

Genome-wide identification of GROWTH-REGULATING FACTORs in *Liriodendron chinense* and functional characterization of *LcGRF2* in leaf size regulation

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Abstract: GROWTH-REGULATING FACTORs (GRFs) play a pivotal role in the regulation of leaf size in plants and have been widely reported in plants. However, their specific functions in leaf size regulation in *Liriodendron chinense* remains unclear. Therefore, in this study, we identified *GRF* genes on a genome-wide scale in *L. chinense* to characterize the roles of *LcGRFs* in regulating leaf size. A total of nine *LcGRF* genes were identified, and these genes exhibited weak expression in mature leaves but strong expression in shoot apex. Notably, *LcGRF2* exhibited the highest expression level in the shoot apex of *L. chinense*. Further RT-qPCR assay revealed that the expression level of *LcGRF2* gradually decreased along with the leaf development process, and also displayed a gradient along the leaf proximo-distal and medio-lateral axes. Furthermore, overexpression of *LcGRF2* in *Arabidopsis thaliana* resulted in increased leaf size, and significantly up-regulated the expression of genes involved in cell division like *AtCYCD3;1*, *AtKNOLLE*, and *AtCYCB1;1*, indicating that *LcGRF2* may influence leaf size by promoting cell proliferation. This work contributes to a better understanding of the roles and molecular mechanisms of *LcGRFs* in the regulation of leaf size in *L. chinense*.

Keywords: *Liriodendron chinense*, GRF transcription factors family, genetic transformation, cell proliferation, leaf size

1. Introduction

Leaves, as important lateral organs of plants, need to experience three intertwined processes, including primordia initiation, polarity establishment, and expansion, to develop into mature leaves with different size and shapes (Byrne, 2005). Leaves originate from founder cells, which are situated in the flank of the shoot apical meristem (SAM). Subsequently, the leaf founder cells undergo specification processes to establish leaf polarity along the adaxial-abaxial, proximo-distal, and medio-lateral axes (Eshed et al., 2001; Hudson, 2000).

Leaf polarity establishment is a crucial process during leaf morphogenesis. Several transcription factor (TF) families and small RNAs participate in determining cell fate during leaf polarity establishment. In the establishment of the adaxial-abaxial axis, the class III *HOMEODOMAIN LEUCINE ZIPPER* (*HD-Zip*), *ASYMMETRIC LEAF1* (*AS1*), and *AS2* were considered as the adaxial determinants (Byrne, 2006; Lin et al., 2003; Yang et al., 2018; Zgurski et al., 2005). In contrast, *AUXIN RESPONSE FACTOR 3* (*ARF3*), *ARF4*, *KANADI* (*KAN*), and *YABBY* (*YAB*) determined the fate of abaxial cells (Eshed et al., 2004; Pekker et al., 2005; Yang et al., 2018). Moreover, two types of small RNAs, microRNA165/166 (miR165/166) and trans-acting small interfering RNA (ta-siRNA), participated in controlling the adaxial-abaxial polarity by targeting *HD-Zip III* genes and *ARF3/ARF4*, respectively (Fahlgren et al., 2006; Zhong and Ye, 2007; Zhou et al., 2007). Furthermore, *AS1/AS2* could promote the expression of *HD-Zip III*, while repress the expression of miR165/166, *KAN* and *YAB* (Fu et al., 2007; Li et al., 2005; Wu et al., 2008). However, *KAN* genes acted in an antagonistic manner to *HD-Zip III* genes, and they could also bind to the promoter of *AS2* to repress its expression (Emery et al., 2003; Wu et al., 2008).

Leaf polarity along the proximo-distal and medio-lateral axes affected blade size (Yang et al., 2018). The dynamic changes in cell proliferation and expansion along the proximo-distal and medio-lateral axes lead to the divergence of leaf size (Gonzalez et al., 2012; Horiguchi et al., 2006; Vercruysse et al., 2020). Notably, the GROWTH-REGULATING FACTOR (GRF) TFs constituted a particularly important part in regulating leaf growth along the proximo-distal and medio-lateral axes (Horiguchi et al., 2005; Kim et al., 2003; Liu et al., 2009; Wang et al., 2011).

GRFs usually contain two conserved domains in the N-terminal region, QLQ (Gln, Leu, Gln) (protein-protein interaction domain) and WRC (Trp, Arg, Cys) (DNA binding domain) (Horiguchi et al., 2005; Kim et al., 2012). Additionally, three less-conserved motifs, TQL (Thr, Gln, Leu), FFD (Phe, Phe, Asp), and GGPL (Gly, Gly, Pro, Leu) were identified in the C-terminal region of GRF proteins (van der Knaap et al., 2000; Zhang et al., 2008). However, the specific functions of these less-conserved motifs still remains unclear.

Since the first member of *GRF* gene was reported in rice (*Oryza sativa*, OsGRF1), this gene family has been widely reported in various land plants, including *Arabidopsis thaliana*, maize (*Zea mays*), moso bamboo (*Phyllostachys edulis*), *Gossypium raimondii*, poplar (*Populus trichocarpa*), and lettuce (*Lactuca sativa*) (Cao et al., 2020; Kim et al., 2003; Shi et al., 2019; van der Knaap et al., 2000; Wang et al., 2020; Zhang et al., 2021; Zhang et al., 2008). *GRFs* play crucial roles in the regulation of cell proliferation and expansion, which are essential for organs development, especially for leaf size regulation. In *A. thaliana*, overexpression of *AtGRF1* and *AtGRF2* increased the size of leaves, while the triple mutants *grf1grf2grf3* displayed smaller leaves (Kim et al., 2003). Compared with the *grf1grf2grf3* triple mutants, the *grf1grf2grf3grf4* quadruple mutants showed much smaller leaves (Kim and Lee, 2006). The changes in leaf size were caused by the alteration of cell size. In poplar, *PagGRF12a* and *PagGRF12b* positively regulated leaf size by promoting both cell proliferation and expansion, while *PagGRF7a* played a negative role in leaf size regulation by repressing cell expansion. (Wang et al., 2020); *GRF5* promoted leaf growth by negatively regulating the expression of *CYTOKININ OXIDASE/DEHYDROGENASE 1 (CKX1)* (Wu et al., 2021). In maize, overexpression of *ZmGRF10* resulted in decreased cell proliferation, which led to a reduction of leaf size (Wu et al., 2014).

Liriodendron chinense, a deciduous tree species of Magnoliaceae, is widely cultivated in southern China for both industrial and landscaping purposes (Tang et al., 2013). The distinct goosefoot-like leaf is an iconic characteristic of *L. chinense*, thus, previous studies mainly focused on investigating the regulatory mechanisms of leaf shape in this species, particularly on genes involved in leaf shape regulation, including Phytohormone, *KNOTTED-LIKE HOMEODOMAIN (KNOX)*, and *CUP-SHAPED COTYLEDON (CUC)* genes (Ma et al., 2020; Ma et al., 2018; Wen et al., 2022). Nevertheless, little attention has been paid to the regulation of leaf size in *L. chinense*, especially the roles of *LcGRFs*. Therefore, this work aims to identify *LcGRF* gene family members in *L. chinense* and analyze their gene structure, conserved motifs, phylogenetic tree, and synteny. Moreover, we selected *LcGRF2* as a target gene that has great potential in leaf development regulation based on the transcriptome data. Through RT-qPCR analysis, self-activating transcriptional activity, subcellular localization, and functional verification, we revealed the roles of *LcGRF2* and its potential mechanism in regulating leaf size. This study extends our understanding of the molecular mechanism of *LcGRFs* in regulating leaf size in *L. chinense*.

