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Genome-Wide Identification and Expression Analysis of the Walnut C-Repeat Binding Factor Gene Family under Low-Temperature Stress

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Abstract: The walnut (*Juglans regia*) is a nut with a high nutritional value and has been recognized throughout the world as an economically important woody plant. However, the walnut is vulnerable to abiotic stresses, especially low-temperature freezes, which can severely impede their growth and development, resulting in substantial financial losses. The *CBF* (C-repeat binding factor) gene, a unique plant transcription factor classified within the AP2/ERF (ethylene response factor) family, plays a vital role in the process of plants coping with abiotic stress, particularly low temperatures. This study utilized bioinformatics techniques to identify eight *JrCBF* genes within the walnut genome, distributed across six chromosomes. The upstream promoter sequences of these genes are rich in cis-regulatory elements related to hormonal responses and non-biotic stresses. Transcriptome data and qRT-PCR analysis revealed that *JrCBF1* and *JrCBF2* were significantly upregulated under low temperatures. *JrCBF1* and *JrCBF2* also responded positively to high-temperature stress. Under drought stress, *JrCBF5* and *JrCBF8* had a significant difference in their expression relative to other genes. To gain further insights into their functionality, subcellular localization experiments were conducted on *JrCBF1* and *JrCBF2*, confirming their nuclear localization. These results provide valuable insights into the specific functions of *CBF* gene in enhancing the resistance of walnut to abiotic stress.

Keywords: walnut; CBF gene family; abiotic stress; expression analysis

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1. Introduction

Low-temperature stress represents a significant environmental factor that restricts plant growth and production, exerting a profound impact on plant development. This effect is particularly pronounced in temperate regions characterized by frequent temperature fluctuations. In response to this challenging environmental factor, a number of plants native to these regions have developed an adaptive mechanism known as cold acclimation [1]. The process of cold acclimation is inherently intricate, encompassing a diverse array of physiological and biochemical transformations. These transformations include modifications in plant cell membranes [2], alterations in the expression of cold-responsive genes and transcription factors [3–5], adjustments in protein profiles [6,7], proline accumulation [8], and repercussions on photosynthesis [9]. More precisely, these physiological and biochemical changes are orchestrated through the low-temperature-induced expression of pertinent functional genes. Currently, the molecular mechanisms of plant response and stress resistance to cold stress have been comprehensively studied, especially in the model plant *Arabidopsis thaliana*. Among them, the most deeply studied regulatory network was the *ICE1* (inducer of *CBF* expression 1)–*CBF*–*COR* (*cold responsive*) pathway [10–12]. Upon exposure to cold stress, rapid expression of *ICE1* occurs, facilitating the upregulation of the *CBF* genes [13–15]. The CBF protein then binds to the CRT/DRE (C-repeat/dehydration-responsive element) cis-element within the promoter region of the *COR* gene downstream and implements multiple levels of regulation involving transcription, translation, modification, and expression [16–18]. This regulatory process culminates in the synthesis of a range

of anti-freeze substances, such as soluble sugars and proline, which significantly enhances the plant's ability to withstand the cold and maintain normal growth.

The *CBF* gene family constitutes a group of transcription factors containing the AP2/ERF (APETALA2/ethylene responsive) DNA-binding domain. These factors are capable of binding to cold-responsive elements (CRT/DRE elements), also known as low-temperature-responsive elements (LTREs), located in the promoter region of *COR* genes, thereby regulating the transcriptional activity of *COR* genes [19]. In *A. thaliana*, six different members form the *CBF* gene family, namely *CBF1/DREB1B*, *CBF2/DREB1C*, *CBF3/DREB1A*, *CBF4*, *DDF1*, and *DDF2* [20–22]. *CBF1*, *CBF2*, and *CBF3* were the key factors that regulate gene expression during cold acclimation signal transduction, which exhibited a rapid and transient induction of expression when confronted with low-temperature signals, while their expression was generally not significantly induced via dehydration and high-salt signals [23–25]. Conversely, *CBF4* was primarily expressed in response to drought stress and ABA signaling, independent of low-temperature induction. However, overexpressing *AtCBF4* activated downstream genes containing C-repeat/dehydration-responsive elements, which were involved in both cold and drought adaptation, simultaneously enhancing the cold and drought resistance of the transgenic plant [21,26]. *AtDDF1* was associated with salt tolerance [22]. Overexpressing *AtDDF1* actively reduced endogenous gibberellic acid (GA) levels under high-salt stress by inducing *GA 2-oxidase* expression, enabling adaptation to stress conditions but resulting in a dwarf phenotype. Furthermore, overexpressing *AtDDF2* also elicited a similar dwarfing phenotype [27].

Walnut is an economically significant tree species highly susceptible to the adverse effects of cold spells, spring inversions, and late frosts, which can severely disrupt its normal growth, development, and yields. *CBF* transcription factors play a key role in response to low temperature stress and can promote plant adaptation to cold environments. However, few studies have been reported on the *CBF* family of walnut in a variety of adversity stresses. Therefore, this study conducted an in-depth analysis on the whole genome level and successfully identified eight *JrCBF* genes. Our examination covered various facets, including their physicochemical properties, gene structures, molecular characteristics, gene chromosome distribution, phylogenetic relationships, stress expression profiles, and subcellular localization analysis. This study established a solid foundation for a more in-depth understanding of the biological function s of the *JrCBF* genes and their roles in responding to abiotic stress, especially cold stress. At the same time, it also provides valuable insights into the mechanism of cold resistance of walnut trees.

