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ARTICLE

Genome-Wide Identification and Expression Pattern Analysis of *LONELY GUY* Gene Family in Walnut (*Juglans regia*)

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ABSTRACT

LONELY GUY (LOG) is a pivotal cytokinin-activating enzyme that plays an important role in plant growth, development, and stress responses. Walnut (*Juglans regia*), an important woody oilseed species, has not yet undergone systematic identification of its *LOG* gene family. In this study, we identified 17 *JrLOG* genes in the walnut genome, which are unevenly distributed across 11 chromosomes. *JrLOG* gene expansion was primarily driven by gene duplication, along with purifying selection. Members of the JrLOG family were categorized into five groups, each exhibiting analogous gene structures, featured motifs, and conserved domains. Transcriptome and quantitative real-time PCR analyses revealed that *JrLOG* gene expression was tissue-specific, developmentally regulated, and responsive to stress situations. Notably, *JrLOG3*, which localizes to the cell membrane, exhibited high expression levels in leaves and responded to both cold and pathogen infection treatments. This study represents the first comprehensive identification of the *LOG* gene family in walnut, offering essential data for further functional studies of this gene family.

KEYWORDS

Cold; LONELY GUY; transcriptome; walnut

Abbreviations

LOG	LONELY GUY
CKs	Cytokinin
qPCR	Quantitative real-time PCR

1 Introduction

Cytokinins (CKs), one of the six major plant hormones, play a crucial role in regulating various biological processes. Their primary function is to stimulate cell division, which is vital for plant growth and tissue formation [1]. CKs also work in conjunction with auxins to control cell differentiation, tissue formation, and stress response [2]. Additionally, CKs interact either antagonistically or synergistically

with other hormones to modulate growth patterns, including branching and the balance between root and shoot growth [3]. CKs also delay leaf senescence by preserving chlorophyll [4]. Under environmental stress, CK levels fluctuate, enabling plants to adapt and enhancing their stress tolerance [5].

CKs are predominantly synthesized in the meristematic tissues of roots, especially at the root apices, and are transported to other parts of the plant via the xylem. The homeostasis of CKs is tightly regulated to ensure normal physiological activities. Several enzymes participate in the synthesis, activation, modification, and degradation of CKs. Among them, LONELY GUY (LOG) plays a pivotal role by directly activating CKs [6] and regulating meristem activity [7]. *LOG* was first identified in rice mutants with defects in meristem maintenance [8]. In plants, *LOG* encodes a cytokinin riboside 5′-monophosphate phosphoribohydrolase, which converts inactive cytokinin nucleotides into active free bases [9]. Furthermore, LOG has been demonstrated to exist in all living organisms [10]. In model plants such as *Arabidopsis thaliana* (Arabidopsis) and *Oryza sativa* (rice), as well as crops like *Triticum aestivum* (wheat), *LOG* genes have been characterized for their roles in regulating plant growth and in responding to environmental stresses [9,11]. Arabidopsi*s* contains nine *LOG* genes, seven of which have been functionally investigated. Various mutations and overexpression techniques have been employed to elucidate the functions of distinct members of the *LOG* gene family at various stages of the plant life cycle and in specific tissues. For instance, the *log3log4log5* triple mutant demonstrated a reduction in shoot apical meristem size, leading to smaller inflorescences, fewer flowers, and seed pods [12]. Mutations in *AtLOG* resulted in shorter root systems [13,14]. In contrast, *LOG* overexpression caused more subtle phenotypes [12]. In poplar, overexpression of *LOG1* significantly increased xylem proliferation [15]. The *GY3* locus, encoding a rice LOG protein, notably enhanced grain yield [11]. Similarly, overexpression of *VlLOG11* from grapevine in tomatoes increased fruit yield and activated CK signaling-related gene expression [16]. In tomatoes, *LOG1* stimulated axillary meristem activation, leading to ectopic branching [17]. While knockout of *Solanum melongena SmLOG1* inhibited the formation of spines on leaves, stems, and fruit sepals [18].

Beyond its role in development, LOG is also critical in mediating responses to environmental and biotic stresses. Overexpression of *Ricinus communis RcLOG5* significantly enhanced drought, salt, and cold tolerance in Arabidopsis [19]. *Gossypium hirsutum* (cotton) *GhLOG3* overexpression enhanced salt tolerance, whereas its downregulation increased sensitivity to salt stress [20]. Under drought and salinity stress, *OsLOG*-overexpressing rice lines showed reduced H_2O_2 accumulation and elevated antioxidant enzyme activity [21]. Additionally, *OsLOGL1* contributed to yield maintenance under high nighttime temperatures [7], and *OsLOG5* regulated rice yield under various environmental and nutrient stress conditions [22].

Walnut (*Juglans regia*) is a widely cultivated nut tree belonging to the family *Juglandaceae*, valued for its nutritional benefits and high-quality wood, making it commercially significant. However, walnut growth and development are frequently challenged by abiotic stresses such as extreme temperature fluctuations and biotic stresses like anthracnose disease. While several genes involved in regulating these processes have been well-characterized, research on the role of *LOG* genes, which are critical for CK activity in plants, remains limited in walnut. Therefore, this study aims to identify the *LOG* gene family members in walnut on a genome-wide scale and systematically analyze their expression profiles, providing fundamental data for further investigation into their functional roles in walnut.

2 Materials and Methods

2.1 Identification of LOG Genes in the Walnut Genome

LOG protein sequences from Arabidopsis were downloaded from the TAIR database and utilized as query sequences to conduct a BLAST search against the protein-coding genes in the walnut genome sequenced by Huang Xuehui's Lab (http://www.xhhuanglab.cn/ (accessed on 20 November

2024)), with an E-value threshold set at 1E−5. The obtained sequences were further examined using Pfam (http://pfam-legacy.xfam.org/ (accessed on 20 November 2024)) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (accessed on 20 November 2024)) to verify the presence of specific domains. The identified candidate genes were renamed according to their chromosomal locations. The physicochemical properties of these walnut LOG (JrLOG) proteins were examined online using the ExPASy (https://www.expasy.org/ (accessed on 20 November 2024)).

2.2 Phylogenetic Analysis and Chromosomal Localization

The LOG protein sequences from rice, Arabidopsis, and walnut were used to perform a multiple sequence alignment analysis. The resulting alignment was used to construct a phylogenetic tree using MEGA7.0 software, employing the Neighbor-Joining (N-J) statistical method with 1000 bootstrap replicates and the Gamma distribution model. Based on genome annotation data, the chromosomal locations of each *JrLOG* gene were visualized using TBtools software.