2. Materials and methods

2.1. Plant materials and growth conditions

The sample trees were obtained from a plantation of *L. chinense* in Jurong County, Zhenjiang City, Jiangsu Province (32° 7' N, 119° 13' E). Petal, pistil, stamen, leaf bud, and S1 leaf (corresponded to newly expanded leaves), S2 leaf (corresponded to larger leaves than those in S1), S3 leaf (corresponded to mature leaves) were collected, and each sample had three biological replicates (Fig. S1). Furthermore, we retrieved different parts of leaves (distal leaf tooth, proximal leaf tooth, distal leaf serration, proximal leaf serration, and leaf center) at different stages (Fig. S1). All samples were quickly frozen using liquid nitrogen and then stored in a -80 °C refrigerator.

A. thaliana was used for transgenic assays. Wide-type (WT) *A. thaliana* (Columbia-0) and transgenic *A. thaliana* were cultivated in a growth chamber under a long-day photoperiod (16 h/8 h, day/night) and 70% relative humidity. Leaves and cotyledons from 35-day-old WT and transgenic plants were collected for the analysis of *GRF2*

expression levels.

2.2 Identification of *GRF* family members in *L. chinense*

The GRF protein sequences of *A. thaliana* were obtained from the Plant Transcription Factor Database (<http://planttfdb.gao-lab.org/>). These sequences were used to identify *GRF* homologous genes in *L. chinense* database (including the proteins from genome data and our full-length transcriptome data) by performing a local BLASTP alignment. Then, we retrieved the Hidden Markov Models (HMM) of the WRC domain (PF08879) and the QLQ domain (PF08880) from Pfam database (<http://pfam.xfam.org/>), which were used for searching the *LcGRFs* from the *L. chinense* database by using HMMER 3.0 software. Finally, genes identified through both BLASTP alignment and HMM search were considered as the putative *LcGRF* genes, then these genes were aligned to Pfam database to further confirm the QLQ and WRC domains. The theoretical isoelectric point (pI), molecular weight (MW) and grand average of hydropathicity (GRAVY) of all *LcGRFs* were obtained on the ExPASy (<http://www.expasy.org/tools/>) website.

2.3. Gene structure and phylogenetic analysis of *LcGRF* genes

The exon-intron structure of *GRF* genes was analyzed using Gene Structure Display Server (<http://gsds.gao-lab.org/>) to visualize the gene structure. Interspecific phylogenetic analysis was conducted to investigate the relationships between GRFs. A total of 72 GRF protein sequences from *A. thaliana*, Chinese pear (*Pyrus bretschneideri*), poplar, grape (*Vitis vinifera*), rice, and maize were downloaded from the Plant Transcription Factor Database. Then clustalW alignment was performed on the 81 GRF proteins, and the alignment result was used to construct a neighbor-joining tree using MEGA-X software (bootstrap value was set as 1,000) (Hall, 2013).

2.4. Multiple alignment and conserved motif analysis

The clustalW alignment was used for the multiple alignment of nine *LcGRFs*, allowing for the identification of sequence characteristics within the QLQ and WRC domains. Additionally, we utilized the MEME tool (<https://meme-suite.org/meme/tools/meme>) to analyze the conserved motifs in *LcGRFs*.

2.5. Synteny analysis of *GRF* genes

Synteny analysis of *GRF* genes was conducted on TBtools software (Chen et al., 2020). For intraspecific synteny analysis of *LcGRFs*, we used the Blast Compare Two Seqs to perform a pairwise comparison of *L. chinense* proteins; then, the annotation file of the *L. chinense* genome was input into the Text Merge for MCScanX for simplifying; finally, the blast results and the simplified genome annotation file were used to analyze the synteny of *L. chinense* genes using the Quick Run MCScanX Wrapper.

For interspecific synteny analysis of *GRFs*, the genome sequences and annotation files of *A. thaliana* and poplar were obtained from the Phytozome 13 database (<https://phytozome-next.jgi.doe.gov/>). The obtained genome sequences and annotation files of *L. chinense*, *A. thaliana*, and poplar were inputted into One Step MCScanX to analyze the synteny between *L. chinense* and *A. thaliana*, as well as *L. chinense* and poplar. Finally, based on the genome annotations, the GRFs of *A. thaliana* and poplar that exhibited collinearity with *LcGRFs* were identified, and the results of the synteny analysis were visualized using Advanced Circos.

2.6. Expression profile analysis of *LcGRF* genes in multiple tissues

The expression levels of *LcGRF* genes in six tissues (bract, sepal, stamen, pistil, mature leaf and shoot apex), were quantified based on the transcriptome data. The expression of each gene was further calculated using the FPKM (fragments per kilobase of transcript per million mapped reads). The TBtools software (Chen et al., 2020) was used to draw the gene expression heatmap.

2.7. RNA isolation, and RT-qPCR assay

The total RNA was extracted from each sample using the RNAPrep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The Evo M-MLV RT Premix for qPCR kit (AG11706, ACCURATE BIOTECHNOLOGY, HUNAN, Co.,Ltd) was used to synthesize cDNA. The SYBR Green Premix Pro *Taq* HS qPCR Kit (AG11701, ACCURATE BIOTECHNOLOGY) was used to perform RT-qPCR on a StepOnePlus™ System (Applied Biosystems, USA), and the amplification process was set according to the kit instructions. All RT-qPCR assay included three technical replications and three biological replicates, and the $2^{-\Delta\Delta C_t}$ method was used to analyze the RT-qPCR results. The *LcACT97* and *LcEIF3* were used as the reference gene for *L. chinense*, and the *AtACT2*

was used as the reference gene for *A. thaliana* (Tu *et al.*, 2019). All primers were provided in Table S1.

2.8 Transactivation assays

The coding sequence (CDS) of *LcGRF2* was subcloned into the GAL4 DNA-binding domain at the *EcoR* I/*Bam*H I sites of the pGBKT7 vector. The correctly sequenced pGBKT7-*LcGRF2* recombinant plasmid and pGBKT7 (negative control) were separately transformed into AH109 cells. Subsequently, the transformed cells were spotted onto SD/-Trp single-deficient culture medium and incubated for 2-4 days. Single colonies were selected, diluted with sterile water to various concentrations (10^0 , 10^1 and 10^2), and then plated on SD/-Trp, SD/-Trp-Ade-His, SD/-Trp-Ade-His+X- α -gal media. Incubation was carried out at 29 °C for 2 days to assess activation activity.

2.9. Plasmid construction, subcellular localization, and genetic transformation of *LcGRF2*

To construct the fusion vector for subcellular localization, the full-length CDS sequences without a stop codon were inserted at the *Hind* III/*Bam*H I sites of the pJIT166 GFP vector driven by CaMV35S promoter. Then, we used young leaves of *L. chinense* seedlings to produce protoplasts following the method described by Huo *et al.* (Huo *et al.*, 2017). The 35S::*LcGRF2*-GFP plasmid and the control (pJIT166 GFP) were separately transformed into protoplasts using the PEG-mediated transformation method (Huo *et al.*, 2017). The subcellular localization results were observed under a ZEISS Imager D2 fluorescence microscope (Carl Zeiss, Germany).

For constructing the overexpression vector, we amplified the full-length coding sequences (CDS) of *LcGRF2* by using the *LcGRF2*-F/*LcGRF2*-R primers that containing the *Kpn* I/*Xba* I sites. The amplified products were then recombined into the pCAMBIA1300 vector containing a 35S promoter using the ClonExpress® Ultra One Step Cloning Kit (Vazyme, Nanjing). The constructed overexpression vector, 35S::*LcGRF2*, was introduced into electro-competent *Agrobacterium tumefaciens* strain GV3101 (provided by Shanghai Weidi Biotechnology Co., Ltd.) Subsequently, the overexpression vector was transformed into *A. thaliana* through the floral-dip method, generating 11 hygromycin-resistant lines (Clough and Bent, 1998). The T3 homozygous of six independent lines exhibiting obvious phenotypes were selected for phenotype analysis and RT-qPCR analysis.