2. Results

2.1. Identification and Protein Characterization of JrCBF Genes

From the walnut genome, a total of eight *JrCBF* genes were identified and designated as *JrCBF1* to *JrCBF8*. Gene sequences are listed in Table S1. The physicochemical properties of the JrCBF proteins were analyzed using the online tool ProtParam. The amino acid lengths of these eight *JrCBF* members ranged from 150 aa (*JrCBF2*) to 266 aa (*JrCBF5* and *JrCBF6*), and the molecular weights of the proteins varied from 16.68 kDa (*JrCBF2*) to 29.46 kDa (*JrCBF8*) (Table 1). Except for the proteins of *JrCBF2* and *JrCBF3*, with theoretical isoelectric points exceeding seven, the other family members exhibited theoretical isoelectric points below seven, indicating that the majority of JrCBF proteins are acidic proteins. The total average protein hydrophobicity values ranged from −0.744 (*JrCBF2*) to −0.331 (*JrCBF6*), all falling below zero, indicating that these proteins are hydrophilic. Notably, all of these proteins exhibited instability coefficients exceeding 50, indicating that they all belonged to unstable proteins. Finally, subcellular localization prediction using YLoc indicated that all JrCBF proteins were localized within the cell nucleus.

To further explore the domain characteristics of *JrCBFs*, we conducted a comparative analysis of the CBF protein sequences from *J. regia*, *A. thaliana*, and *Populus trichocarpa*. This analysis revealed that all of these sequences exhibited AP2 structural domains and motifs typical of the *CBF* family (Figure 1). The striking similarity in these structures across the

three species underscored their remarkably high degree of conservation throughout the evolutionary process.

Table 1. Physicochemical properties of JrCBF proteins in walnut.

Gene Name	Gene ID	Protein Length (aa)	MW (kD)	pI	GRAVY	Instability Index	Predicted Location
JrCBF1	JreChr03G13399	212	23.25	5.26	−0.563	52.72	Nucleus
JrCBF2	JreChr04G12265	150	16.68	10.00	−0.744	59.52	Nucleus
JrCBF3	JreChr09G12450	218	24.00	8.59	−0.477	54.02	Nucleus
JrCBF4	JreChr11G11425	249	27.63	5.53	−0.482	57.99	Nucleus
JrCBF5	JreChr12G11046	253	28.82	5.42	−0.743	56.81	Nucleus
JrCBF6	JreChr13G10824	266	29.15	6.44	−0.331	56.14	Nucleus
JrCBF7	JreChr13G10825	266	29.45	5.82	−0.512	55.73	Nucleus
JrCBF8	JreChr13G10826	265	29.46	6.19	−0.486	60.50	Nucleus

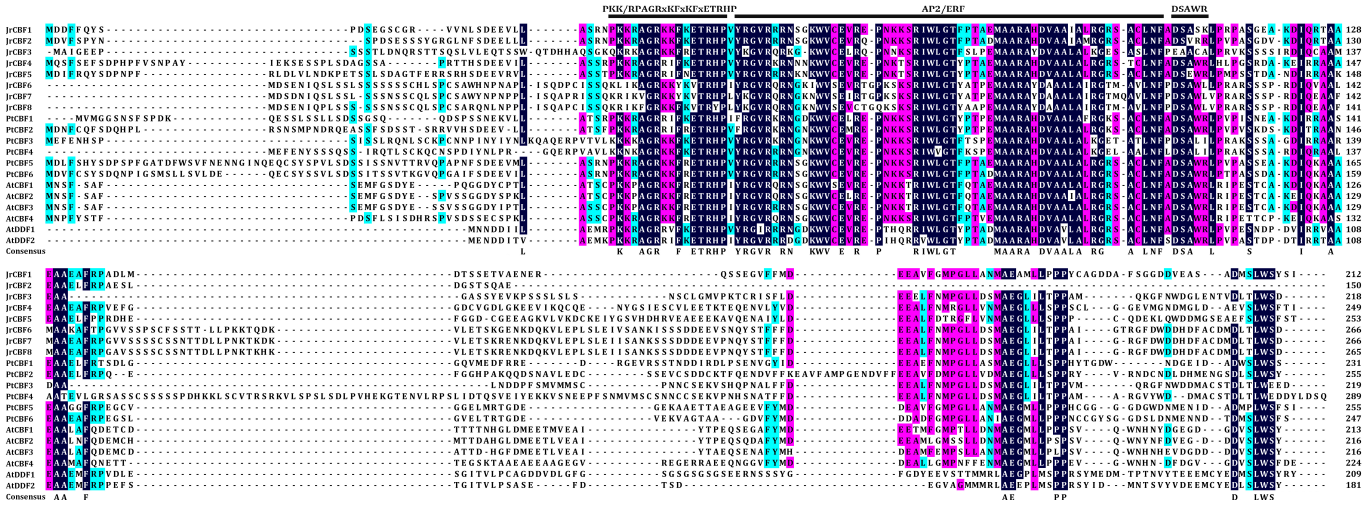

Figure 1. Multiple sequence alignment between JrCBF proteins, PtCBF proteins in *P. trichocarpa*, and AtCBF proteins in *A. thaliana*. Dark blue represents the same amino acid residues, pink represents 80% similarity, and light blue represents 40% similarity.

2.2. Gene Structure and Conserved Motif Analysis of JrCBF Genes

To elucidate the structural characteristics of the *CBF* genes, we analyzed the introns, exons, structural domains, and conserved motifs of the *JrCBF*, *PtCBF*, and *AtCBF* sequences. The CBF proteins were categorized into three subgroups (Figure 2A), revealing shared similarities within each subgroup. Eight *JrCBF* genes exclusively consisted of exons without any introns (Figure 2B). The AP2 structural domain was consistently present in all of these CBF protein sequences (Figure 2C). Conserved motifs among *CBF* family members (*JrCBFs*, *AtCBFs*, and *PtCBFs*) were examined via MEME (Figure S1). All 20 CBF protein sequences featured motif 2, motif 1, and motif 4, indicative of these regions as common, conserved functional motifs shared among *CBF* family proteins (Figure 2D). Motif 7 was notably conserved at the 5′ terminus of 14 CBF proteins. It is important to note that the quantity and arrangement of motifs in other *JrCBF* genes remained largely consistent, with the exception of *JrCBF2*, which lacked motif 3 and motif 5 towards the end. This distinction indicated a difference in *JrCBF2* functionality (Figure 3D).