2.3 Gene Structure and Conserved Domain Analysis

The gene structures of *JrLOG* genes, including coding sequences and introns, were visualized using the GSDS online tool (https://gsds.cgrpoee.top/ (accessed on 20 November 2024)). Conserved motifs in the JrLOG proteins were detected using the MEME suite (https://meme-suite.org/meme/ (accessed on 20 November 2024)) (Table S1).

2.4 Gene Collinearity Analysis

Collinearity relationships among walnut chromosomes, as well as between walnut, Arabidopsis, and rice, were analyzed using the MCScanX program. Gene duplication events for *JrLOG* in walnut were visualized with Advanced Circos. The nonsynonymous (Ka) and synonymous (Ks) substitution rates, along with their ratio (Ka/Ks), were calculated using the KaKs Calculator software (Table S2).

2.5 Promoter Cis-Regulatory Element Analysis

The 1500 bp upstream sequences from the start codon of each *JrLOG* gene were extracted from the walnut genome and submitted to the PlantCARE database (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (accessed on 20 November 2024)) for the prediction of *cis*-regulatory elements. The number and distribution of these elements were visualized after statistical analysis.

2.6 Plant Materials and Treatments

Two-year-old, uniformly growing *Juglans regia* ‘Lvling’ walnut trees were used as experimental materials and randomly divided into two groups. For cold treatments, one group was placed in a growth chamber at 4°C, while the control group was kept at 25°C. Walnut leaves were collected at 0.5, 1, and 2 h post-treatment, immediately frozen in liquid nitrogen and stored at −80°C.

2.7 Gene Expression Analysis

The expression profiles of *JrLOG* genes in various walnut tissues were based on RNA-Seq data from 19 different tissues [23]. The expression patterns of *JrLOG* in walnut endopleura during different developmental stages were derived from transcriptomic data covering 35 to 147 days post-flowering [24]. The expression levels of *JrLOG* in response to *Colletotrichum gloeosporioides* infection were analyzed using published RNA-Seq data [25]. The response of *JrLOG* to cold stress was further explored using quantitative real-time PCR (qPCR) following the protocol described by Zheng et al. [26]. The primers used are listed in Table S1.

2.8 Gene Cloning, Sequence Analysis, and Subcellular Localization

The coding sequence of *JrLOG3* was cloned from walnut leaf cDNA following the method outlined by Jia et al. [27]. The predicted amino acid sequence was employed for multiple sequence alignment and phylogenetic analysis, as described in Section 2.2, by comparing it with LOG proteins from different species with known functions. The *JrLOG3* coding region was inserted into the pCambia2300 vector, creating a JrLOG3-EGFP expression cassette driven by the *35S* constitutive promoter. The recombinant vector was then introduced into tobacco (*Nicotiana benthamiana*) epidermal cells via *Agrobacterium*-mediated transformation. After 3 days of dark incubation, the transformed tobacco cells were examined for green fluorescent protein (GFP) signals using confocal microscopy, following the previously described procedure [26].

3 Results

3.1 Identification of Walnut LOG Genes

Nine *AtLOG* genes were identified and documented in the *Arabidopsis thaliana* genome. BLASTP searches were performed against the walnut genome protein database to discover *LOG* genes using the nine AtLOG protein sequences. Seventeen potential *JrLOG* genes were identified by manual inspection and verification using the NCBI conserved domain database (Table 1). These *JrLOG* genes were designated sequentially based on their chromosomal locations (*JrLOG01–JrLOG17*). The 17 *JrLOG* genes were distributed across 11 chromosomes of the walnut genome, with chromosome 13 containing the highest number of *LOG* genes (3), while chromosomes 8, 9, 10, and 14 each contained two genes. Chromosomes 5, 6, 7, 12, 15, and 16 each housed a single *JrLOG* gene (Table 1).

The relationships among the *LOG* genes were elucidated by constructing a neighbor-joining tree based on the LOG protein sequences from Arabidopsis, rice, and walnut. According to the phylogenetic tree (Fig. 1), these proteins were classified into six groups, labeled Group I to Group VI, with all walnut LOG proteins clustering into Groups I to V.

3.2 Features and Structural Analysis of JrLOG Genes

The lengths of *JrLOG* genes ranged from 1326 to 4317 bp. All members of the *JrLOG* gene family contained 4 to 6 introns. Specifically, *JrLOG10* had 4 introns, *JrLOG4* contained 5, while the remaining members had 6 introns. The number and distribution of introns were relatively conserved within each phylogenetic cluster (Fig. 2A,B). For instance, the introns in Group I genes were notably short, while those in Group IV were much longer. This conservation of gene structure within evolutionary clades suggested that these genes have been subjected to similar evolutionary pressures.

Physicochemical property analysis of the JrLOG proteins revealed that their amino acid lengths ranged from 157 to 302, with estimated molecular weights between 17.13 and 33.15 kDa. The theoretical isoelectric points (pI) ranged from 5.04 to 8.61, encompassing both acidic and basic values. The instability index of these proteins varied between 30 and 50, with more than half of the members exhibiting an index greater than 40, indicating that JrLOG proteins were generally unstable (Table 1).

MEME analysis indicated that, except JrLOG10 and JrLOG4, all members possessed three characteristic motifs. JrLOG4 contains two specific structural domains, while JrLOG10 had only one (Fig. 2C). Notably, protein domain analysis further revealed that all members contained a conserved PpnN domain, a core component of nucleotide monophosphate nucleosidases (Fig. 2D). Multiple sequence alignment confirmed that the amino acid sequences within these three motifs were highly conserved, except in JrLOG10 and JrLOG4 (Fig. 2E).