2.10. Phenotypic analysis of transgenic *A. thaliana*

Five rosette leaves were collected from 35-day-old WT *A. thaliana* plants and transgenic plants. The retrieved leaves were used to measure the length and width of the blade as well as the length of the petiole. Each measurement was performed with at least nine replicates per line.

2.11. Statistical Analysis

Data analysis was conducted using the one-way ANOVA Tukey's test. All data are expressed as mean $\pm$ standard deviation (SD) of three replicates. Statistical significance was determined with Student's t-test. $P < 0.05$ was regarded as significant (*), and $P \leq 0.01$ was considered highly significant (**).

3. Results

3.1. Identification of *GRF* genes in *L. chinense*

We identified nine *LcGRF* genes (Table 1). Notably, *GRF1* and *GRF3* gene had two copies, resulting in the formation of two gene pairs (*LcGRF1a/LcGRF1b* and *LcGRF3a/LcGRF3b*). The length of *LcGRFs* ranged from 234 (*LcGRF3*) to 600 (*LcGRF1*) amino acids (aa), with molecular weight (MW) ranging from 25,341.77 (*LcGRF3*) to 64,728.04 Da (*LcGRF1*). The isoelectric point (pI) of *LcGRFs* varied from 6.47 (*LcGRF7*) to 9.51 (*LcGRF4*). Additionally, all *LcGRFs* were found to be hydrophilic (GRAVY value below zero). (Table 1).

Table 1 Physicochemical information on *LcGRF* genes

Gene	Amino acid length (aa)	Mw (Da)	pI	GRAVY
<i>LcGRF1a</i>	600	64728.04	8.09	-0.509
<i>LcGRF1b</i>	600	64728.04	8.09	-0.509
<i>LcGRF2</i>	574	61273.36	8.40	-0.948
<i>LcGRF3a</i>	234	25341.77	9.45	-0.360
<i>LcGRF3b</i>	234	25341.77	9.45	-0.360
<i>LcGRF4</i>	404	43998.95	9.51	-0.733

<i>LcGRF5</i>	348	39369.60	9.11	-0.930
<i>LcGRF6</i>	341	37791.84	8.47	-0.848
<i>LcGRF7</i>	561	60058.72	6.47	-0.551

3.2. Gene structure and phylogenetic analysis of *LcGRF* genes

Nine *LcGRF* genes were classified into four groups, and the genes corresponding to each group had similar gene structure (Fig. 1A). *LcGRF1a*, *LcGRF1b*, and *LcGRF2* shared a similar number of exons with *LcGRF4* and *LcGRF7*, consisting of four exons (Fig. 1A). In contrast, the remaining four *LcGRF* genes only had three exons. Notably, *LcGRF3a* and *LcGRF3b* were grouped together, while *LcGRF5* and *LcGRF6* formed a separate cluster (Fig. 1A).

To investigate the phylogenetic relationships between LcGRFs and GRFs from other plant species, we constructed a phylogenetic tree of GRFs from *L. chinense* (9 LcGRFs), *A. thaliana* (9 AtGRFs), *Py. bretschneideri* (10 PbGRFs), poplar (19 PtGRFs), grape (8 VvGRFs), rice (12 OsGRFs), and maize (14 ZmGRFs). The 81 GRF proteins were classified into four subfamilies (subfamily A to D), consistent with the results of intraspecific phylogenetic analysis (Fig. 1B). Each subfamily contained GRF proteins from *L. chinense*, *A. thaliana*, *Py. bretschneideri*, poplar and grape, suggesting that LcGRFs had putative orthologous proteins in these plants (Fig. 1B). Moreover, subfamily B comprised 14 GRFs, including LcGRF4 and LcGRF7 (Fig. 1B). Interestingly, subfamily B did not include any OsGRFs and ZmGRFs, indicating that this subfamily may be specific to dicotyledonous plants (Fig. 1B). Furthermore, in the phylogenetic analysis, LcGRFs showed closer relationships with GRFs from grape compared to other dicotyledonous species (Fig. 1B). By contrast, most of LcGRFs had distant relationships with OsGRFs and ZmGRFs (Fig. 1B). This observation may be attributed to the fact that rice and maize are monocots. Notably, except for OsGRF10 and OsGRF12, other OsGRFs and ZmGRFs formed an independent evolutionary branch that was distinct from the evolutionary branch of dicotyledons within each subfamily (Fig. 1B). This suggested that GRF may have differentiated accompany with the differentiation of monocots and dicotyledon.

