvardhini et al. 2018), grape (Cheng et al. 2018), and soybean (Wang et al. 2019). Both groups contain genes from moss, indicating an early origin of *GRF* genes before the divergence of land plants. The green alga *C.reinhardtii* has two *GRF* genes and they were included in the non-ε group for them containing four and one introns, respectively.

In *A.trichopoda, O.sativa, Z.mays, C.arabica* and *C.sinensis,* the majority of GRF isoforms were included in the non-ε group, while in lower plants and Eudicots, the number of GRF isoforms in ε group was equal to that in non-ε group (Figs. 1, 3). These results indicate different patterns of expansion of GRF proteins in monocots and eudicots, respectively. 26 GRF isoforms were identified in tea (Fig. 3). While only 8 and 11 GRF isoforms were identified in cacao and coffee, respectively (Fig. 4). The tea genome experiences two rounds of whole-genome duplications (WGD) that occurred ~ 30 to 40 and ~ 90 to 100 Mya since the γ-event ~140 Mya (Wei et al. 2018; Xia et al. 2017). However, the coffee and cacao genomes display no sign of WGD in its lineages since the γ triplication at the origin of the core eudicots (Argout et al. 2011; Denoeud et al. 2014) (Fig. 4). The different expansion patterns of *GRF* isoforms among these three economical plants suggesting functional variations *GRF* among tea, coffee and cacao.

Structure Analysis of CsGRF Genes and CsGRF Proteins

To butter understand the gene structural evolution, the exon–intron organization of *CsGRF* genes were analyzed with the online service GSDS based on their genome and coding sequences (Fig. 5). The ε group has six to seven

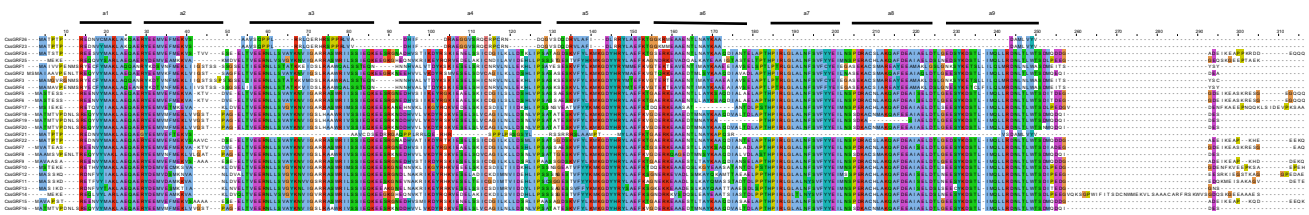


Fig. 2 Sequence alignment of tea GRF proteins. Nine α-helices were marked as α1-α9