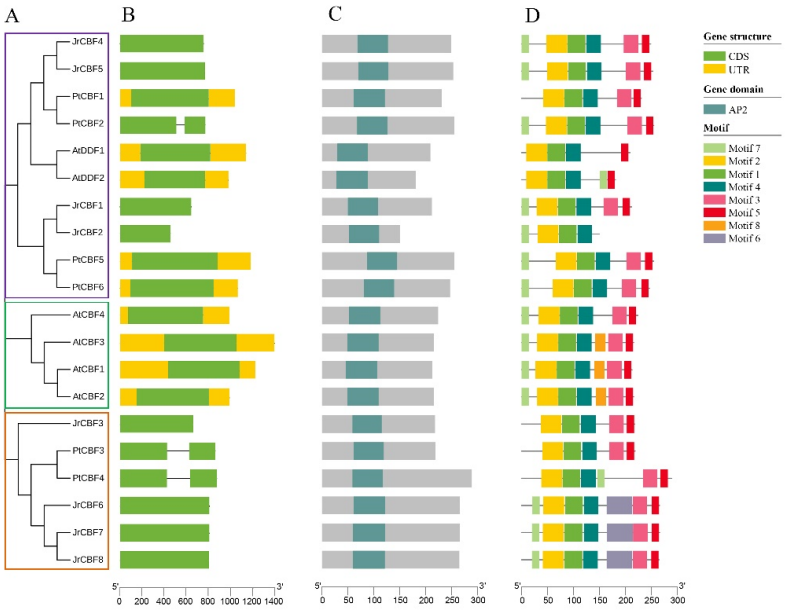

Figure 2. Phylogenetic tree, conserved motif, domain, and gene structure in *JrCBFs*, *AtCBFs*, and *PtCBFs*. (**A**) The phylogenetic tree was constructed using the neighbor-joining method. (**B**) The composition and position of exons and introns in *CBF* genes. (**C**) Domains in CBF proteins. (**D**) Conserved motifs in CBF proteins.

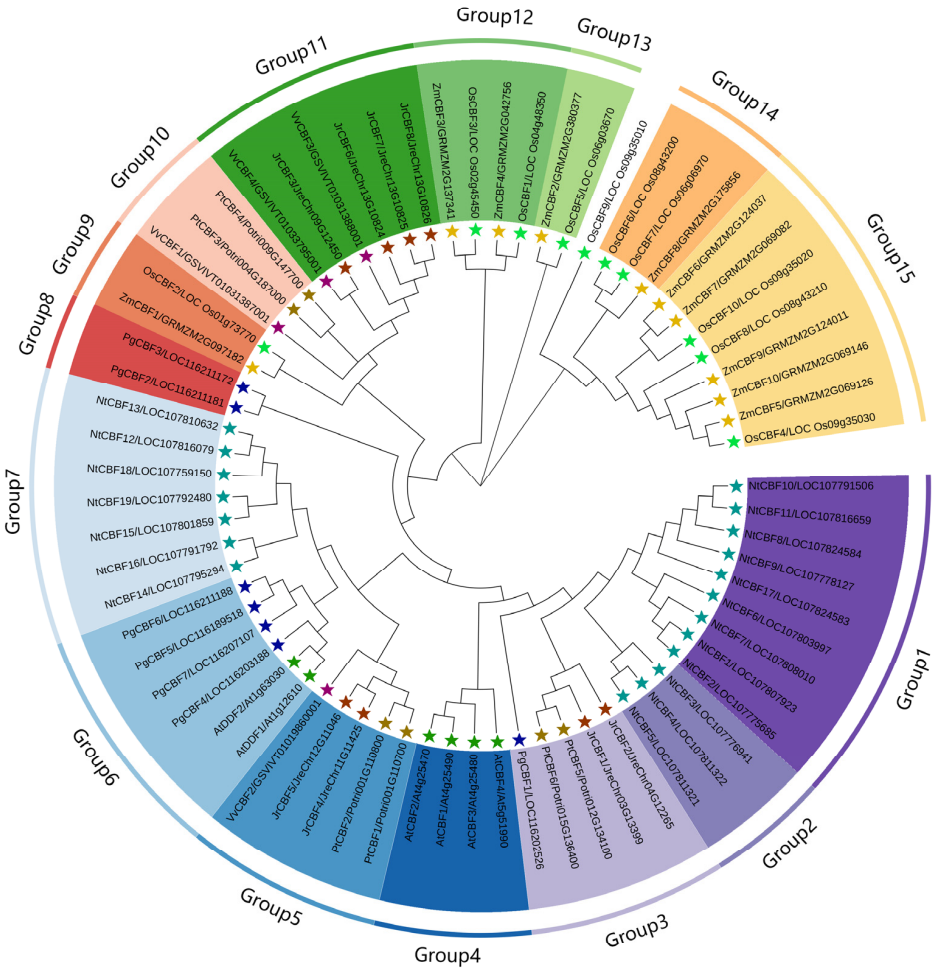

Figure 3. The phylogenetic tree of CBF family proteins from *Punica granatum*, *Zea mays*, *Oryza sativa*, *Nicotiana tabacum*, *Vitis vinifera*, *J. regia*, *P. trichocarpa*, and *A. thaliana*.

2.3. Phylogenetic Analysis of the JrCBF Genes

To elucidate the phylogenetic relationships between CBF proteins, we investigated the eight characterized CBF members from walnut, six CBF members from poplar, seven CBF members from pomegranate, ten CBF members from maize, four CBF members from grape, six CBF members from *Arabidopsis*, ten CBF members from rice, and nineteen CBF members from tobacco. The phylogenetic tree divided the 70 CBF proteins into 15 groups (Figure 3). *JrCBF1* and *JrCBF2* were classified within group 3, while *JrCBF4* and *JrCBF5* were categorized within group 5. *JrCBF3*, *JrCBF6*, *JrCBF7,* and *JrCBF8* were clustered in group 11. The distribution of the six CBF/DREB 1 proteins in *Arabidopsis* aligned with prior research, located in groups 4 and 6 [28,29]. The classification results of the *JrCBF* genes revealed that they were closer to the *PtCBF* and *VvCBF* genes than to the *AtCBFs*, indicating that the *JrCBF* genes were more closely related to the adaptive evolution of woody plants. In contrast, the phylogenetic relationship between *JrCBF*s and *PtCBF*s and *VvCBFs* was closer than that with pomegranate *PgCBFs* in woody plants.