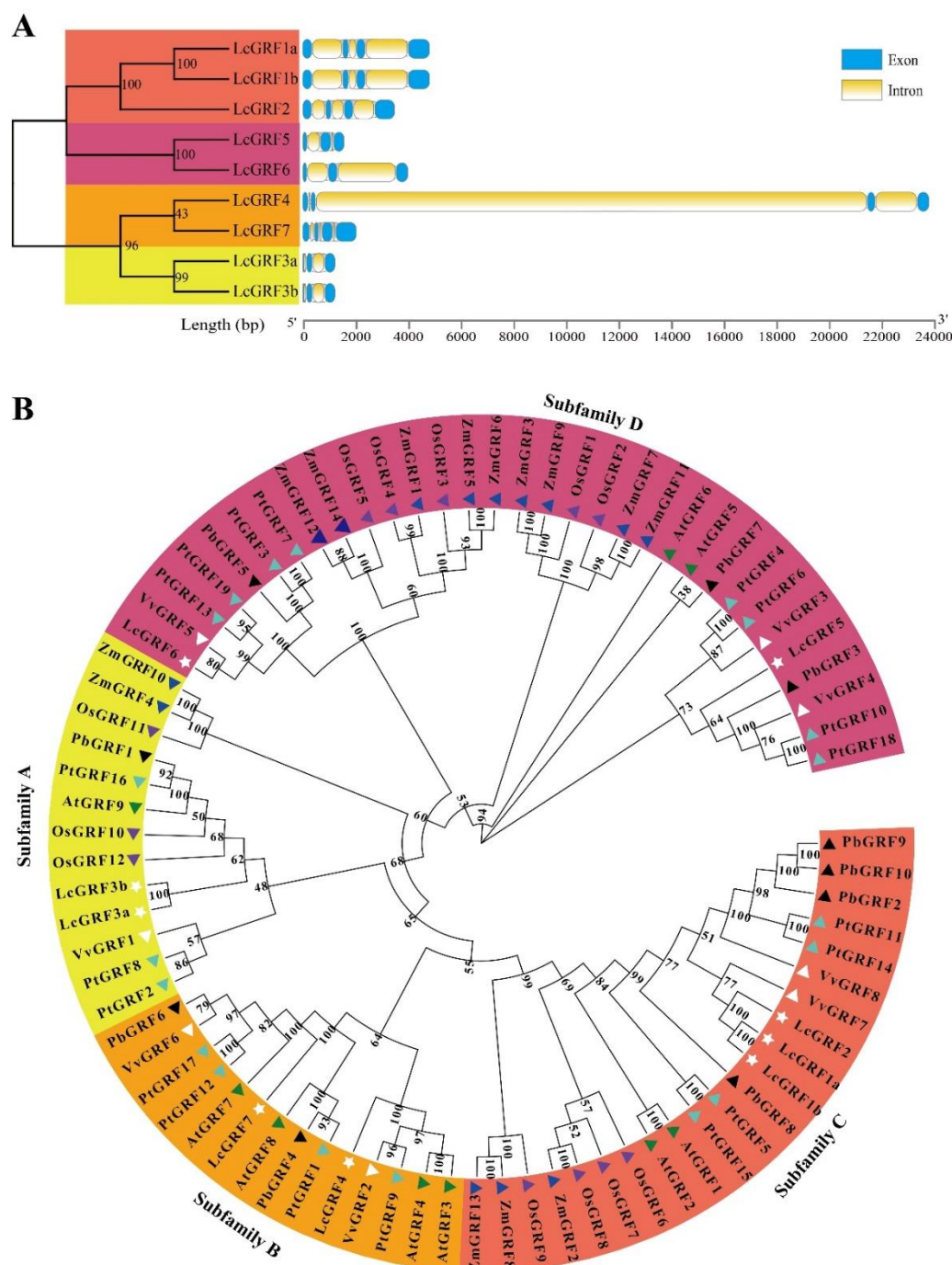


Fig. 1 Gene structure of *LcGRF* genes and phylogenetic tree of *LcGRFs*. The neighbor-joining method in MEGA-X was used to constructed the phylogenetic tree (Bootstrap values was set as 1,000). (A) Gene structure of *LcGRF* genes and phylogenetic tree of *LcGRFs*. (B) Phylogenetic analysis of GRFs from *L. chinense*, *A. thaliana*, *Py. bretschneideri*, poplar, grape, rice, and maize. Red stars represent GRFs from *L. chinense* (9 *LcGRFs*). White, black, turquoise, green, purple, and blue triangles represent GRFs of grape (8 *VvGRFs*), *Py. bretschneideri* (10 *PbGRFs*), poplar (19 *PtGRFs*), *A. thaliana* (9 *AtGRFs*), rice (12 *OsGRFs*), and maize (14 *ZmGRFs*) respectively. All 81 GRFs could be divided into four subfamilies.

3.3. Multiple sequence alignments and conserved motifs analysis

Revealing the conserved domains of *LcGRFs* is crucial for understanding their functions. Therefore, we performed multiple sequence alignments of the nine *LcGRFs*. Our analysis revealed that all *LcGRF* proteins contained Q-L-Q residues, which are characteristic of the QLQ domain (Fig. 2A). Moreover, a WRC domain accompanied by a zinc finger motif (C₃H) was highly conserved in all *LcGRFs* (Fig. 2B). Furthermore, we observed that the TQL domain was exclusively present in members of subfamily C (*LcGRF1a*, *LcGRF1b*, and *LcGRF2*) and subfamily D (*LcGRF5* and *LcGRF6*) (Fig. 2C and D). This finding indicated that the functions of *LcGRFs* in

subfamilies C and D may differ from those in subfamilies A and B.

To further investigate the sequence characteristics of LcGRFs in different subfamilies, we analyzed the conserved motifs in the nine LcGRF proteins. A total of five conserved motifs were identified, and LcGRFs in the same subfamily contained similar conservative motif composition (Fig. 2E). Notably, motif 1 and motif 2 were appeared in all LcGRF proteins, corresponding to the WRC domain and the QLQ domain, respectively (Fig. 2E). Although motifs 3, 4, and 5 were present in most of LcGRF proteins, their functions remained unknown (Fig. 2E). Moreover, we noticed that LcGRF3a and LcGRF3b exhibited truncated C-terminal, which may potentially affect the function of LcGRF3a and LcGRF3b in *L. chinense* (Fig. 2E).

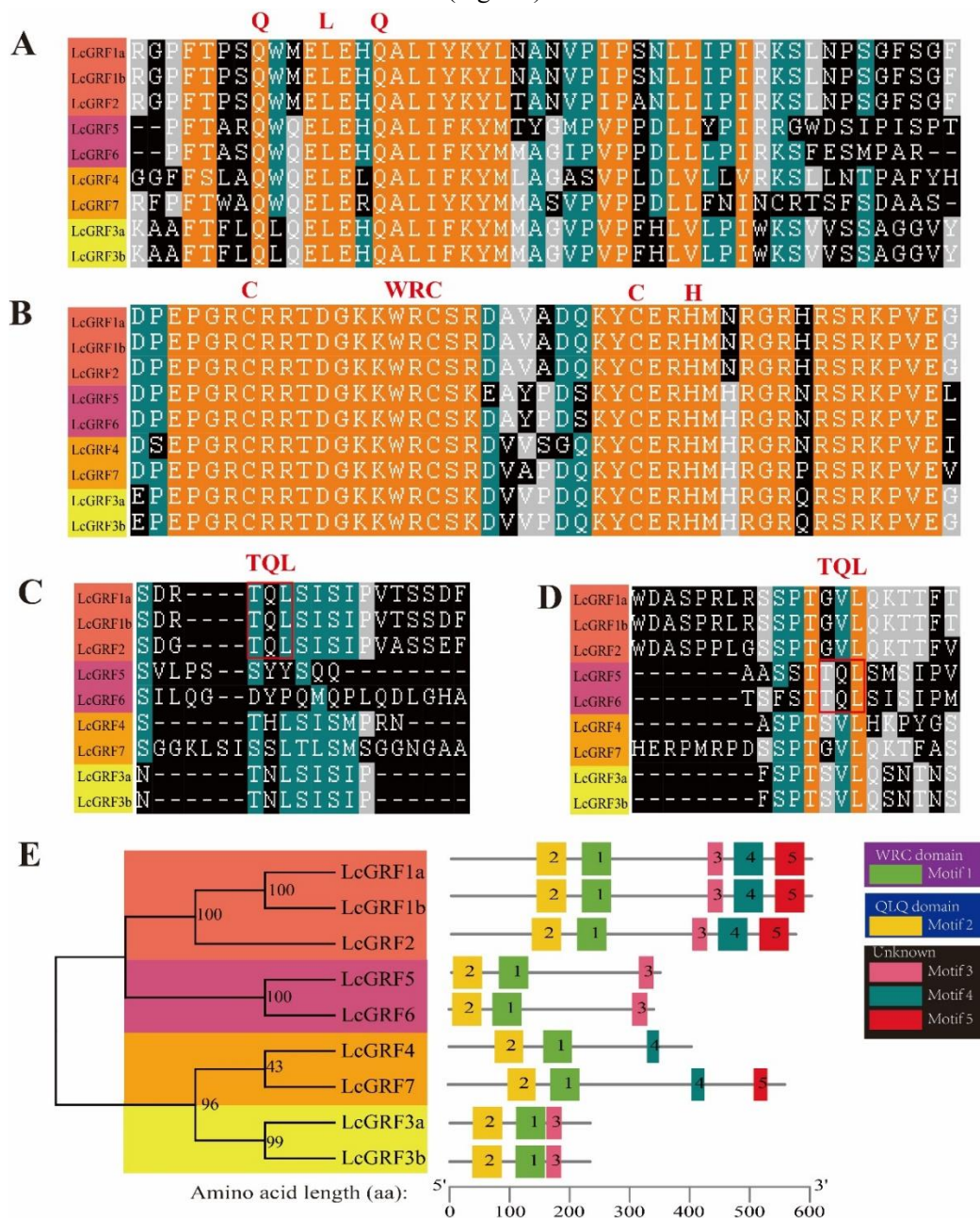


Fig. 2 Multiple alignment and conserved motif analysis of LcGRFs. (A)-(C) QLQ, WRC, and TQL domains of LcGRF proteins. (D) Conserved motifs in LcGRF proteins. (E) Distribution of 5 conserved motifs in the LcGRF proteins. Differently colored squares indicate different motifs.