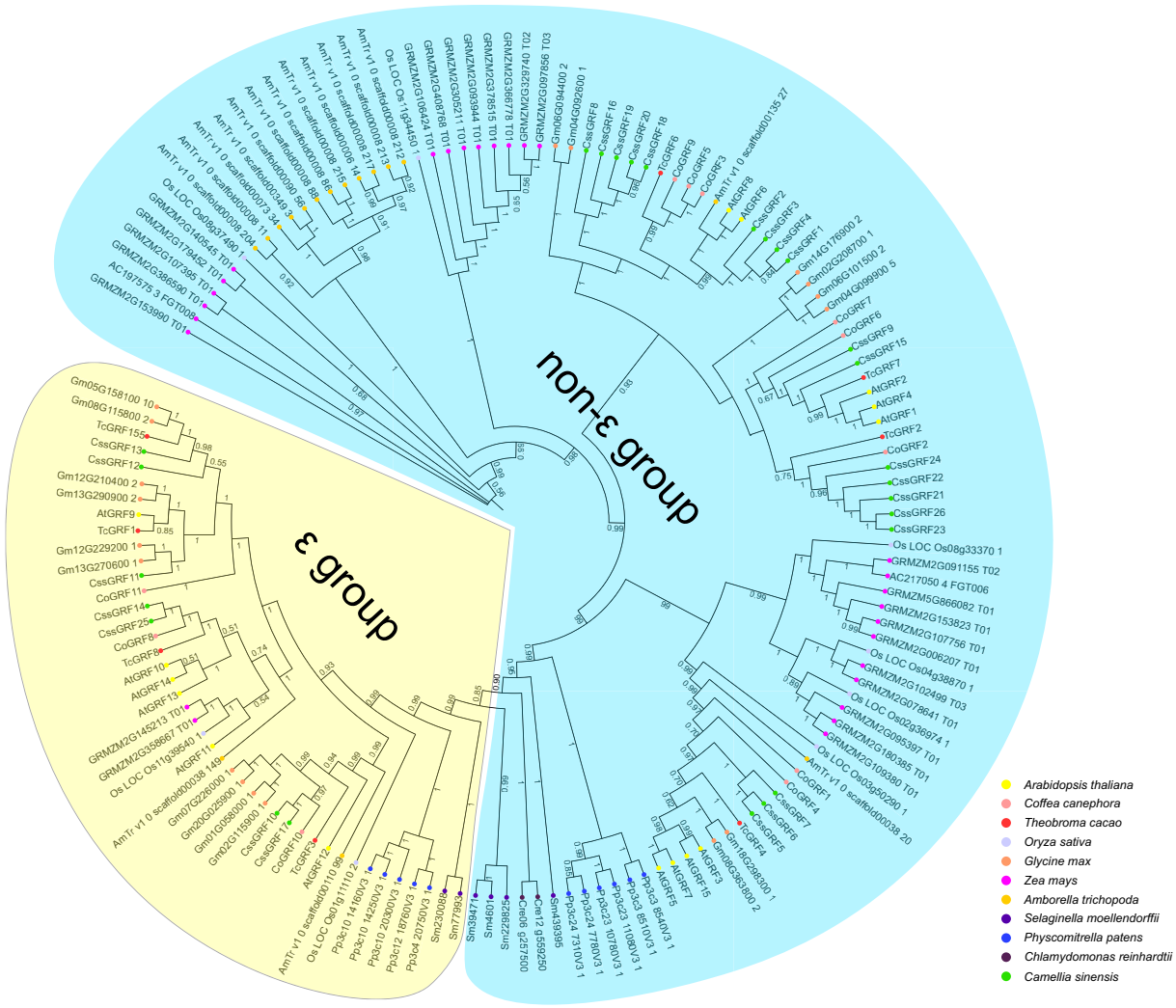


Fig. 3 Phylogenetic tree of *GRF* in plant species based on Bayesian analysis of coding nucleotide sequences. Section colours indicate epsilon and non-epsilon types of isoforms. Cs: *Camellia sinensis*, Ca: *Coffea arabica*, Thecc: *Theobroma cacao*, At: *Arabidopsis thaliana*, Loc Os: *Oryza sativa*, Gm: *Glycine max*, Pp: *Physcomitrella patens*, GRMZM: *Zea mays*, AmTr: *Amborella trichopoda*, Sm: *Selaginella moellendorffii*, Cre: *Chlamydomonas reinhardtii* (Color figure online)

introns, while the majority of the non-ε group has tree introns, except for *CsGRF1*, *CsGRF3*, *CsGRF4*, *CsGRF18*, *CsGRF20* genes have no introns (Fig. 5). Exon–intron gene structure correlates with division into epsilon and non-epsilon groups. The exon/intron pattern in terms of the number of introns and exon length were obviously different in the two groups of *GsGRF* genes, suggesting the diversity of *CsGRF* genes during the evolution. To investigate the protein sequence features of the CsGRFs, 7 motifs were predicted by the MEME tool (Fig. 5). Majority of CsGRF (18/26, 69%) contained all seven motifs, while the others members contained variable numbers of motifs with CsGRF21, CsGRF23, CsGRF26 only had motifs 4 and 5. The differences in the type and number of motifs in CsGRFs indicate the structural basis for the diversity in protein function.

Chromosome Distribution and Synteny Analysis of CsGRFs

Twenty-three *CsGRF* genes were distributed unevenly on nine chromosomes, and we named the *CsGRF* genes according to their positions on chromosomes (Fig. 6). The chr1 contained six *CsGRF* genes, and chr2, 6, 8 only contained one *CsGRF* gene. Segmental duplication played an essential role in *CsGRF* expansion in Arabidopsis, rice, Vitis, Soybean and Populus (Cheng et al. 2018; Tian et al. 2015; Wang et al. 2019; Wu et al. 1997; Yashvardhini et al. 2018). To better understand the evolution of *CsGRF* genes, we conducted a synteny analysis (Fig. 7A). 14 *CsGRF* isoforms exhibiting segmental duplication events between different chromosomes and contigs (*CsGRF5*/*CsGRF7*,