2.4. Cis-Acting Component Analysis in the Promoter of the JrCBF Genes

Cis-acting elements within the promoter region exhibited a significant impact on the regulation of gene expression. In order to identify cis-regulatory elements within the promoter region of *JrCBFs*, we selected the upstream 2-kilobase (kb) region of *JrCBFs* for a search in the PlantCARE database. Our analysis revealed the widespread presence of cis-elements associated with plant hormone responses and abiotic stress in this region (Figure 4). The promoter region of JrCBF genes encompassed various plant hormone-responsive elements, including jasmonic acid (MeJA), salicylic acid (SA), auxin and abscisic acid (ABA). Furthermore, elements associated with abiotic stress responses, such as anaerobic induction essential elements (AREs), low-temperature response elements (LTRs), and drought-inducible MYB-binding sites (MBSs), were identified. Additionally, we noticed that light-responsive elements were common in the promoters of most *JrCBF* genes. These findings suggested that the expression of *JrCBF* genes may be influenced by a variety of cis-regulatory elements.

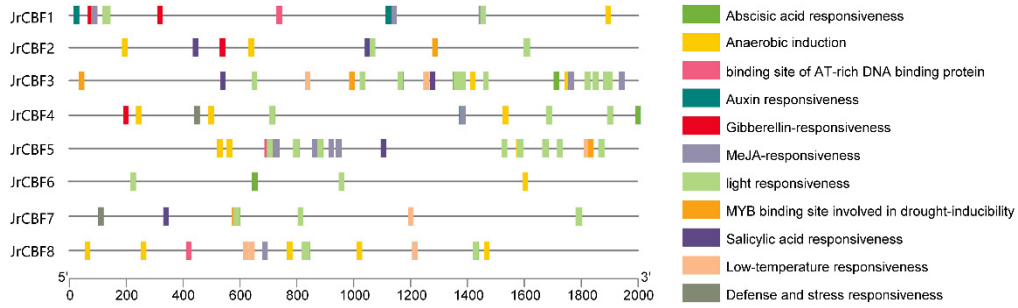

Figure 4. Cis-acting elements within the 2000-bp upstream region of *JrCBF* genes. Different colors represent different cis-acting elements.

2.5. Chromosomal Location and Collinearity Analysis of the JrCBF Genes

We conducted chromosome mapping analysis to understand the distribution of *JrCBF* genes more deeply and marked their positions on each chromosome. The results revealed that eight *JrCBFs* were unevenly distributed on six chromosomes (Figure 5). Chromosome 3, 4, 9, 11, and 12 each contained one *JrCBF* gene. There were three *JrCBF* genes on chromosome 13, namely *JrCBF6*, *JrCBF7* and *JrCBF8*, which were tandem duplications to form a gene cluster. Gene duplication events were widespread across various species and played a critical role in creating new functional genes and driving species evolution [30]. There were seven segmental duplication pairs on the chromosomes of walnut, of which *JrCBF1*, *JrCBF2*, *JrCBF4*, and *JrCBF5* were segmental duplication gene pairs to each other, suggesting that these genes may possess functional similarities (Figure 5). These findings

indicated that segmental duplication events were the primary factor contributing to *CBF* gene amplification in walnut, followed by tandem duplication.

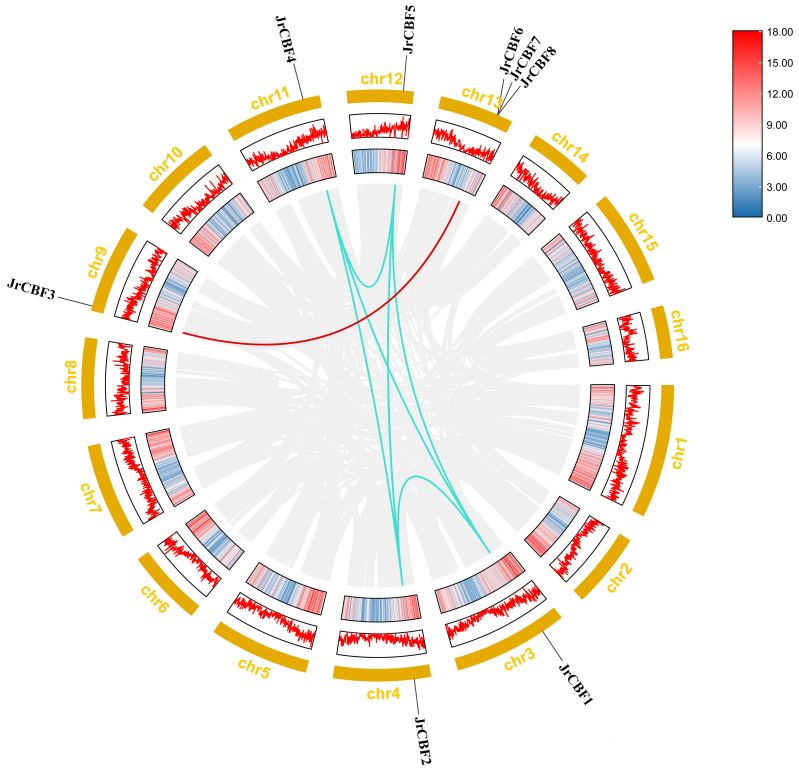

Figure 5. Chromosomal distribution and inter-chromosomal relationships of *JrCBF* genes. Red lines and blue lines connect duplicate *JrCBF* gene pairs. The heat map in the inner square represents gene density, while the outer orange squares represent chromosomes.