3.4. Synteny analysis of *LcGRF* genes

Above analysis had revealed the phylogenetic relationships of *LcGRF* genes from intraspecific and interspecific perspectives. However, to gain a deeper understanding of the evolutionary events of *LcGRF* genes, we conducted synteny analysis. Before performing the synteny analysis, we first confirmed the chromosomal location

of *LcGRF* genes. Our findings revealed that the nine *LcGRF* genes were located on five chromosomes and one contig (contig 255). Notably, the 8th chromosome harbored the largest number of *LcGRF* genes (3 *LcGRF* genes) (Fig. 3A). Two duplicated gene pairs, *LcGRF1a/LcGRF2* and *LcGRF1b/LcGRF2*, were identified within *L. chinense* (Fig. 3A), indicating that these three *LcGRF* genes were arose before the divergence of *LcGRF* genes.

A previous study reported that *L. chinense* was more ancient than *A. thaliana* and poplar (Chen *et al.*, 2019). Through a whole genome-wide synteny analysis, we found that 33.33% (3/9) of *LcGRF* genes showed collinearity with 44.44% (4/9) of *AtGRF* genes, indicating that the majority of *LcGRF* genes (66.67%) originated after the divergence of *L. chinense* and *A. thaliana* (Fig. 3B). However, in poplar, a significant proportion of *PtGRF* genes (63.16% or 12/19) displayed synteny with a high percentage of *LcGRF* genes (77.78% or 7/9) (Fig. 3C), which was caused by segmental duplication events. This indicated that *GRF* family occurred gene expansion in poplar. Moreover, *LcGRFs* showed a closer genetic distance to *PtGRFs* than to *AtGRFs* (Fig. 1B). These findings suggested that the evolution of *GRF* genes in woody plants may be different with that in herbaceous plants.

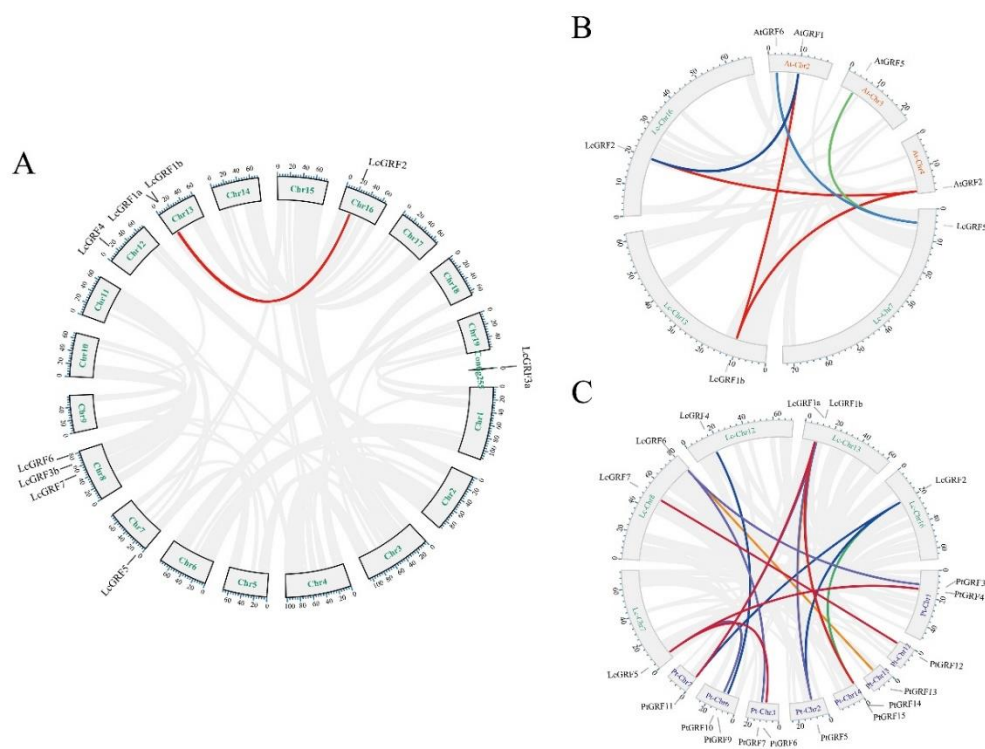


Fig. 3 Intraspecific and interspecific synteny analysis of *LcGRF* genes. (A) Intraspecific synteny analysis of *GRF* genes in *L. chinense*. Red line indicates there is a collinearity between two given *LcGRF* genes. (B) Interspecific synteny analysis of *GRF* genes in *L. chinense* and *A. thaliana*. Lc-Chr represents the chromosome from *L. chinense* (green font). At-Chr represents the chromosome from *A. thaliana* (orange font). Color lines indicate synteny between two given *GRF* genes. We only displayed chromosomes that carried *GRF* genes. The major scale of the chromosome is 10 Mb. (C) Synteny analysis of *GRF* genes from *L. chinense* and poplar. Lc-Chr represents the chromosome from *L. chinense* (green font). Pt-Chr represents the chromosome from poplar (purple font). Color lines indicate synteny between two given *GRF* genes. We only displayed chromosomes that carried *GRF* genes. The major scale of chromosome is 20 Mb.

3.5. Expression profiles of *LcGRF* genes in different tissues

Studying the expression profiles of *LcGRF* genes is the foundation for revealing their roles in *L. chinense*. By analyzing the transcriptome data from our previous study, we examined the expression profiles of *LcGRF* genes in seven different tissues (Tu *et al.*, 2021). The heatmap showed that all *LcGRF* genes exhibited the lowest expression level in mature leaf, while displaying the highest expression level in shoot apex (Fig. 4A). Especially, among all *LcGRF* genes, *LcGRF2* showed the highest expression level in shoot apex (Fig. 4A). Notably, the shoot apex is the region where leaves and flowers originate.

To further explore the expression pattern of *LcGRF2*, we performed RT-qPCR to assess its expression in leaves at different stage and reproductive tissues. As shown in Fig. 4B, *LcGRF2* displayed a robust expression in flower

bud. Moreover, the expression level of *LcGRF2* gradually decreased during the development of lobed leaves (S1 leaf to S3 leaf). This indicated that *LcGRF2* may regulate the development of lobed leaves.

However, the question of whether *LcGRF2* regulates leaf shape or leaf size still remains unclear. Thus, we analyze the expression level of *LcGRF2* in various regions of leaves at different stages. Overall, the expression levels of *LcGRF2* in different leaf regions decreased along with the leaf development (Fig. 4C). At each developmental stage, *LcGRF2* showed a higher expression level in the distal region of leaves (distal leaf tooth and distal leaf serration) compared to the proximal region (proximal leaf tooth and proximal leaf serration). Additionally, in both the distal and proximal regions of leaves, there were no significant differences in the expression levels of *LcGRF2* between leaf tooth and serration (Fig. 4C). This indicated that *LcGRF2* may not participate in the regulation of leaf shape in *L. chinense*. Moreover, we observed a distinct difference in the expression level of *LcGRF2* between the center area and outer area of leaves. However, the differences in *LcGRF2* expression level between these two regions, as well as between the distal and proximal regions, decreased along with leaf development (Fig. 4C). These findings indicated that the gradient expression pattern of *LcGRF2* along the proximal-distal axis (from high to low) and medio-lateral axis (from low to high) of leaves may be closely associated with the regulation of leaf size.