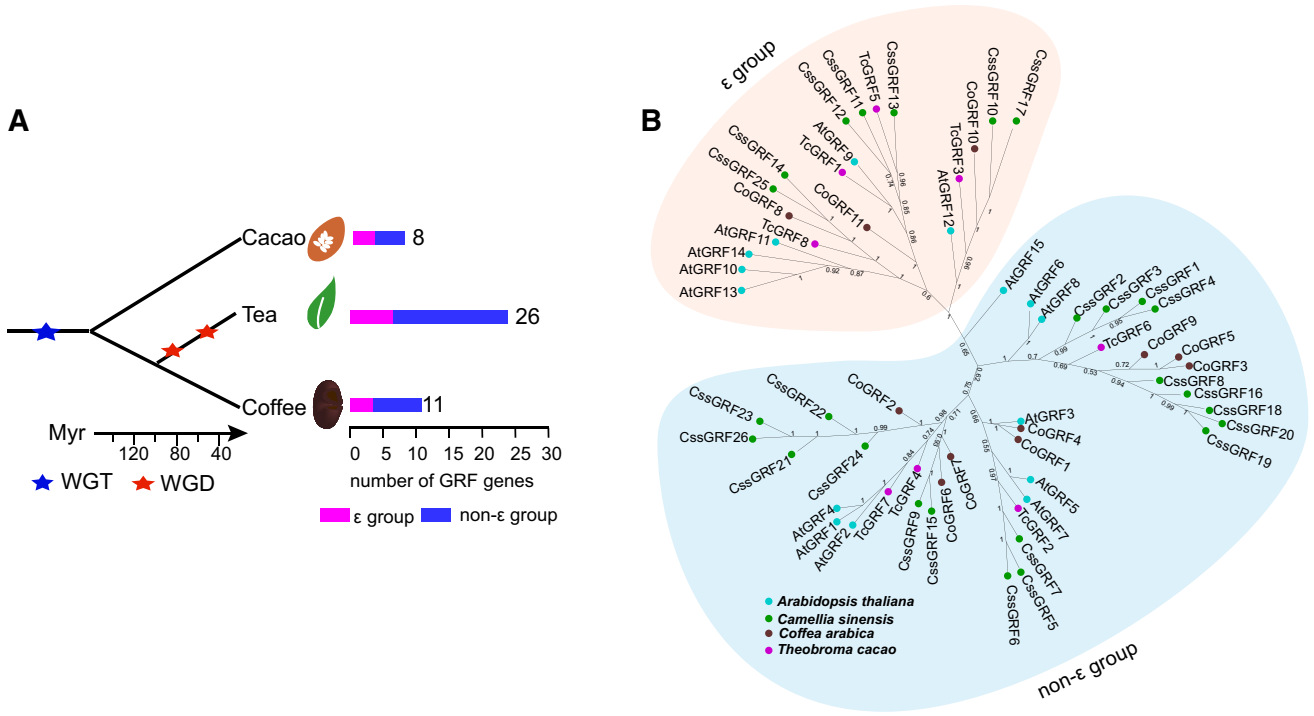


Fig. 4 Evolution of *GRF* genes in tea, coffee and cacao. **A** Distribution of genes encoding GRFs among tea tree, coffee and cacao genomes. **B** Phylogenetic tree of *GRF* genes from tea, coffee, cacao and Arabidopsis based on Bayesian analysis