We conducted genome-to-genome collinearity analysis among walnut, *Arabidopsis*, and poplar. The results revealed that the number of homologous transcription factors between *JrCBFs* and *PtCBFs* exceeds that of *AtCBFs* (Figure 6). Specifically, there were five pairs of *CBF* homologous genes between walnut and *Arabidopsis*, and thirteen pairs between walnut and poplar. Furthermore, the majority of *JrCBF* genes had 2–3 corresponding homologous sequences in *PtCBF* genes (Figure 6). This indicated that these genes may play a significant role in the evolution of the *CBF* gene family.

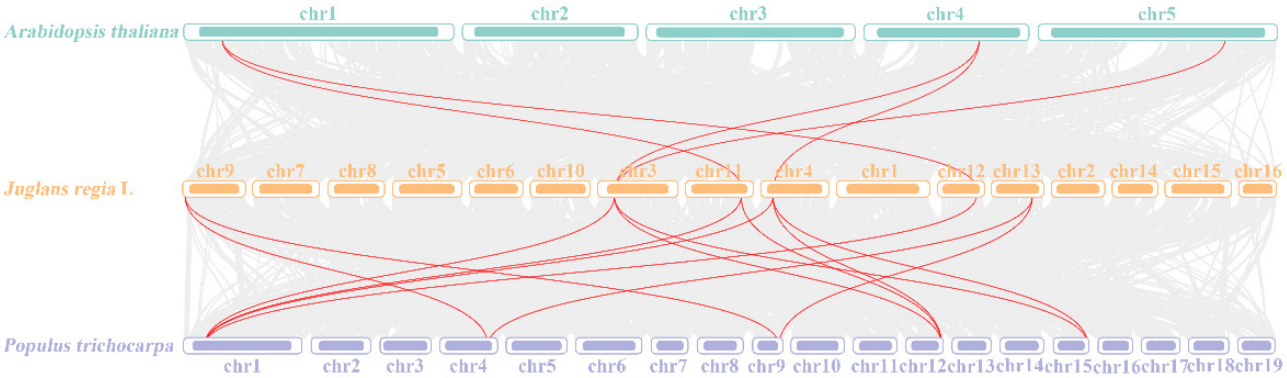

Figure 6. Collinear relationship between *J. regia*, *A. thaliana*, and *P. trichocarpa*. The red line represents the collinearity between *CBF* genes, and the gray line represents the collinearity of other genes in the genome.

2.6. Expression Pattern of JrCBF Genes under Low-Temperature Stress

In order to evaluate the expression pattern of *JrCBF* genes under low-temperature stress, we analyzed the published transcriptome data. The results unveiled a diverse range of responses exhibited by each *JrCBF* gene under cold conditions. *JrCBF1* and *JrCBF2* exhibited an initial increase in expression after 3 h of cold treatment (Figure 7), followed by a subsequent decrease, highlighting their quick response to low-temperature stress in walnut. On the other hand, *JrCBF4* and *JrCBF5* displayed an elevation in expression during the early stages of the low-temperature treatment, reaching their peak at 12 h, and then gradually declining (Figure 7), indicating their significant role in combating low-temperature stress. In contrast, *JrCBF3*, *JrCBF6*, *JrCBF7*, and *JrCBF8* exhibited minimal levels of expression across all samples, suggesting their insensitivity to the low-temperature treatment.

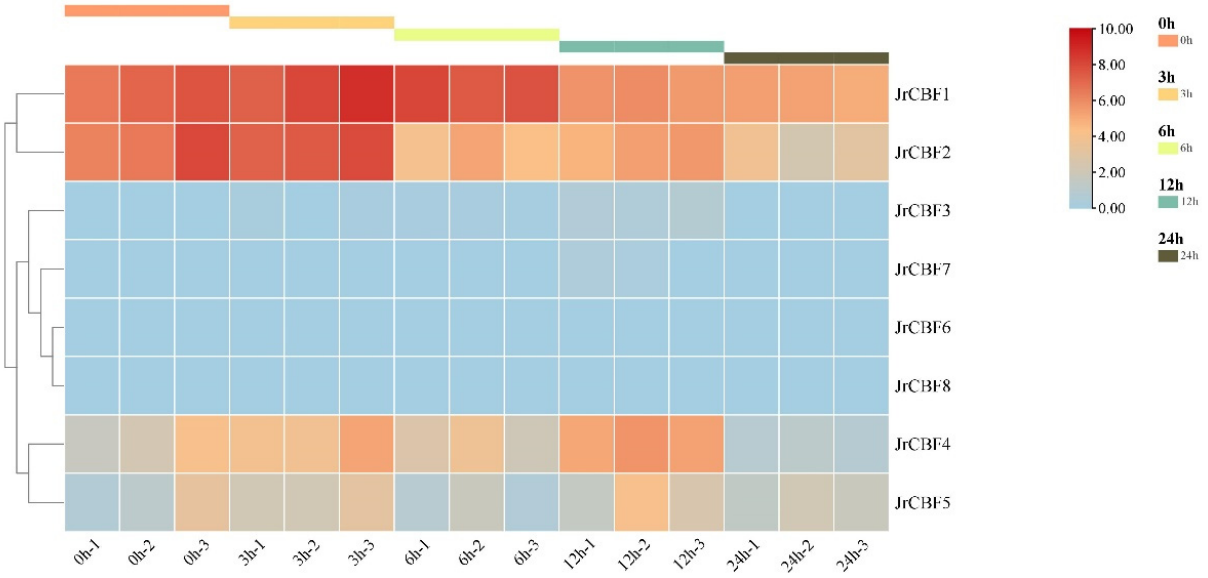

Figure 7. Expression profiles of *JrCBF* genes under low-temperature stress in walnut. Color scale represents FPKM values. Red blocks indicate high expression levels, and light blue blocks indicate low expression levels.