Table 1: Information of *JrLOG* family members

Name		Gene position					Protein information						
	Gene ID	Chr	Start	End	Strand	Length	Amino acid	MW	pI	Instability index	Aliphatic index	Aromaticity	Gravy
JrLOG01	JreChr05G12746	chr5	7297663	7299170	–	1508	229	25.24	6.31	41.18	94.89	0.066	−0.106
JrLOG02	JreChr06G11008	chr6	24200507	24201997	+	1491	237	26.26	8.52	36.72	96.2	0.076	−0.027
JrLOG03	JreChr07G11437	chr7	28747076	28749574	–	2499	225	24.56	6.52	39.18	92.71	0.067	−0.108
JrLOG04	JreChr08G12069	chr8	7120184	7122708	+	2525	157	17.13	8.64	47.47	91.91	0.051	−0.175
JrLOG05	JreChr08G12153	chr8	7778521	7779890	+	1370	219	24.03	5.98	31.98	95.21	0.064	−0.105
JrLOG06	JreChr09G10522	chr9	1601921	1606237	–	4317	210	23.33	5.8	43.08	94.67	0.076	−0.101
JrLOG07	JreChr09G11108	chr9	25953735	25957901	–	4167	218	23.85	5.9	42.62	90.73	0.069	−0.178
JrLOG08	JreChr10G10240	chr10	1284455	1288137	–	3683	218	24.08	5.25	39.4	95.64	0.073	−0.150
JrLOG09	JreChr10G11059	chr10	26869578	26873837	+	4260	215	23.73	5.9	40.5	92	0.070	−0.200
JrLOG10	JreChr12G11464	chr12	25096242	25098492	+	2251	301	33.15	6.61	46.23	85.88	0.093	−0.216
JrLOG11	JreChr13G10245	chr13	13029608	13033021	–	3414	212	23.29	6.32	50.08	92.88	0.075	−0.126
JrLOG12	JreChr13G11278	chr13	27854449	27856912	–	2464	200	21.90	5.12	44.15	97	0.070	0.077
JrLOG13	JreChr13G11675	chr13	542565	544018	+	1454	216	23.57	6.1	30.83	93.01	0.074	−0.097
JrLOG14	JreChr14G11040	chr14	24497953	24501646	–	3694	218	23.82	5.04	41.51	95.69	0.073	0.036
JrLOG15	JreChr14G11110	chr14	288907	290232	+	1326	206	22.36	5.83	40.21	93.74	0.063	−0.075
JrLOG16	JreChr15G11818	chr15	4206082	4208432	–	2351	235	25.82	5.82	30.06	88.34	0.081	−0.107
JrLOG17	JreChr16G10096	chr16	1218274	1220439	–	2166	208	22.99	5.88	35.46	94.66	0.077	−0.002

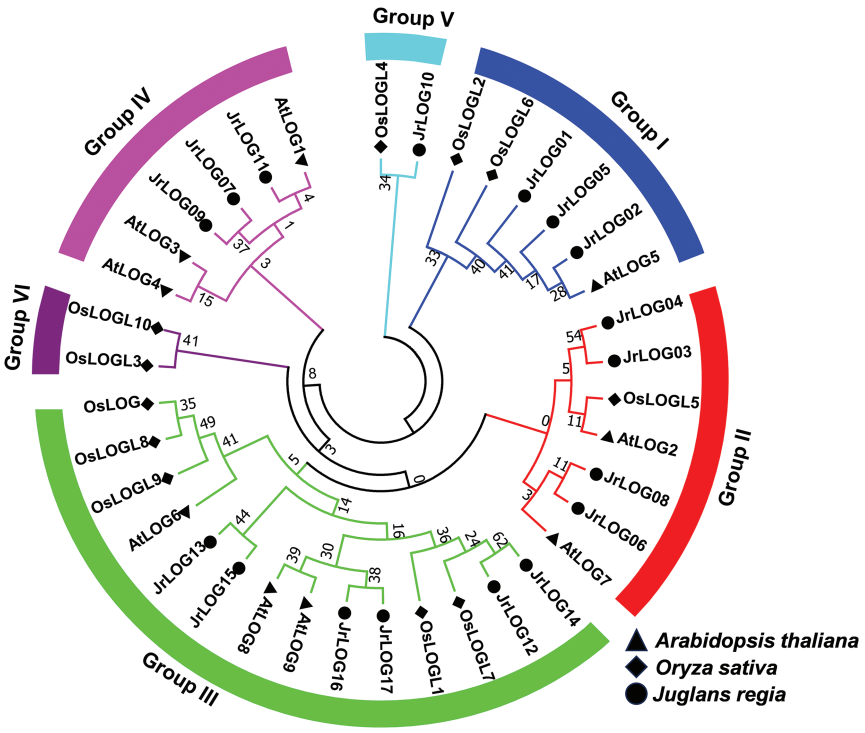

Figure 1: Neighbor-joining tree among *LOG* genes from walnut, rice, and Arabidopsis

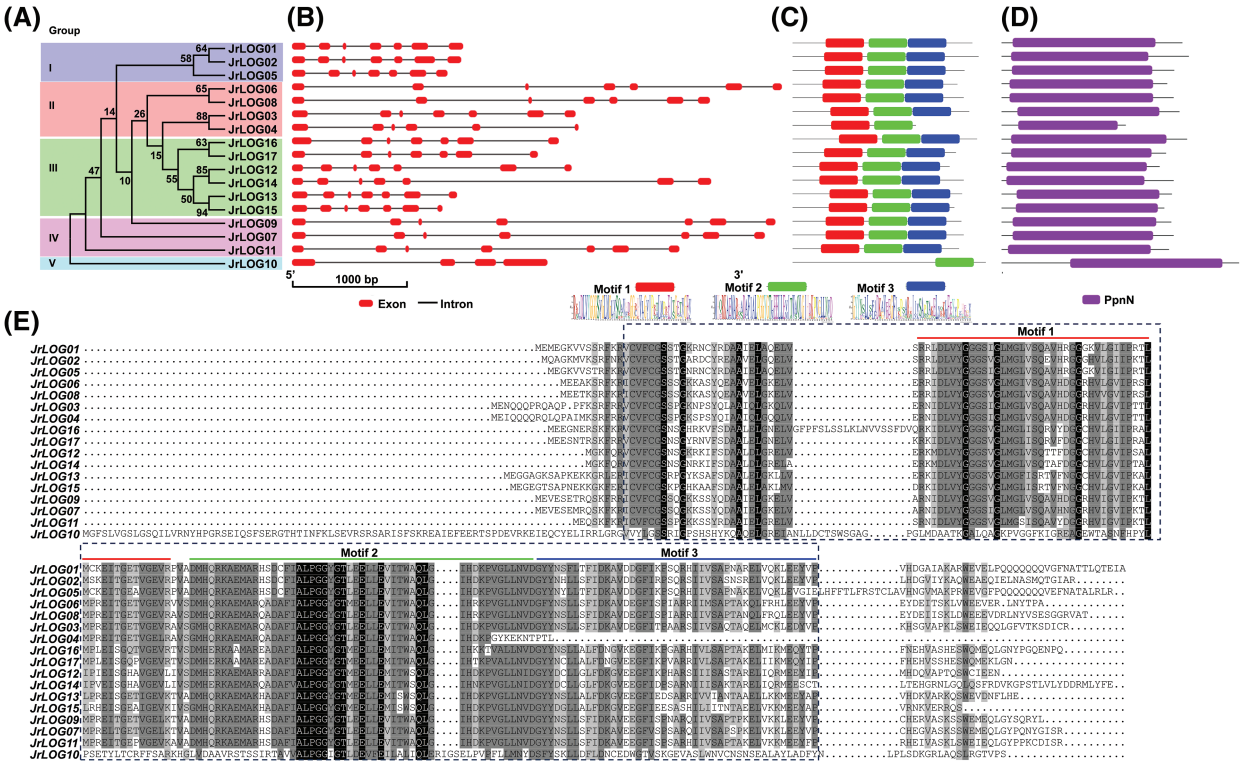

Figure 2: Phylogenetic relationship, gene, and protein features analysis of the JrLOG. (A) Neighbor-joining tree among LOG members from walnut. (B) and (C) show the protein feature motif and conserved domain, respectively. (D) Gene structure. (E) Protein multiple sequence alignment

3.3 Collinearity Analysis

Gene collinearity analysis was performed to uncover the conservation and evolutionary patterns of gene family members across different species. Although *JrLOG* members were distributed across various chromosomes in differing numbers (Fig. 3A), nine pairs of collinear genes were detected among *JrLOG* members in walnut (Fig. 3B). Except for *JrLOG10*, all other members exhibited collinear relationships. Notably, these collinear genes were not located on the same chromosome. Additionally, collinearity relationships were observed among the three Group I members, *JrLOG01*, *JrLOG02*, and *JrLOG05*.