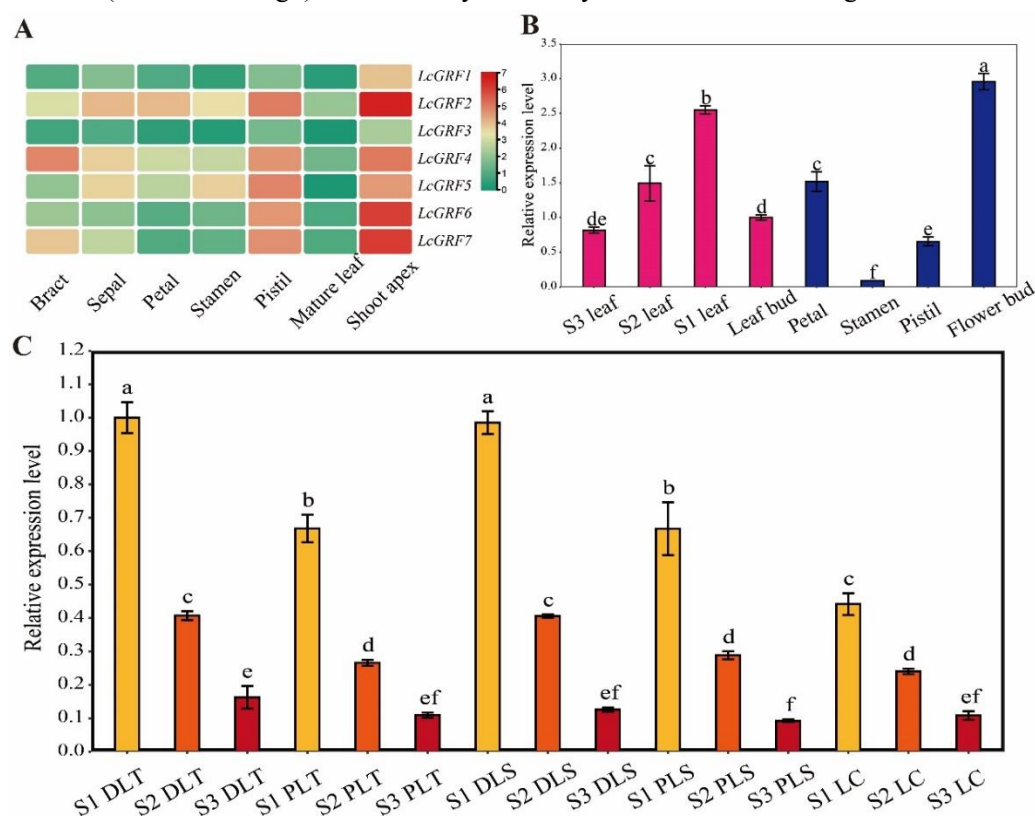


Fig. 4 Expression profiles of *LcGRF* genes. (A) Heatmap of the expression levels of *LcGRF* genes in different tissues. The $\log_2(\text{FPKM})$ values of *LcGRF* genes were used to construct heatmap, which were provided by transcriptome sequencing data. (B) RT-qPCR analysis of *LcGRF2* in different stage leaves and flower tissues. (C) Relative expression levels of *LcGRF2* in different parts of leaves at different stages. DLT: distal leaf tooth; PLT: proximal leaf tooth; DLS: distal leaf serration; PLS: proximal leaf serration; LC: leaf center. Bar plots with the same color indicate that they are from the same development stage. Note: All data represent means $\pm$ SD of three repetitive experiments. Different lowercase letters (a, b, c, d, e and f) above the bars represent significant differences ($P < 0.05$).

3.6. Transcriptional activity of *LcGRF2*

Transcriptional activity is an important feature of transcription factors. The results showed that pGBKT7-*LcGRF2* grew normally in triple -deficient media and displayed blue color on SD/-Trp-Ade-His+X- α -gal solid medium (Fig. 5A), which indicated that the *LcGRF2* protein was transcriptionally active.

3.7. Subcellular localization of *LcGRF2* gene

To gain a better understanding of the function of *LcGRF2* in *L. chinense*, we analyzed the subcellular localization of *LcGRF2* by performing transient transformation using the 35S::*LcGRF2*-GFP vector. As a control,

the 35S::GFP showed green fluorescence throughout the protoplasts (Guo et al., 2023; Huo et al., 2017a). However, the 35S::LcGRF2-GFP fusion protein only showed green fluorescence in the nucleus of *L. chinense* protoplast (Fig. 5B). These results demonstrated that LcGRF2 was localized to the nucleus.

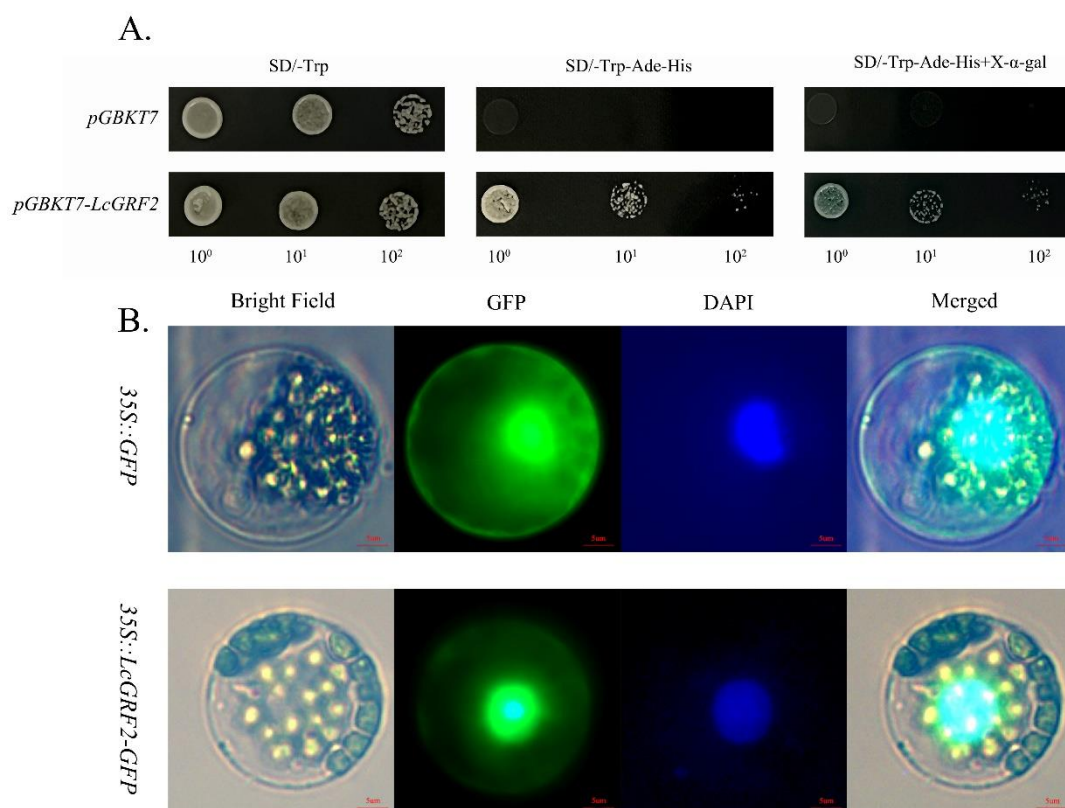


Fig. 5 Transcriptional activity and subcellular localization of LcGRF2. (A) Analysis of the LcGRF2 transcriptional activity. pPGKT7 as a negative control and the Wconcentration of bacterial solution was diluted to 10⁰, 10¹ and 10². (B) Subcellular localization of LcGRF2 in *L. chinense* protoplast. The 35S::GFP was used as a control, and the green fluorescence of the 35S::GFP was appeared throughout the protoplasts interior (first column figure). The green fluorescence of the 35S::LcGRF2-GFP fusion protein was only detected in nucleus (second column figure). DAPI was used as a nuclear marker. Bar = 5 μm.