2.7. Expression Patterns of JrCBF Genes under Different Abiotic Stresses

We employed qRT-PCR to detect the expression levels of *JrCBF* genes under low-temperature stress (4 °C). Under the low-temperature treatment, the expression levels of *JrCBF1* and *JrCBF2* significantly exceeded those of other *JrCBF* genes. Their initial expression levels rapidly increased, reaching their peak after 6 h of treatment, and then gradually declined (Figure 8A). *JrCBF4* and *JrCBF5* displayed an initial increase in expression, reaching their peak after 24 h, followed by a decline, indicating their lower sensitivity to low temperatures compared to *JrCBF1* and *JrCBF2*. Furthermore, the expression patterns of *JrCBF3*, *JrCBF6*, *JrCBF7*, and *JrCBF8* revealed no significant differences compared to the control group, consistent with the transcriptomic results.

We also investigated whether the *JrCBF* genes responded to other abiotic stresses by employing high-temperature (38 °C) and drought (20% PEG) treatments. Under the high-temperature treatment, the expression levels of *JrCBF1* and *JrCBF2* gradually increased, reaching their peak at 48 h, suggesting their potentially crucial regulatory roles in high-temperature stress (Figure 8B). In contrast, the expression levels of *JrCBF3*, *JrCBF4*, *JrCBF6*, and *JrCBF7* significantly decreased after treatment. *JrCBF5* exhibited an initial decrease, followed by an increase, and then another decrease in its expression levels. The expression of *JrCBF8* initially decreased within the first 3 h of treatment and then steadily increased (Figure 8B). However, when compared to the control group, the difference was not significant, indicating its lower sensitivity to high temperatures. Under the drought treatment, *JrCBF5* and *JrCBF8* exhibited a notable response to drought. Their expression

levels initially decreased, then reached their peak at 48 h. Conversely, the six remaining genes demonstrated reduced expression under drought stress, reaching their lowest levels at 6 h or 9 h and gradually recovering to normal levels (Figure 8C).

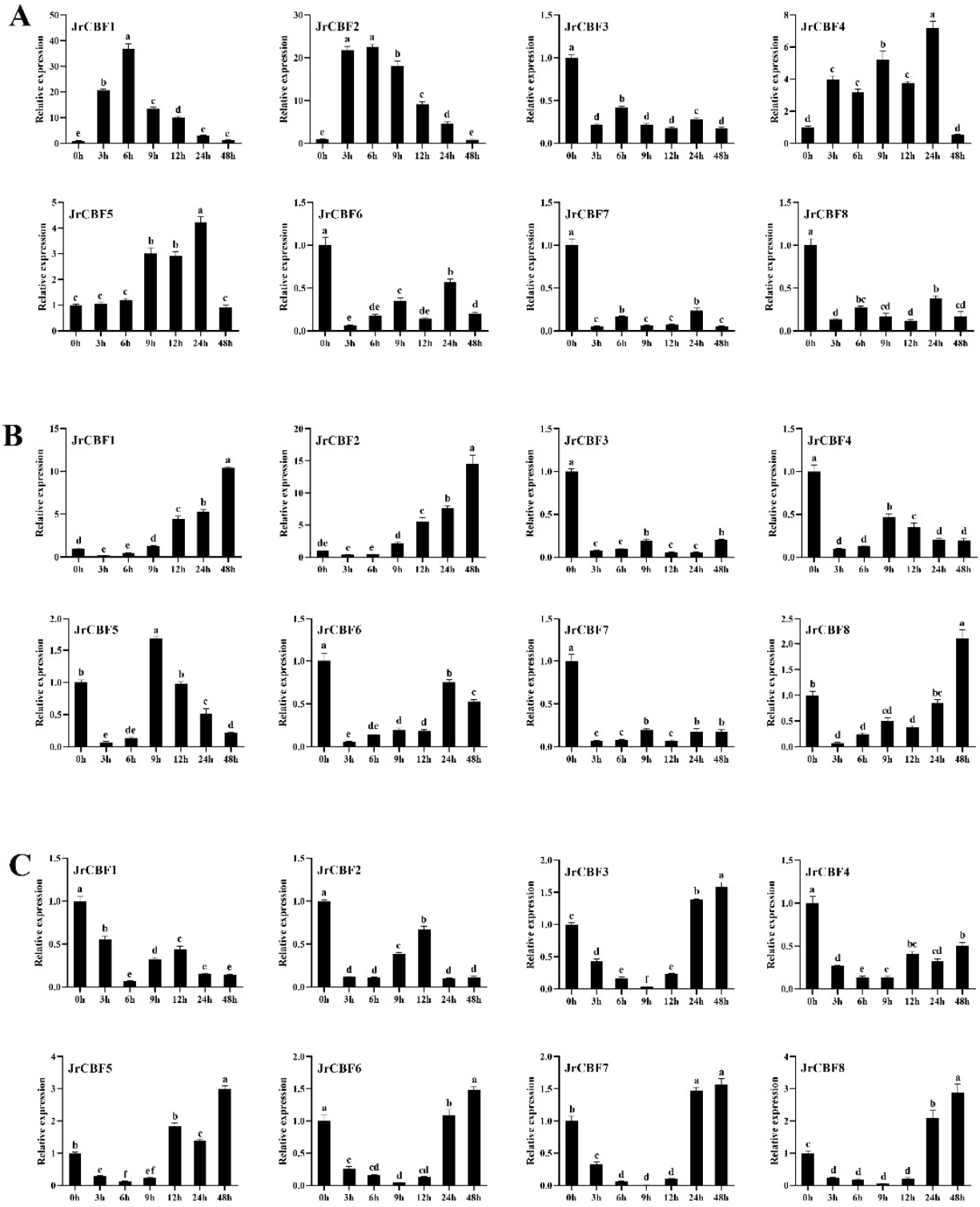

Figure 8. Expression patterns of *JrCBFs* under different abiotic stresses. (**A**) Low-temperature treatment (4 °C). (**B**) High-temperature treatment (38 °C). (**C**) 20% PEG treatment simulates drought. The significance of the difference was indicated with different lowercase letters ($p < 0.05$).