Thirteen pairs of collinear *LOG* genes were identified between walnut and Arabidopsis (Fig. 3C), while only seven collinear pairs were detected between walnut and rice (Fig. 3D). This suggests that *LOG* genes in walnut share a closer evolutionary relationship with those in Arabidopsis than with rice.

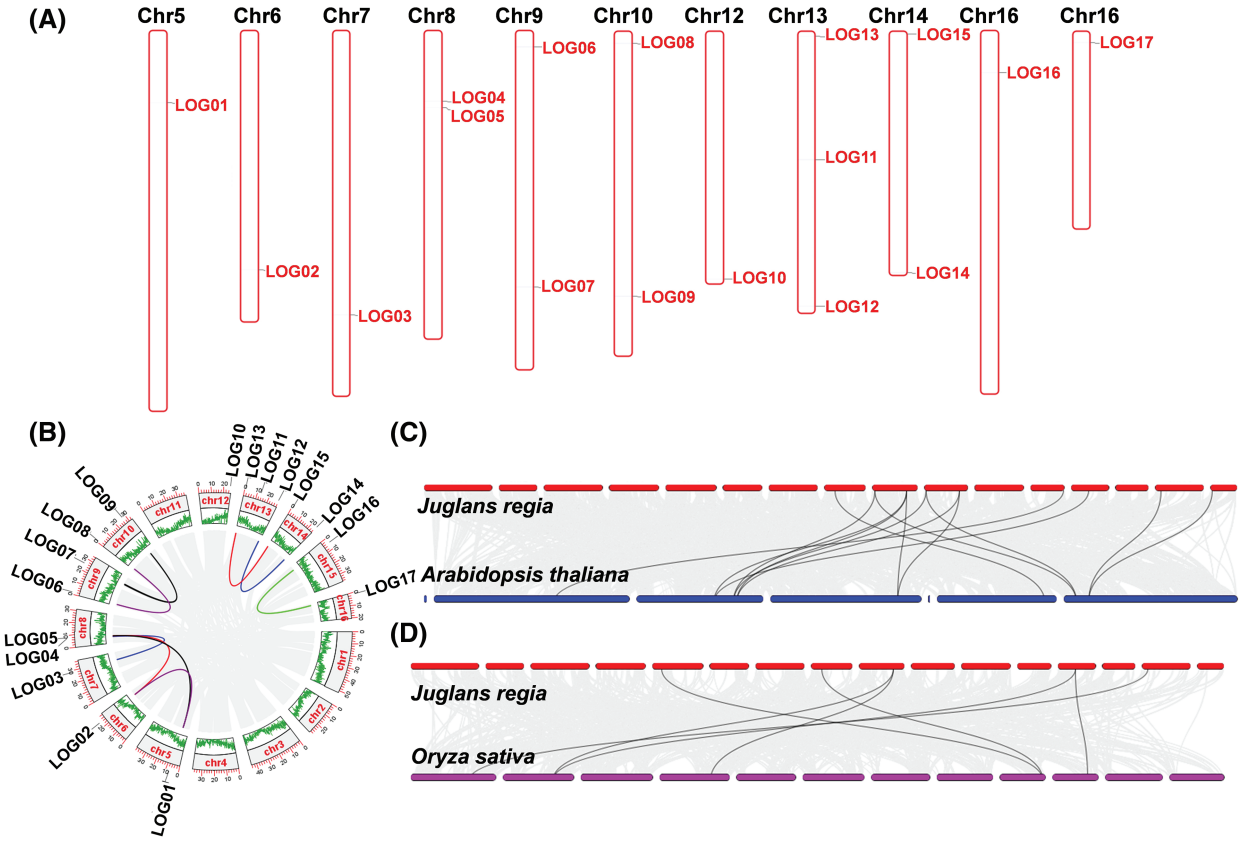

Figure 3: Chromosome localization and collinearity analysis of *LOG* genes. (A) Diagram showing the physical location of *JrLOG* genes on walnut chromosomes. (B) Collinearity analysis among *JrLOG* gene members in walnut. (C) Collinearity analysis of *LOG* genes between walnut and Arabidopsis. (D) Collinearity analysis of *LOG* genes between walnut and rice

3.4 Cis-Regulatory Element Analysis of the Promoter Region

Cis-regulatory elements (*CREs*) play a critical role in understanding gene regulation, phenotype expression, and plant adaptability. To gain insights into the regulatory mechanisms of *JrLOG* genes, we analyzed the potential *CREs* in their promoter regions (Fig. 4).