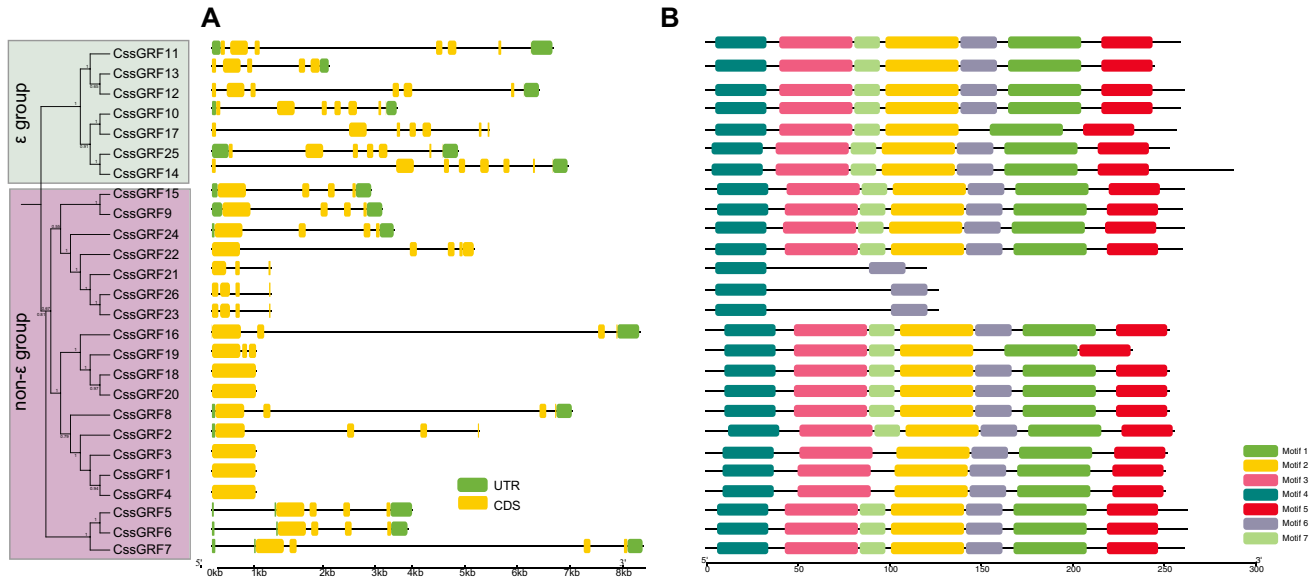


Fig. 5 Conserved motifs and gene structure in CsGRF14s. **A** Exon/intron structures of *CsGRF14* genes. **B** Conserved motifs of the CsGRF14s. Each motif is represented by a number in colored box

*CsGRF*6/*CsGRF7*, *CsGRF*8/*CsGRF16*, *CsGRF*9/*CsGRF15*, *CsGRF*10/*CsGRF17*, *CsGRF*12/*CsGRF13*, *CsGRF*22/*CsGRF15*, *CsGRF*23/*CsGRF26*) (Fig. 7A). 8 isoforms were located on one block and lacked duplicates on their corresponding blocks (*CsGRF*1, *CsGRF*11, *CsGRF*14, *CsGRF*18, *CsGRF*19, *CsGRF*20, *CsGRF*21, *CsGRF*24). These results suggested that *CsGRF* genes were also evolved from segment duplication. Similar results were also identified in *GRF* genes in *T.cacao* and *C.canephora*.