2.8. Subcellular Localization of JrCBF1 and JrCBF2

To explore the subcellular localization of *JrCBF1* and *JrCBF2*, we transiently co-expressed the JrCBF1-GFP and JrCBF2-GFP fusion constructs, carried in the pICH86988 vector, along with a nucleolus marker (FIB2:mCherry) in tobacco leaves. The distribution of green fluorescent protein was observed using confocal laser scanning microscopy. The green fluorescence emitted by JrCBF1:GFP and JrCBF2:GFP was observed within the cell nucleus, encompassing both the nucleolus and nucleoplasm, consistent with the predicted results (Figure 9).

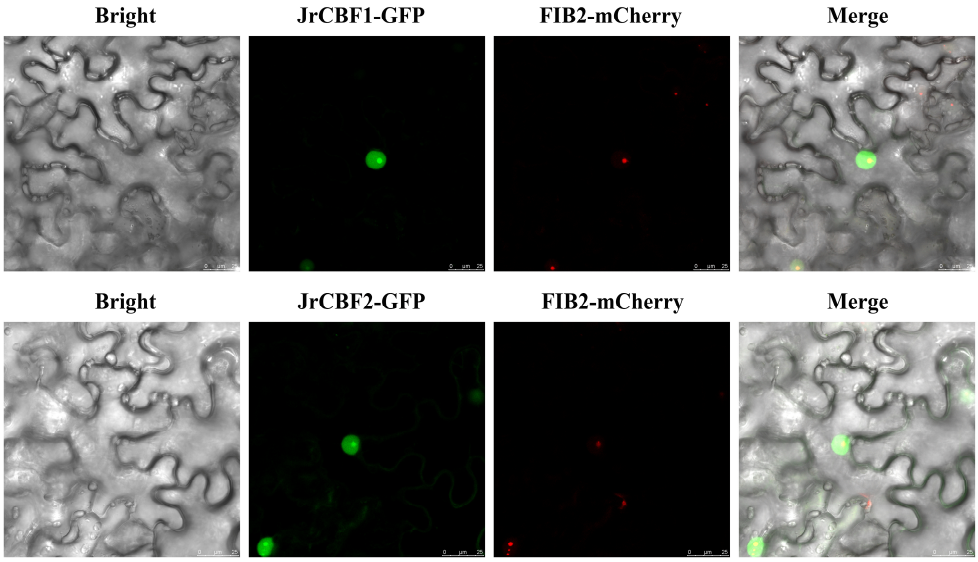

Figure 9. Subcellular location of JrCBF1 and JrCBF2 proteins in lower epidermal cells of tobacco leaves. The bar value in the figure is 25μm.

3. Discussion

Walnut is widely grown around the world, although they are somewhat sensitive to low temperature environments. When walnut is exposed to low temperatures, their normal growth and development can be affected, which can have some unfavorable economic consequences [31]. The *CBF* (C-repeat/DRE binding factor) gene family is closely related to plant cold tolerance, and it is mainly induced and expressed under cold stress. It can improve the cold resistance of plants by increasing the expression of several genes related to the cold resistance mechanism of plants [32,33]. This regulation mechanism enables plants to better adapt to low-temperature environments, thus ensuring their normal growth and development. Through this in-depth study of the walnut *CBF* gene family, we can understand the response mechanism of walnut to low-temperature stress and provide an important theoretical basis for their cold-resistant breeding.

By comparing the structural and motif differences between different genes, we can infer their genetic relationship, evolution process, and role in biological evolution. In this study, we identified eight *JrCBF* genes in walnut. Physicochemical analysis revealed that these walnut CBF proteins were characterized as unstable and predominantly acidic, suggesting their potential functionality within acidic subcellular environments. Notably, all eight *JrCBF* gene-encoded proteins were found to contain the highly conserved AP2 structural domain (Figure 2C). Interestingly, a structural analysis of the *JrCBF* genes revealed that none of the eight *JrCBF* genes contained introns (Figure 2B). This finding aligned with previous research conducted on species such as *Betula platyphylla* [34], cucumber [35], and *Liriodendron chinense* [36], indicating that the *CBF* gene family generally lacks introns or has only a few. This characteristic may enable these genes to respond more swiftly to environmental changes by requiring less splicing and transcription [37]. Such genetic simplicity equips them with the capacity to rapidly adapt to diverse stressful conditions. CBF protein-conserved motif analysis revealed that motif 2, motif 1, and motif 4 were

highly conserved in all 20 CBF protein sequences (Figure 2). These three motifs were the characteristic sequence PKK/RPAGRxKFxETRHP, the AP2/ERF-binding domain, and the characteristic sequence DSAWR located downstream of the binding domain (Figure S1). *CBF* was a subfamily of the DREB family, with an AP2 domain, which can specifically bind to CRT/DRE elements [38] and induce the expression of genes related to ABA, drought, and cold responses. In addition, the PKKPAGR sequence may be critical for the transcriptional activity of *CBF.* In *A.thaliana*, mutations in the PKKPAGR sequence of *AtCBF1* weakened the ability of the CBF1 protein to bind to the DNA recognition element (CRT/DRE) on the promoter of the *COR15a* gene, decreased *COR15a* gene expression, and the plants exhibited more sensitivity to cold stress [39]. The PKKPAGR motifs were extremely important for the exercise of the CBF protein in the function of the transcription factor, which was crucial for the plants to adapt to the cold environment. DSAWR was rarely reported and studied, and we are not clear about its specific biological function for the time being.

Furthermore, JrCBF proteins displayed a close relationship with CBF proteins from poplar and grape (Figure 3), indicating that the *CBF* family relationship between woody plants was closer than that of herbaceous plants. Through phylogenetic tree and collinearity analysis, we observed that *JrCBF1* shares homology with *AtCBF2* and *AtCBF4*, while *JrCBF2* also exhibited homology with *AtCBF2* (Figure 6). Furthermore, our collinearity analysis revealed a linear relationship between *JrCBF1* and *JrCBF2* (Figure 5). These findings indicated that *JrCBF1* and *JrCBF2* were functionally similar to *AtCBF2* and *AtCBF4*, providing an explanation for the positive responses of *JrCBF1* and *JrCBF2* to cold stress.