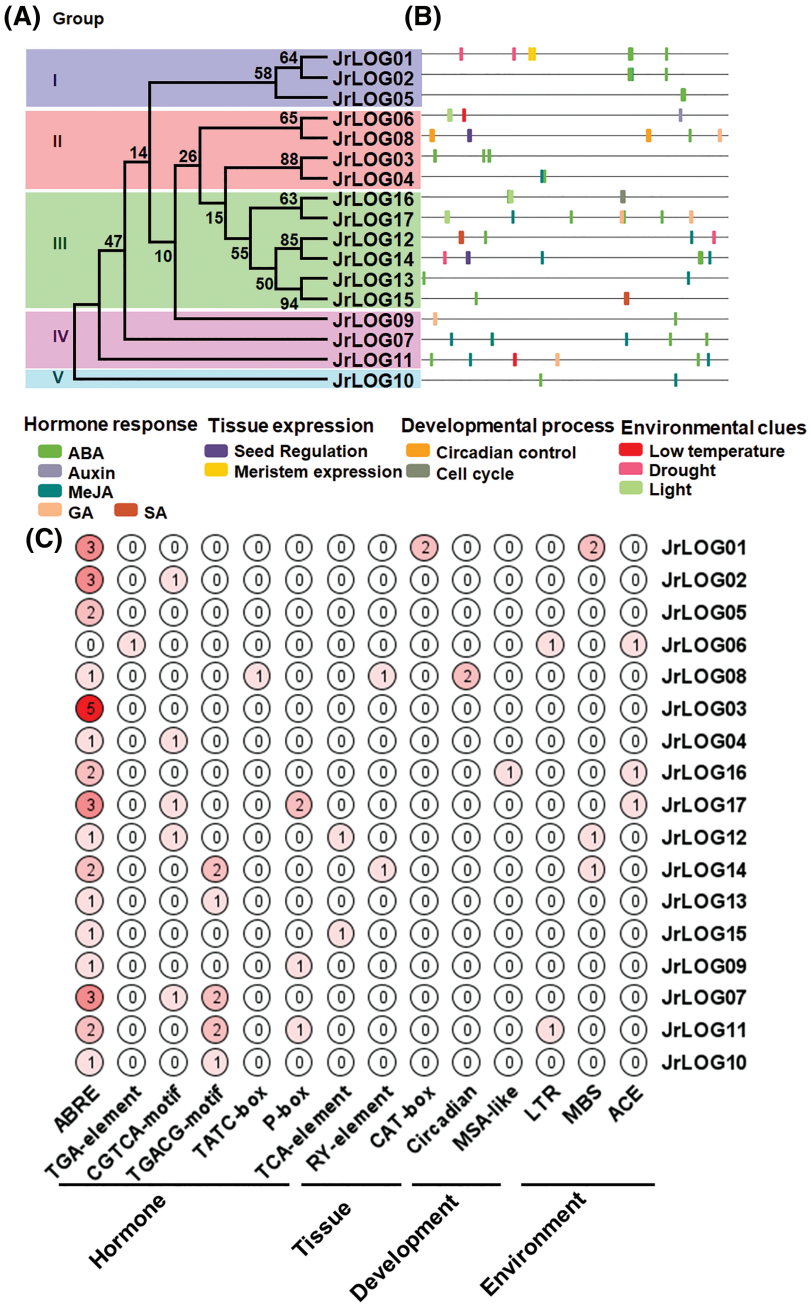

Figure 4: Predicted *cis*-regulatory elements (*CREs*) within the *JrLOG* promoters. (A) and (B) illustrate the clades of *LOG* family members and the distribution of diverse *CREs* within their promoters, represented by various colors in the graph. (C) presents a heatmap displaying the number of different *CREs* elements within different *JrLOG* promoters. ABRE, ABA-responsive element; TGA-element, auxin-responsive element; CGTCA-motif/TGACG-motif, MeJA-responsiveness; TATC-box/P-box, gibberellin-responsiveness; TCA-element, Salicylic acid responsiveness; RY-element, Seed-specific regulation; CAT-box, Meristem expression; Circadian, Circadian control; MSA-like, Cell cycle; LTR, Low-temperature responsiveness; MBS, Drought-inducibility; ACE, Light responsiveness

Several elements associated with plant hormone responses were identified, including those responsive to ABA (ABRE), auxin (TGA-element), MeJA (TGACG-motif and CGTCA-motif), GA (P-box and TATC-box), and SA (TCA-element), which were present in many members of the gene family. Notably, all members, except *JrLOG6*, contained ABA-responsive elements, with *JrLOG3* exhibiting the highest number of ABRE motifs. Interestingly, auxin-responsive elements were found exclusively in the *JrLOG6* promoter. Additionally, tissue-specific expression elements were identified, such as seed-specific expression elements (RY-element) in *JrLOG08* and *JrLOG14*, and meristem-specific elements (CAT-box) in *JrLOG01*. *CREs* related to developmental processes were also detected, including two circadian control elements in *JrLOG08* and cell cycle-related MSA-like elements in *JrLOG16*. Moreover, several *CREs* associated with environmental responses were predicted, such as those related to low temperature (LTR), drought (MBS), and light response (ACE-element). Interestingly, the distribution of *CREs* was not conserved among members of the same evolutionary clade, suggesting that *JrLOG* genes may be involved in a diverse array of biological processes.

3.5 Tissue-Specific and Developmental Stage Expression Analysis of JrLOG Genes

Tissue-specific expression analysis (Fig. 5A) revealed that *JrLOG3*, *JrLOG10*, *JrLOG11*, and *JrLOG16* were highly expressed in the leaf mature (LM), leaves (LE), and leaf young (LY). *JrLOG04* exhibited notably high expression in the pistillate flower (FL), while *JrLOG07* showed strong expression exclusively in vegetative buds (VB). *JrLOG01* and *JrLOG09* were highly expressed in hull dehiscing (HU) and hull peel (HP), respectively, whereas *JrLOG13* had high expression in hull immature (HL). Additionally, *JrLOG08* and *JrLOG17* were strongly expressed in catkins (CK). *JrLOG12* was the only gene with high expression in both the callus exterior (CE) and callus interior (CI). *JrLOG02* and *JrLOG06* exhibited elevated expression levels in somatic embryo (SE), while *JrLOG05* and *JrLOG14* were specifically highly expressed in roots (RT).

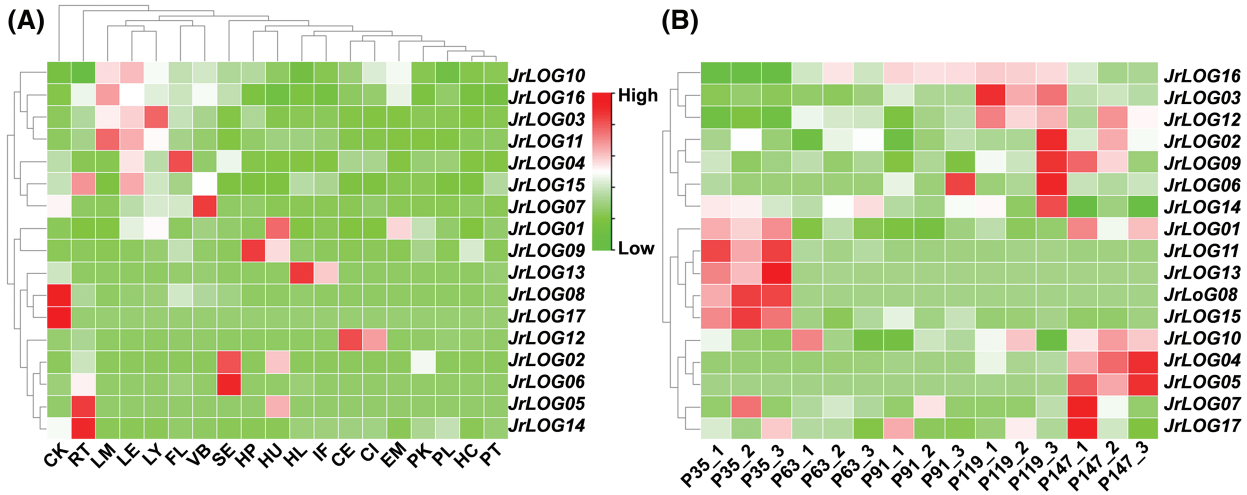

Figure 5: Expression characteristics of *JrLOG* gene in different tissues and development stage. (A) *JrTLP* expression profiles in different tissues. CE, callus exterior; CI, callus interior; CK, catkins; EM, embryo; FL, pistil late flower; HC, hull cortex; HL, hull immature; HP, hull peel; HU, hull dehiscing; IF, fruit immature; LE, leaves; LM, leaf mature; LY, leaf young; PK, packing tissue mature; PL, pellicle; PT, packing tissue immature; RT, root; SE, somatic embryo; VB, vegetative bud. (B) *JrTLP* expression profiles during the developmental stages of walnut endopleura. The heat map was generated based on the transcriptomic data