3.8. Overexpression of LcGRF2 induces leaf enlargement in *A. thaliana*

To further investigate the function of LcGRF2, we transformed the 35S::LcGRF2 vector into *A. thaliana* and after screening on selective medium and DNA detection, a total of 11 T1-positive transgenic plants were obtained (Fig. S2). To streamline our workload, we took 6 positive transgenic lines exhibiting obvious phenotypes for subsequent RT-qPCR analysis and ultimately identified three lines with the highest expression levels (Fig. 6A). The three transgenic lines displayed enhanced growth vigor compared to WT plants (Fig. 6B-E). Especially, the transgenic plants exhibited enlarged leaves, with significantly longer leaf length compared to WT plants (Fig. 6F-J). However, overexpressing LcGRF2 did not lead to a significant increase in petiole length in the transgenic plants, while it significantly increased the blade length (Fig. 6K and L). Moreover, the three LcGRF2 OE lines showed a wider blade than WT plants (Fig. 6M).

Previous studies have reported that GRFs can influence leaf size by affecting either cell number or cell size (Kim et al., 2003; Kim and Lee, 2006; Lee et al., 2009; Wang et al., 2020). In order to figure out whether the enlarged leaves in transgenic plants resulted from an increase in cell number or cell size, we analyzed the expression levels of three genes associated with the cell cycle (*AtCYCD3;1*, *AtKNOLLE*, and *AtCYCB1;1*) and two genes related to cell expansion (*AtEXP10* and *AtSAUR53*). RT-qPCR analysis revealed that the expression levels of *AtCYCD3;1*, *AtKNOLLE*, and *AtCYCB1;1* were significantly higher in transgenic plants compared to WT plants (Fig. 7A-C). However, there was no significant increase in the expression levels of *AtEXP10* and *AtSAUR53* in transgenic plants (Fig. 7D and E). These findings indicated that overexpression of LcGRF2 could increase blade size by promoting cell proliferation rather than cell expansion.

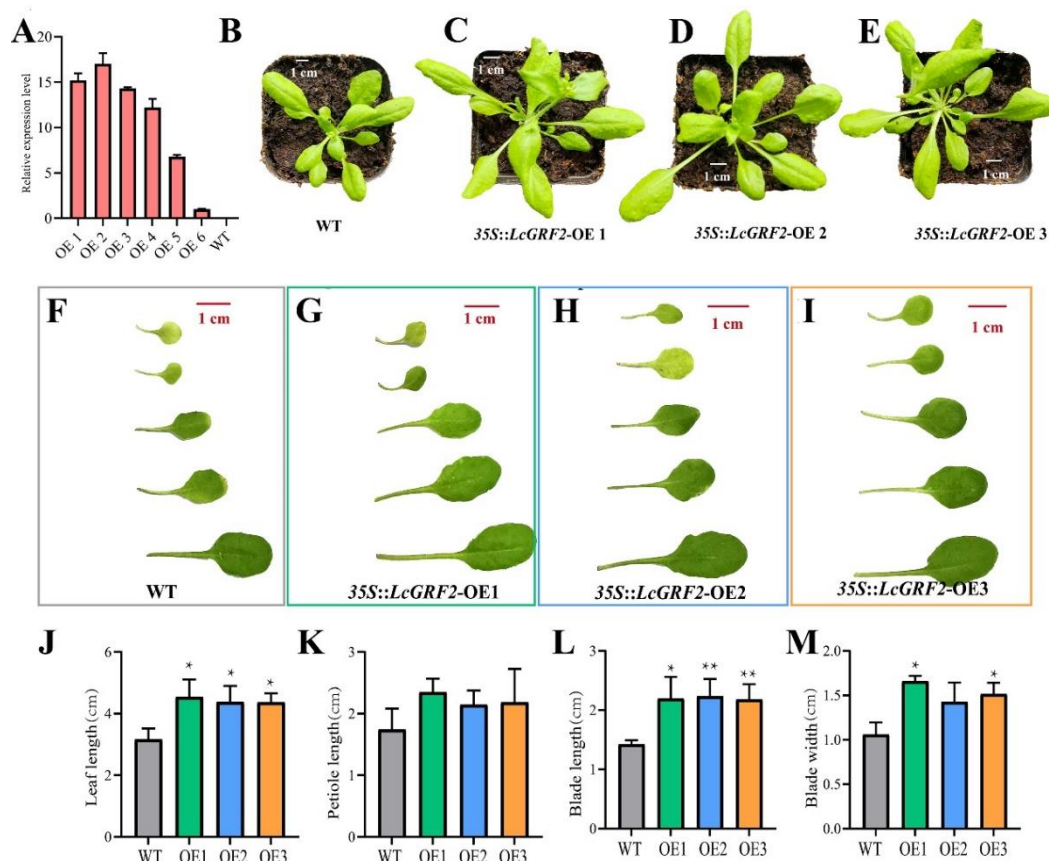


Fig. 6 Phenotypic observation and statistical analysis of 35-day-old *35S::LcGRF2* transgenic plants. The T test was used to evaluate the significance of differences between WT and T3 transgenic plants, * indicates significant difference between WT and transgenic plants ($P < 0.05$), and ** indicates highly significant difference between WT and transgenic plants ($P < 0.01$). (A) Detection of the expression level of *LcGRF2* in WT plants and six *35S::LcGRF2* overexpression lines. (B)-(E) Phenotype of WT plant and three *35S::LcGRF2* overexpression lines. (F)-(I) The first to fifth leaves of WT, *35S::LcGRF2* overexpression line 1, 2 and 3, respectively. (J)-(M) Statistics of leaf length, petiole length, blade length, blade width. Values are means $\pm$ SD ($n = 9$).

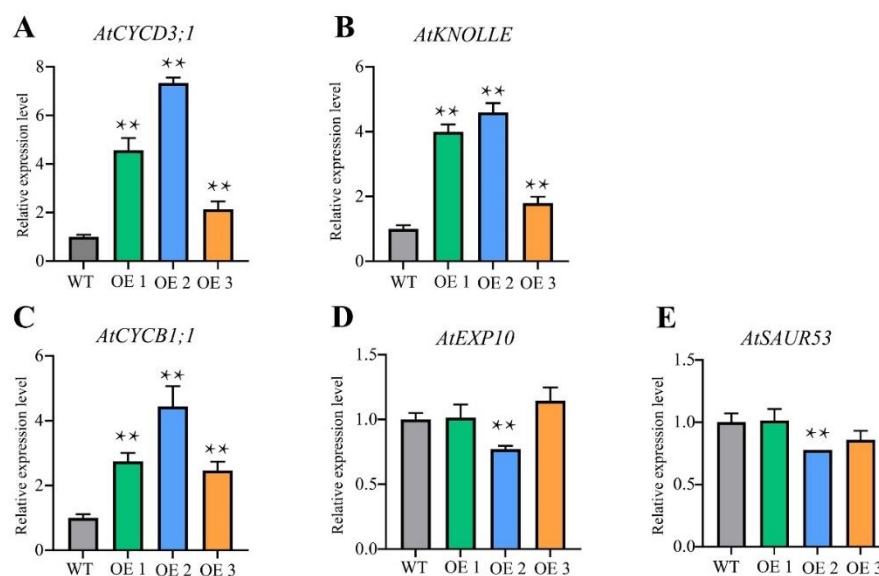


Fig. 7 RT-qPCR analysis of five genes in WT plants and three *35S::LcGRF2* overexpression lines. Values are means $\pm$ SD ($n = 3$), all data were retrieved from three repetitive experiments. The T test was used to evaluate the significance of differences between WT and transgenic plants, * indicates significant difference in expression level between WT and transgenic plants ($P < 0.05$), and ** indicates highly significant difference in expression level between WT and transgenic plants ($P < 0.01$). (A)-(C) RT-qPCR analysis of three cell cycle genes, *AtCYCD3;1*, *AtKNOLLE*, and *AtCYCB1;1*, in WT and transgenic plants. (D) and (E) RT-qPCR analysis of two

cell expansion-related genes, *AtEXP10* and *AtSAUR53*, in WT and transgenic plants.