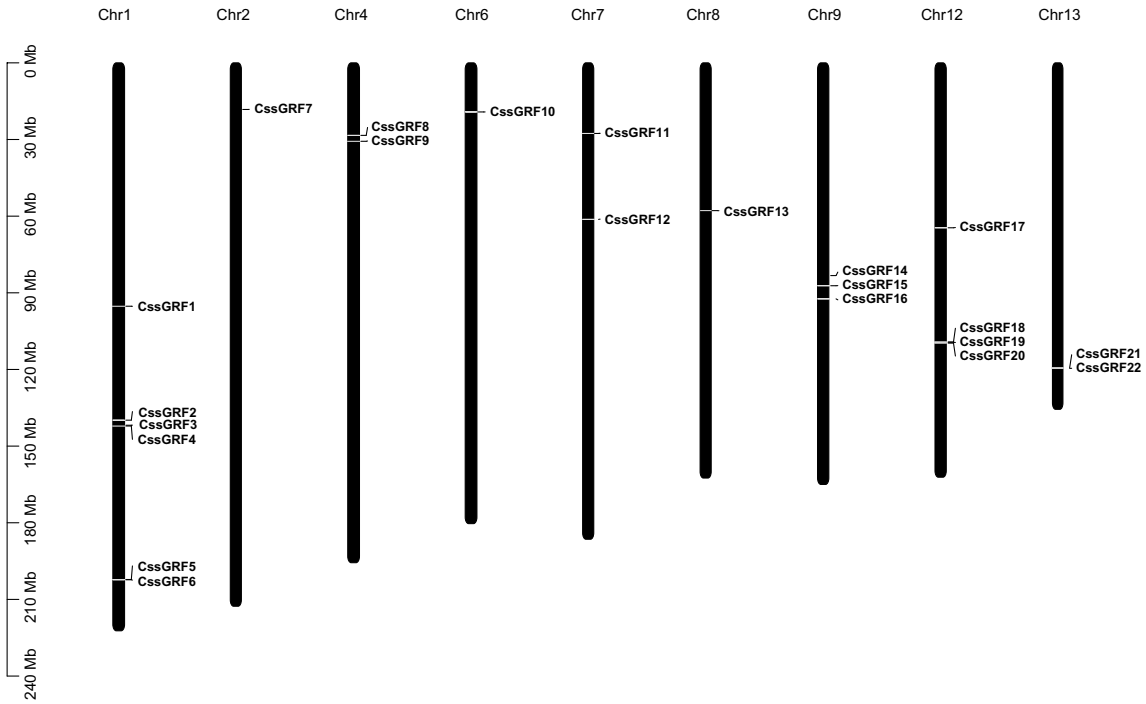


Fig. 6 Genomic localization of *CsGRF14* genes. Twenty-two *GRF14* genes were located on 9 out of 15 chromosomes

To compare the origin and evolutionary relatedness among tea, Arabidopsis, *T.cacao* and *C.canephora*, the shared synteny was analyzed (Fig. 7B). In tea, 10 *GRF* genes (38%) were found to have syntenic pairing with 11 Arabidopsis *GRF* genes (73%). In *C.canephora*, 9 *GRF* genes (81%) were found to have syntenic pairing with 6 T.*cacao GRF* genes (75%). These results furthered indicated that segmental duplication contributed predominantly to expansion of *GRF* genes.

Expression Pattern of CsGRF Genes in Different Tissues and Under Abiotic and Biotic Stresses

The expression patterns of *CsGRF* genes in different tea tissues (e.g., apical bud, flower, fruit, young leaf, mature leaf, old leaf, root and stem) were performed based on the previous RNA-seq data generated by Wei and co-workers (Wei et al. 2018) (Fig. 8). Only two *GRF* genes (*CsGRF18* and *CsGRF19*) did not expressed in that dataset, and the other 24 *CsGRF* genes were expressed in more than one but not all tissues. In addition, most *CsGRF* genes showed a distinct tissue-specific expression pattern, suggesting the diversity of their roles (Fig. 8). For instance, six genes (*CsGRF4/7/9/11/16/24*) displayed high expression in the flower. Two genes (*CsGRF5/22*) were specifically expressed in old leaf. *CsGRF1* and *CsGRF12* had a significantly transcript accumulation in fruit and root, respectively. These tissue-specific expression patterns were consistent with the previous research in *AtGRFs*, *OsGRFs*, *GmGRFs*, *VvGRFs* and *PtGRFs* (Cheng et al. 2018; Tian et al. 2015; Wang et al. 2019; Yashvardhini et al. 2018), indicating that *CsGRF* genes had extensive functional divergence.

Different abiotic and biotic stresses such as cold, drought, salinity, and pest adversely affect plant growth and development. Considerable evidence has shown that GRF proteins can regulate the expression of many other stress-related genes and play vital roles in response to both abiotic and biotic stresses (Denison et al. 2011; Yang et al. 2019). To explore the roles of *CsGRF* genes in tea plant under diverse environmental conditions, the expression patterns of *CsGRF* genes in different stresses (cold, PEG, NaCl, *Ectropis oblique* damage) were examined using qRT-PCR (Fig. 9). Ten genes showed no or very low expression in leaves were not further analyzed. The expression levels of most *CsGRF* genes were changed, and all of them showed significantly different expression patterns in response to different stresses (Fig. 9). The expression of *CsGRF14* and *CsGRF17* genes were specially upregulated under cold stress, while the expression of *CsGRF10* and *CsGRF16* were specially downregulated under cold and *E.oblique* stresses, respectively (Fig. 9). Expression levels of *CsGRF21* were increased under drought, salt and *E.oblique* stresses, while expression levels of *CsGRF5* were decreased under these stresses. *CsGRF9* was significantly upregulated under abiotic stresses (cold, salt, drought), while *CsGRF9* was upregulated under cold stress and downregulated under drought and salt stresses. These results indicate the different