The walnut endopleura stores nutrients, protects the seed, and influences germination. Therefore, the expression profiles of *JrLOG* genes during seed endopleura development were also analyzed (Fig. 5B). *JrLOG01*, *JrLOG08*, *JrLOG11*, *JrLOG13*, and *JrLOG15* displayed high expression during the early stages of endopleura development, whereas the remaining members exhibited increased expression closer to fruit maturation. Notably, *JrLOG3* was significantly upregulated after the fruit hardening stage.

3.6 JrLOG Genes Expression in Responses to Anthracnose Infection

Upon inoculation with anthracnose, the expression levels of *JrLOG* genes fluctuated noticeably (Fig. 6). *JrLOG05* was upregulated in genotype F423 genotype only 48 h post-infection, whereas, in the F26 genotype, its upregulation was delayed until 72 h and showed only a slight increase. In contrast, *JrLOG10* in F26 exhibited two distinct expression peaks at 48 and 120 h post-infection, whereas in F423, it exhibited a consistently low level of expression. Additionally, *JrLOG17* in F26 exhibited significant induction at both 24 and 48 h post-infection compared to F423. Notably, despite a general downregulation in transcription, *JrLOG3* maintained relatively high expression in F26 at 24 h post-infection compared to its expression in F423. These findings suggest potential differences in the stress response mechanisms between the two genotypes.

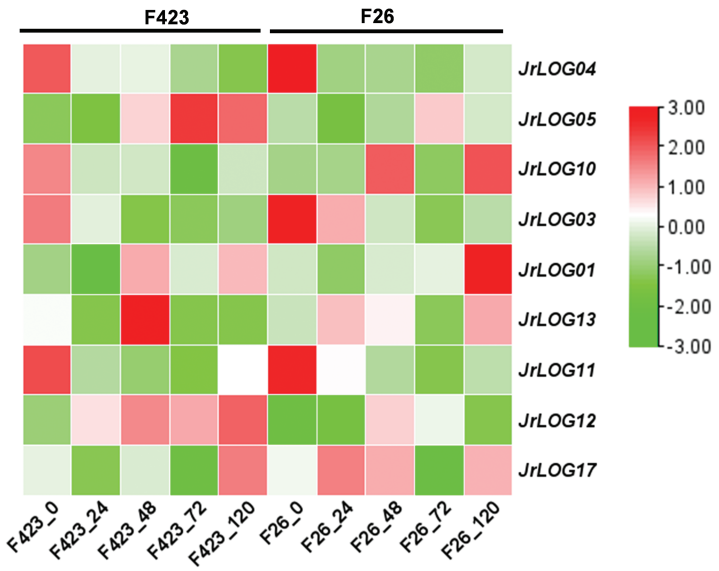

Figure 6: *JrLOG* gene expression profiles in F26 and the F423 fruits in response to anthracnose infection. The heat map was generated based on the transcriptomic data

3.7 JrLOG Genes Expression in Responses to Cold Stress

Under cold stress, the expression of *JrLOG* gene family members exhibited substantial variations (Fig. 7). *JrLOG5*, *JrLOG8*, *JrLOG14*, and *JrLOG15* appeared to be unresponsive to cold treatment, while *JrLOG6*, *JrLOG12*, and *JrLOG13* showed a general tendency towards downregulation in their expression levels after cold treatment, despite also experiencing fluctuations. Additionally, some genes displayed fluctuating expression patterns without any specific trend, including *JrLOG6*, *JrLOG9*, *JrLOG10*, *JrLOG16*, and *JrLOG17*. No significant differences in transcription levels were detected for *JrLOG1*, *JrLOG2*, *JrLOG4*, and *JrLOG7* at 0.5 h and 1 h post-cold treatment; however, significant induction in expression was observed at 2 h. Notably, *JrLOG3* and *JrLOG11* exhibited strong transcriptional activation as early as 0.5 h post-cold treatment and were induced at all sampling time points.