4. Discussion

GRFs are plant-specific transcription factors (TFs) that play a crucial role in leaf development. In this work, our focus was on characterizing the gene structure, chromosome localization, conserved domains, synteny and functions of *GRFs* in *L. chinense*. A total of 9 *GRF* genes were identified in the whole genome of *L. chinense*. Notably, the number of *GRFs* in *L. chinense* was higher than that in tea plant (*Camellia sinensis*, 6 *GRFs*), cucumber (*Cucumis sativus*, 8 *GRFs*), and grape (8 *GRFs*), but lower compared to rice (12 *GRFs*), poplar (19 *GRFs*), Chinese pear (10 *GRFs*), maize (14 *GRFs*), moso bamboo (18 *GRFs*), peach (*Prunus persica*, 10 *GRFs*), and foxtail millet (*Setaria italica*, 10 *GRFs*) (Cao et al., 2016; Chen and Ge, 2022; Liu et al., 2022; Shi et al., 2019; Wu et al., 2017; Zhang et al., 2008; Zhou et al., 2018). Species undergo different gene duplication events, which may contribute to the variation in *GRF* gene numbers. In *L. chinense*, *GRFs* have undergone two segmental duplication events. In contrast, moso bamboo, peach, and foxtail millet have experienced 10, 3, and 6 duplication events, respectively. While no *GRF* gene duplication event has been identified in cucumber (Chen and Ge, 2022; Liu et al., 2022; Shi et al., 2019; Zhou et al., 2018).

GRFs typically consist of two conserved domains in the N-terminal region, QLQ and WRC domains. The C-terminal region of GRFs generally exhibits lower conservation compared to the N-terminal region and this region often contains three less-conserved motifs (FFD, TQL, and GGPL). In our study, we found that all LcGRFs possessed the QLQ and WRC domains (Fig. 2A). However, no FFD and GGPL motifs was identified in LcGRFs. Interestingly, we found that five LcGRFs in the C and D subfamilies contained the TQL motif. In poplar, a different pattern was observed, with only five GRFs harboring the TQL motif, eight GRFs containing the FFD motif, and 15 GRFs containing the GGPL motif (Wang et al., 2020). In Moso bamboo, it has been reported that six GRFs possess both FFD and TQL motifs (Shi et al., 2019). These findings indicated that the appearance of TQL, FFD, and GGPL motifs may be related to specific functions of GRFs, but the essential functional significance of these motifs in GRFs is still unknown. Additionally, we observed that LcGRF3a/b had a truncated C-terminal and were grouped into subfamily A with AtGRF9 (Fig. 1). AtGRF9 activated the expression of *OBP3-RESPONSIVE GENE 3 (ORG3)*, leading to negative regulation of leaf growth (Omidbakhshfard et al., 2018). ZmGRF10 also contained a truncated C-terminal, and overexpression of *ZmGRF10* in maize produced smaller leaves through repressing cell proliferation (Wu et al., 2014). Given the similarity in the truncated C-terminal region, it is plausible that LcGRF3a/b shares similar functions with AtGRF9 and ZmGRF10, but further functional verifications is required.

Gene expression patterns provide valuable insights into the potential roles of genes in plants. The expression profiles of *GRFs* have been widely investigated. *GRFs* are commonly expressed in organs characterized by vigorous cell proliferation, such as root tips, SAM, young stems, young leaves, and early flower buds. (Kim et al., 2003; Lu et al., 2020; Wu et al., 2017). In *L. chinense*, most LcGRFs exhibit high expression levels in shoot apex and pistil, including *LcGRF2* (Fig. 2A). *LcGRF2* also showed a high expression level in flower buds, suggesting a potential role for LcGRFs in regulating flower organ development. In *A. thaliana*, the highest levels of *GRFs* were detected in pistil, which gradually decrease as flower development progresses. (Liang et al., 2014). Furthermore, the expression level of *LcGRF2* decreased during lobed leaf development (Fig. 4B). This trend has also been observed in *A. thaliana* and rice (Debernardi et al., 2014; Lu et al., 2020). Additionally, an expression gradient of *LcGRF2*, ranging from high to low, was detected along the proximal-distal axis of leaf (Fig. 4C). A contrary gradient expression of *GRFs* along the proximal-distal axis was observed in *A. thaliana*, where the transcript levels of *AtGRFs* were decreased from the distal region to the proximal region of leaf (Horiguchi et al., 2005). This expression pattern of *GRFs* along the proximal-distal axis regulated the allometric pattern of leaf growth (Das Gupta and Nath, 2015). These findings suggested that *L. chinense* has a different leaf growth pattern along the proximal-distal axis compared to *A. thaliana*.

As important regulators of growth and development, *GRFs* participated in the regulation of leaf development by influencing cell proliferation and/or cell expansion. In *A. thaliana*, overexpression of *AtGRF2* led to enlarged leaves due to an increase in cell size (Kim et al., 2003). In overexpressed *AtGRF5* lines, transgenic plants showed larger leaves than WT plants, primarily attributed to an increase in cell number (Horiguchi et al., 2005). In poplar

(*P. alba* × *P. glandulosa*), overexpression of *PagGRF7a* resulted in the down-regulation of cell expansion-related genes like *EXPA11a* and *EXPA11b*, which produced smaller leaves than WT plants. While *PagGRF12a* and *PagGRF12b* acted as positive regulators of leaf size, and in the overexpressed lines of these genes not only increased the expression of cell division-related genes (*CYCB1;1a* and *CYCB1;1b*) but also decreased the expression of cell expansion-related genes (Wang et al., 2020). *LcGRF2* showed distinct expression patterns along both the proximal-distal and the medio-lateral axis, indicating that *LcGRF2* may be closely related to leaf size development. Since establishing an efficient and stable genetic transformation system for *L. chinense*, a woody plant rich in numerous compounds, is exceptionally challenging, and we have not yet developed an efficient and stable genetic transformation system for *L. chinense* so far. Hence, we applied the *A. thaliana* transformation system to investigate the function of the *LcGRF2*. Overexpression of *LcGRF2* in *A. thaliana* produced enlarged leaves, which was caused by the up-regulation of cell division-related genes (*AtCYCD3;1*, *AtKNOLLE*, and *AtCYCB1;1*) (Fig. 7). These findings strongly indicated that *LcGRF2* may regulate leaf size by affecting cell proliferation.

5. Conclusion

In this study, we identified nine *LcGRF* genes in *L. chinense* genome. By analyzing the transcriptome data, we found that *LcGRF2* exhibited significant potential in regulating leaf size. Expression analysis revealed a gradual decrease in *LcGRF2* expression during leaf development, and its expression displayed a gradient pattern along the proximal-distal and medio-lateral axes of leaves. Furthermore, overexpression of *LcGRF2* in *A. thaliana* enlarged leaf size by increasing cell numbers. These findings indicated that *LcGRF2* regulated leaf size by controlling cell proliferation along both the proximal-distal and medio-lateral axis of leaves.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Data availability

The supplementary material is available. Tables S1, Figures S1 and Figures S2 can be found in File S1.

Supplementary materials

Table S1 Primers for RT-qPCR

Fig. S1 Materials for RT-qPCR analysis.

Fig. S2 The gel images for detecting positive transgenic lines in the T1 generation

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