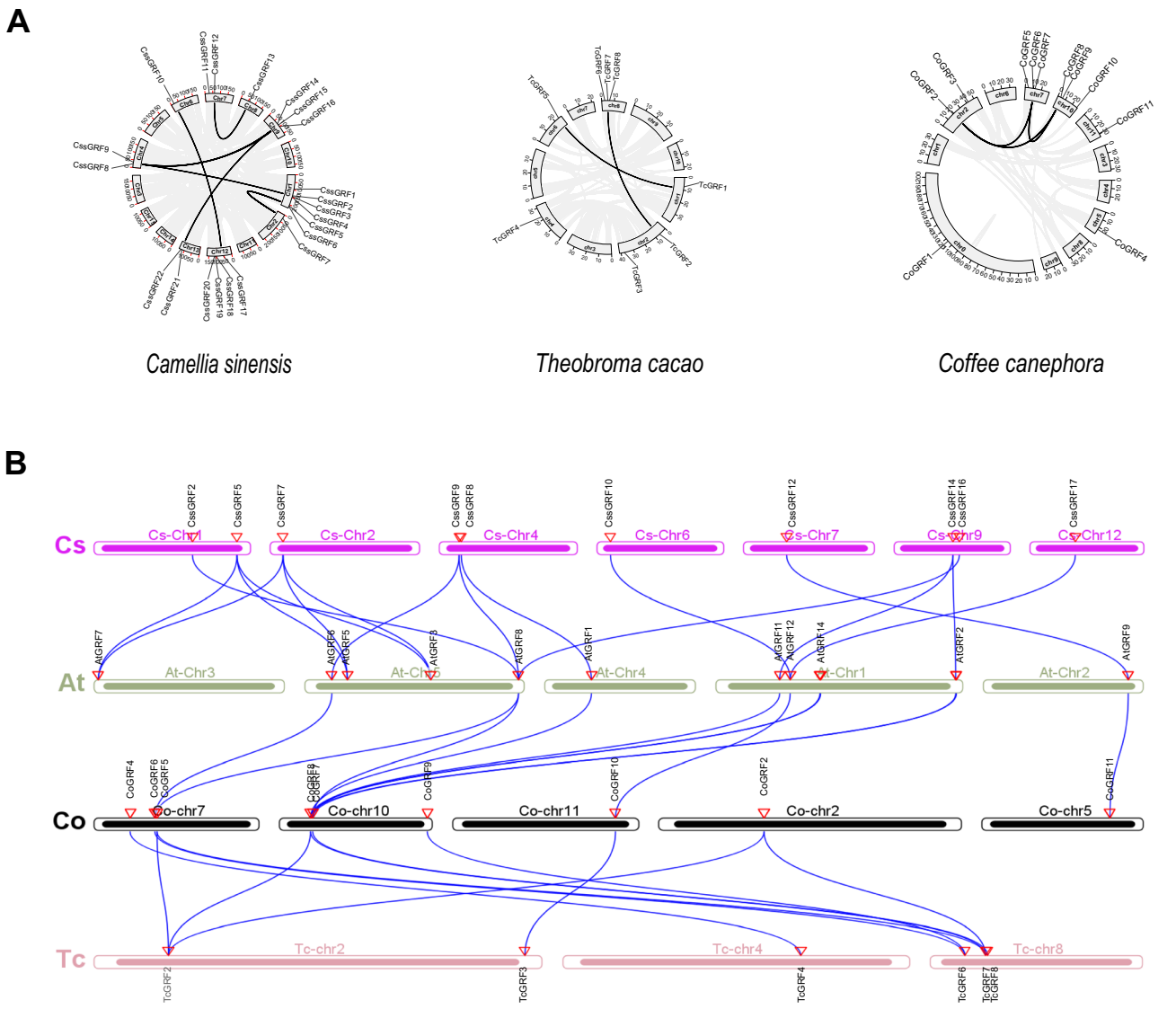


Fig. 7 Synteny analysis of *GRF* genes in *Camellia sinensis*, *Theobroma cacao*, and *Coffee canephora*. **A** Synteny analysis of the *GRF* genes in *C.sinensis*, *T.cacao*, and *C.canephora*, respectively. **B** Synteny relationship of *GRF* genes among Arabidopsis, *C.sinensis*, *T.cacao*, and *C.canephora*. The syntenic pairs were prepared according to the homology between them. The positions of all the *GRF* genes are depicted in the chromosomes. Vertical lines highlight orthologs

roles of *CsGRFs* in different stress conditions and reveal that the response mechanism is complex and diverse.