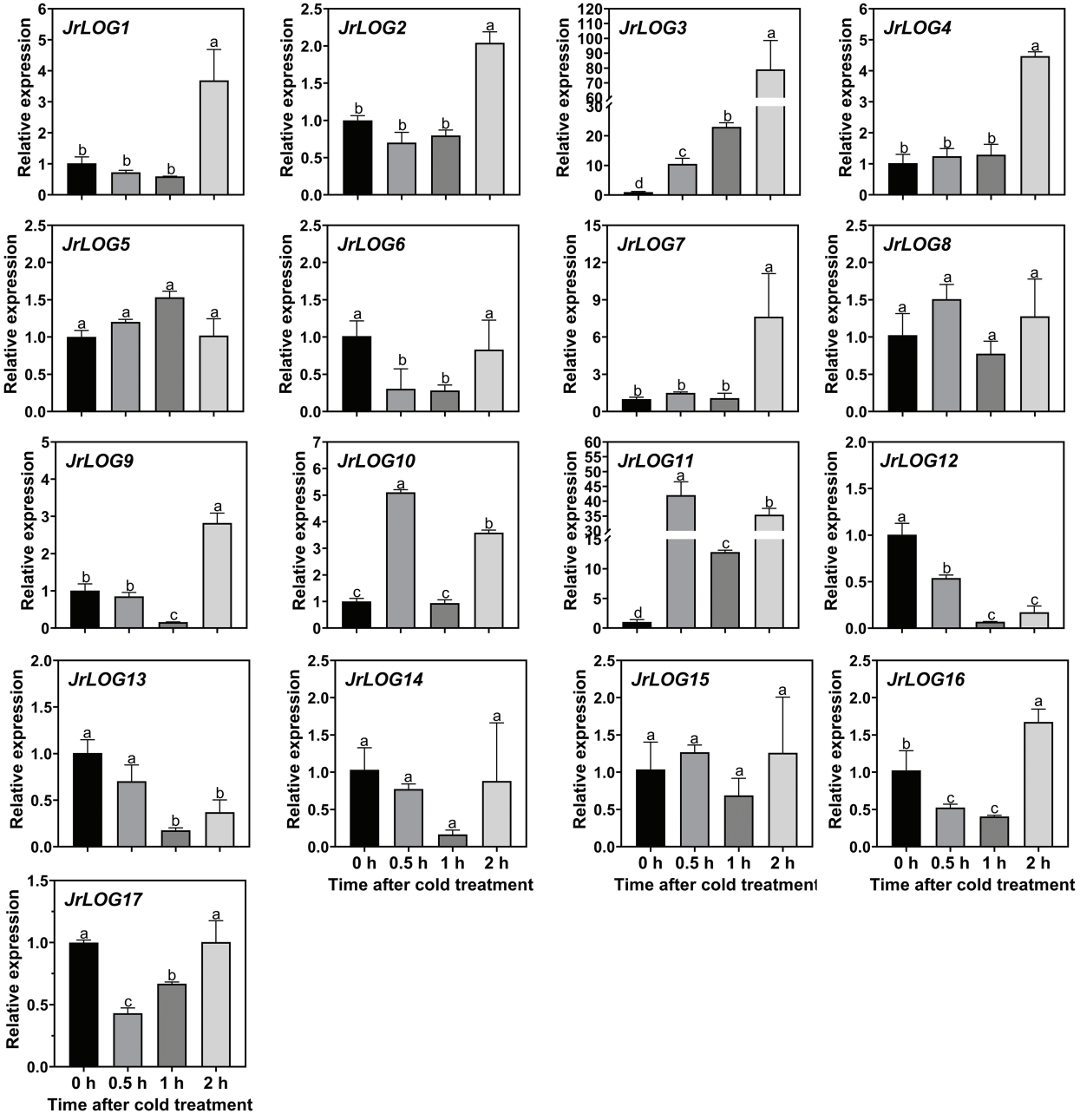

Figure 7: *JrLOG* gene expression in response to cold stress. Samples were collected at 0, 0.5, 1, and 2 h after cold at 4°C. Each value represents the mean ± SD of three replicates. Different letters mean significant difference at the 0.05 level

3.8 Gene Cloning and Subcellular Localization of JrLOG3

Using leaf tissue as the material, the expected band size for *JrLOG3* was successfully amplified through via transcription PCR (Fig. 8A). Sequencing confirmed that the coding region of *JrLOG3* is 678 bp in length, encoding a 225-amino-acid protein with a predicted molecular weight of 25.4 kDa. Phylogenetic analysis indicated that JrLOG3 was closely related to LOG proteins from other species, notably GhLOG3 from

cotton and SlycLOG1a from tomato (Fig. 8B). Multiple sequence alignment demonstrated that *JrLOG3* shares a high degree of conservation with these LOG proteins, including the catalytic sequence 'PGGxGTxE', a key feature situated at the core of the protein's three-dimensional structure. This catalytic motif was entirely conserved across all analyzed proteins (Fig. 8C,D). Subcellular localization analysis revealed that GFP fluorescence driven by JrLOG3 was exclusively expressed in the cell membrane (Fig. 8E).

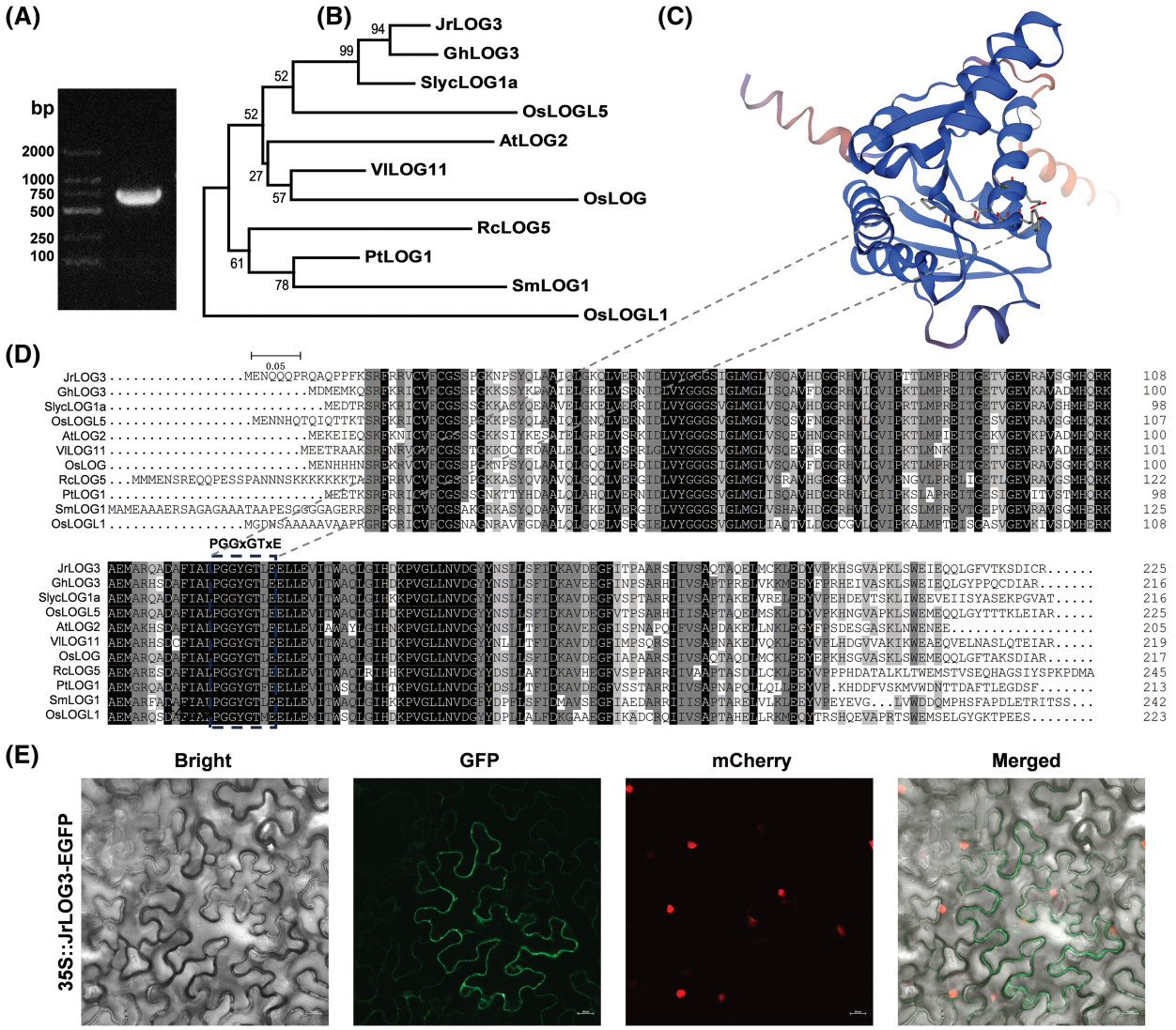

Figure 8: Gene cloning and analysis of *JrLOG3*. (A) Cloning of the *JrLOG1* gene, with agarose gel electrophoresis showing the position of the target band. (B) Phylogenetic analysis of JrLOG3 concerning functionally characterized LOG proteins across diverse species. (C) Three-dimensional structure prediction of JrLOG3 protein. (D) Multiple sequence alignment analysis of JrLOG3 about functionally characterized LOG proteins across diverse species. (E) Subcellular localization analysis of JrLOG3 in tobacco epidermal cells. Scale bar, 20 μm

4 Discussion

4.1 Identification of JrLOG Genes

LOG genes have been identified in several plant species; however, the *LOG* gene family in walnut (*Juglans regia*), an economically important woody oil tree species, has not been systematically explored. In this study, we identified 17 *LOG*-encoding genes in the walnut genome through homology-based comparisons. The *JrLOG* members were categorized into five subgroups using phylogenetic analysis (Fig. 1 and Fig. 2A). It appears that these genes have undergone duplication events (Fig. 3), likely associated with a whole-genome duplication approximately 20 million years ago [28]. Consequently, As a result, *JrLOG* genes are located on several chromosomes, comprising nine pairs of syntenic genes (Fig. 3B). Similarly, the expansion of the *RcLOG* gene family in castor bean (*Ricinus communis*) was driven by gene duplication events [19]. In walnut, this expansion was accompanied by purifying selection, as indicated by the Ka/Ks ratios of all syntenic gene pairs being much less than 1 (Table S2). Closely grouped *JrLOG* genes display similar gene architectures, amino acid sequences, conserved motifs, and domain characteristics (Fig. 2). Nonetheless, *JrLOG10* lacks synteny with other members, suggesting it may have evolved as an independent lineage, leading to the loss of at least three exons and the absence of two characteristic motifs.

Moreover, closely related members, such as *JrLOG3* and *JrLOG4* (Fig. 2A), although forming a pair of syntenic genes (Fig. 3B), exhibited exon loss during gene duplication (Fig. 2B). This loss resulted in *JrLOG4* lacking a C-terminal motif (Fig. 2C) and retaining only a diminished PpnN domain (Fig. 2D). Similarly, some members of the *KNOX* gene family have evolved without the C-terminal domain, forming distinct clades with functions similar to those of other members [29]. Notably, even closely related *JrLOG* genes displayed variation in the number and types of *CREs* in their promoters (Fig. 4), potentially explaining the balance between conservation and variability within the *JrLOG* family. Thirteen pairs of syntenic *LOG* genes were identified between walnut and Arabidopsis (Fig. 3C), whereas only seven pairs were found between walnut and rice (Fig. 3D), likely due to the closer evolutionary relationship among dicotyledonous plants compared to that between dicots and monocots.

4.2 Expression Analysis of JrLOG Genes

The *LOG* gene family plays a critical role in plant growth and development [12]. Thus, we systematically characterized the expression profiles of the *JrLOG* genes across various tissues and developmental stages. The abundance of *JrLOG3, JrLOG10, JrLOG11*, and *JrLOG16* at various phases of leaf development indicates that these genes are essential for this process. Their expression may enhance endogenous CK levels, which could improve leaf size and regulate plant architecture [30]. Its possible function in floral development and reproduction was indicated by the specific and strong expression of *JrLOG04* in pistillate flowers, an organ that is essential for walnut output. These results are in agreement with those from Arabidopsis, whose *LOG* genes control the development of floral meristems and are expressed in flowers [12]. In rice, *LOG1* influences CK levels in young panicles, which modulates grain yield [11].

Seed coats, essential for seed germination, respond positively to exogenous CK treatments [31]. The high expression of *JrLOG1*, *JrLOG9*, and *JrLOG13* in the hull suggests their possible involvement in hormone regulation related to seed germination [32]. CK application has been shown to enhance shoot regeneration efficiency in peanuts (*Arachis hypogaea* L.) [33] and to support callus growth by elevating endogenous CK levels [34]. *JrLOG12* was specifically expressed in callus tissue, implicating it in tissue regeneration and cellular reprogramming [35]. Additionally, in *Medicago truncatula*, *MtLOG1* and *MtLOG2* regulate lateral root formation [36], raising the possibility that the root-specific expression of *JrLOG5* and *JrLOG14* in walnut may serve a similar function.

CKs also play a critical role in plant immunity against pathogens. For instance, infection by *Verticillium longisporum* alters CK levels, while in legumes, CK responses vary following bacterial inoculation [37]. In disease-resistant walnut germplasm, *JrLOG3* maintained high expression 24 h after pathogen infection, suggesting its potential role in walnut disease resistance (Fig. 6). Under cold stress, *JrLOG3* and *JrLOG11* were upregulated, with *JrLOG03* exhibiting rapid and continuous activation (Fig. 7). Conversely, several *JrLOG* genes were downregulated and displayed fluctuating expression patterns under cold stress, possibly as a strategy to slow growth in adverse conditions [38]. Given the significant expression of *JrLOG3* in leaves and its responsiveness to both biotic and abiotic stresses, we cloned and analyzed this gene further. The cloned *JrLOG3* is closely related to *GhLOG3* from cotton (Fig. 8B), which induces adversity stress resistance [20]. While transient and stable expression of LOG fusion proteins in rice, Arabidopsis, and *Chlamydomonas reinhardtii* indicated localization in the cytoplasm and nucleus [9], *JrLOG3* exhibited subcellular localization to the cell membrane (Fig. 8E). Similar to other LOG proteins, JrLOG3 contains the conserved "PGGxGTxxE" sequence, which contributes to AMP stability, indicating a shared catalytic mechanism. Consequently, *JrLOG3* is a strong candidate for further investigation into its role in cytokinin regulation, organ development (e.g., leaf expansion), and stress tolerance.

5 Conclusions

This study identified 17 *JrLOG* genes from the walnut (*Juglans regia*) genome through bioinformatics analysis and categorized them into five distinct groups. The gene structures, amino acid sequences, unique motifs, and conserved domains demonstrated considerable conservation within each group. Across multiple tissues and developmental stages, as well as in response to various biotic and abiotic stressors, the *JrLOG* genes displayed distinct yet varied expression patterns. *JrLOG3* was localized to the cell membrane, demonstrated elevated expression in leaves, and reacted to both abiotic and biotic stresses. These findings establish a robust basis for the continued investigation of *JrLOG* genes and underscore potential candidates for genetic modification to improve stress tolerance in walnut.

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