Discussion

GRFs Genes Have Been Conserved During Speciation

Plant GRFs play important roles in the developmental regulation and in response to environmental stresses. Although, many plant GRFs have been identified, the evolutionary origin and phylogenetic relationship of GRFs are not yet fully explored. In this study, a comprehensive phylogenetic analysis of the plant GRFs from algae, Bryophyta, Pteridophyta and angiosperms was performed (Fig. 3). All the *GRFs* genes were divided into two groups (ε group and non-ε group), which is in accordance with previous studies in Arabidopsis, rice, grape and soybean (Cheng et al. 2018; Wang et al. 2019; Yashvardhini et al. 2018). In the ancient terricolous plants, there are 11 and 6 GRF isoforms in *Physomitrella patens* and *Selaginella*

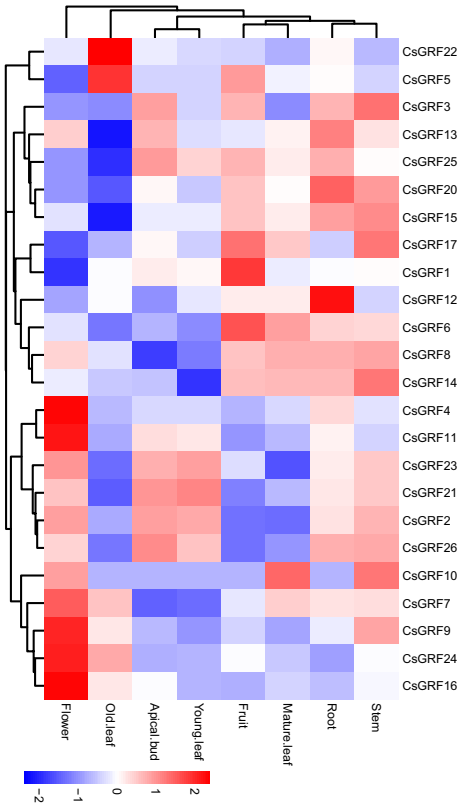


Fig. 8 Expression patterns of *GRF* genes in different tissues of tea plant

moellendorffii, respectively (Figs. 1, 3). In neonatal tericolous plants, there are 17, 8, 25, 11, 26, 18, 8 and 15 GRF isoforms in *Amborella trichopoda*, *Oryza sativa*, *Zea mays*, *Coffea canephora*, *Camellia sinensis*, *Glycine max*, *Theobroma cacao* and *Arabidopsis thaliana*, respectively. These results indicate that the number of GRF was expanded in land plants. Based on the phylogenetic analysis, plant GRFs were originated during the early evolutionary history of algae (Fig. 3).

26 GRF isoforms were identified from tea genome (Figs. 1, 3), and this number was substantially more than those identified in other plants, including coffee and cacao (Figs. 1, 3, 4). In previous studies, the tea genome was shown to experience two WGD events, one occurred ~ 30 to 40 million years ago (Mya) and the second occurred ~ 90 to 100 Mya (Wei et al. 2018; Xia et al. 2017). The high GRFs numbers in tea genome, indicating that the WGD duplication might be the main mechanism of *GRF* gene family expansion during the evolution of tea. Previous studies have revealed that segmental duplication events contributed to *GRF* gene duplication in rice, grape and soybean (Cheng et al. 2018; Wang et al. 2019; Yashvardhini et al. 2018). The synteny analysis also showed that segment duplication was the major gene duplication for GRF expansion in tea. Regulatory genes and signaling genes were showed to be retained after duplication comparted to the genome-wide average, and many genes associated with secondary metabolisms and disease resistance were significantly amplified in the tea genome (Wei et al. 2018; Xia et al. 2017). GRFs play important roles in regulating complex environmental signaling pathways and networks, and allowing crosstalk between different pathways. Therefore, the GRFs have been conserved throughout evolution.

Functional Divergence in CsGRF Genes

Many researches have suggested that plant *GRF* gens are signal moderators, its transcription were regulated by various environmental signals, consistent with their diverse roles in growth, development and stress responses (Denison et al. 2011). Most tea *GRF* genes were differentially expressed in the different tissues of tea plant (Fig. 5), indicating that *CsGRFs* play different roles in different organs or tissues. This phenomenon is also reported in other species, including Arabidopsis, rice, grape, maize and soybean (Cheng et al. 2018; Denison et al. 2011; Wang et al. 2019; Yashvardhini et al. 2018).

The transcription of the *GRFs* genes was either induced or inhibited by cold, drought, salt and *E.oblique* (Fig. 9), indicating that *CsGRFs* play important roles in plant response to various abiotic and biotic stresses. In addition, we found that many close *CsGRF* genes exhibit the opposite expression trend under different stress. For example, *CsGRF9* was upregulated under cold, drought and salt stresses, while *CsGRF10* was only down-regulated under cold stress. *CsGRF24* was upregulated under cold, drought and *E.oblique* stresses, while *CsGRF16* was only down-regulated under *E.oblique* stress. *CsGRF2* and *CsGRF21* were upregulated under salt stress, while its expressions display an opposite trend under *E.oblique* stress with *CsGRF2* was down-regulated while *CsGRF21* was up-regulated. Therefore, *GRFs* have different expression patterns in different tissues, various abiotic and biotic stresses, suggesting that the *CsGRF* genes are involved in the tea plant growth, development and responses to stresses, and different isoforms may play specific roles in specific processes. The complex and diverse expression patterns of *CsGRF* genes suggested that they potentially related to environmental adaptation and phytochemical properties within the tea lineage.

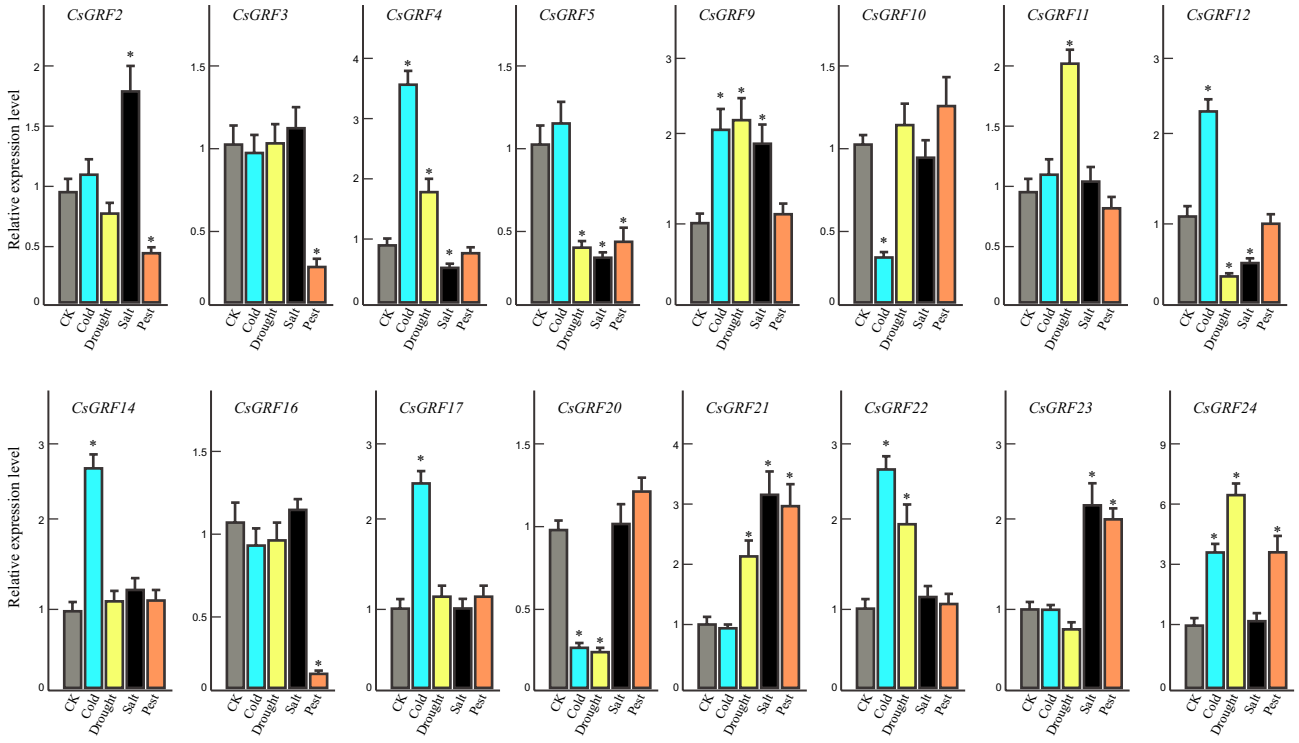


Fig. 9 Expression analysis of *CsGRF* genes in response to cold, drought, salt and *E. oblique* treatments by qRT-PCR. Expression data were normalized to *CsPTB* gene expression level and every *CsGRF* genes under control was normalized as '1'. The mean expression value was calculated from three replications. The standard deviations of three biological replicates are represented by the error bars. Asterisks on top of the bars indicating statistically significant differences ($*p < 0.05$)

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Author Contributions Z-BZ and Z-GF designed the research. Z-BZ, X-KW and SW wrote the manuscript. X-KW, SW, QG performed the identification of *GRF* genes, protein structure, evolution analysis, and expression analysis. WZ participated in manuscript preparation and revision.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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