The analysis of cis-regulatory elements within the promoter region indicated that *JrCBFs* may be involved in multiple responses and played a role in plant hormone responses and abiotic stress defenses. Light-responsive elements widely existed in the promoter region of all *JrCBF* genes, which indicated that they may be regulated via light. The signal pathway of plant photoreceptors played an important role in the response of plants to abiotic stress [40]. In *Arabidopsis*, the activity of the *CBF* pathway was influenced by the photoperiod. During warm, long-day seasons, this pathway was inhibited by the *phytochrome B* (*PHYB*) and photoreceptor-interacting factors *PIF4* and *PIF7*. As daylight duration decreased, this inhibition was relieved, leading to enhanced *CBF* pathway activity and increased cold resistance in plants [41]. Therefore, it is plausible that *JrCBF* genes may participate in light signal transduction, regulating plant growth and development. Among the hormone-responsive elements, ABRE was found to be the most abundant cis-regulatory element, indicating that abscisic acid (ABA) was a crucial plant hormone in the regulation of walnut *CBF* genes for cold resistance. ABA regulated *CBFs'* expression by activating *OST1* (*open stomata 1*) kinase [42] and inducing the transcription factor *MYB96* [43]. Walnut may have a similar regulatory pathway, suggesting that *JrCBFs* may be induced via ABA signaling.

The *JrCBF* genes exhibited varying response patterns to different abiotic stresses. In this study, *JrCBF1* and *JrCBF2* displayed an upregulation trend under low-temperature treatment, indicating their high sensitivity to cold stress and potential involvement in cold stress regulation, warranting further in-depth research. Moreover, only some *JrCBF* genes (*JrCBF1*, *JrCBF2*, *JrCBF4*, and *JrCBF5*) showed an upregulation trend after low-temperature treatment, a phenomenon consistent with studies on *Brassica rapa* [44], *Acer truncatum*, and *Acer yangbiense* [45]. However, it is worth noting that in other plants like cucumber [35], dandelion [46], and ryegrass [47], all *CB F* genes were highly expressed after cold treatment. This variation may be due to different species experiencing distinct evolutionary pressures, resulting in changes in the regulatory elements or sequences of *CBF* genes [48,49], leading to variations in the evolution and expression patterns of the *CBF* gene family across different plants. In this study, we also investigated the expression patterns of *JrCBF* genes under high-temperature and drought treatments. Under the high-temperature treatment, *JrCBF1* and *JrCBF2* displayed significant upregulation, indicating their high sensitivity to temperature and potentially crucial roles in temperature stress, aligning with previous findings in plants like tea [50], potato [51], and lettuce [52]. In the case of drought stress, *JrCBF5* and *JrCBF8*

exhibited significant upregulation after 48 h of treatment, suggesting their involvement in drought stress regulation. However, the extent of upregulation of *JrCBF5* and *JrCBF8* was not particularly pronounced, indicating that a longer duration of drought treatment may be needed to more clearly determine the gene expression patterns. These findings suggest that the *CBF* gene may play an important regulatory role in various abiotic stress responses of walnut, which provides an important theoretical basis for the follow-up study on how the *CBF* gene regulates the responses of walnut under abiotic stress.

4. Materials and Methods

4.1. Genome-Wide Identification of JrCBF Genes in Walnut

Walnut genome data were downloaded from (http://www.xhhuanglab.cn/data/juglans.html, accessed on 20 April 2023), and *Arabidopsis* genome data were obtained from TAIR (https://www.arabidopsis.org/, accessed on 20 April 2023). Six AtCBF protein sequences were used to initially identify walnut *CBF* candidate genes (E value < 1.00×10^{-10}) via BlastP. The corresponding AP2 domain (PF00847) was downloaded from the Pfam database (http://pfam-legacy.xfam.org/, accessed on 20 April 2023), and candidate gene family members containing the AP2 structural domain (E value $\leq 1 \times 10^{-5}$) were identified using HMMER v3.3.2 software. Then, the NCBI-CDD database (https://www.ncbi.nlm.nih.gov/cdd/, accessed on 20 April 2023) and Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 20 April 2023) were used to screen conserved structural domains. *CBF* gene family feature sequences were analyzed to identify *JrCBF* gene members.

The CBF protein series' respective length, isoelectric point, and relative molecular mass were determined through online Expasy software (https://web.expasy.org/protparam/, accessed on 22 April 2023), and subcellular localization predictions were generated with YLoc (https://abi-services.cs.uni-tuebingen.de/yloc/webloc.cgi, accessed on 22 April 2023). CBF proteins from three different species (*A. thaliana*, *P. trichocarpa*, and *J. regia*) were selected. Subsequently, we conducted a multiple sequence comparison of their protein sequences using MEGA X (64-bit) software with default parameters. The resultant maps of the multiple sequence comparison for *JrCBFs* were further enhanced using GeneDoc 2.7 software.

4.2. Gene Structure and Phylogenetic Tree

We obtained the genome information of *P. trichocarpa*, which was stored in the Phytozome database (https://phytozome-next.jgi.doe.gov/info/Ptrichocarpa_v3_0, accessed on 6 May 2023). At the same time, the genomic information of *A. thaliana* was obtained from the TAIR database. To deeply analyze the protein sequence of the *CBF* gene family, we used the MEME online tool (https://meme-suite.org/meme/, accessed on 6 May 2023) to find motifs [53]. In addition, we also used Clutalx 2 [54] for multiple alignment of amino acid sequences. Finally, we constructed the phylogenetic tree using MEGA-X and adopted the adjacency method (NJ) as the construction method. The specific parameters were as follows: the "Poisson model" as the mode, "Complete deletion" for gap handling, and 1000 bootstrap replicates with a random seed for the confidence values. Eight species (*Punica granatum*, *Zea mays*, *Oryza sativa*, *Nicotiana tabacum*, *Vitis vinifera*, *J. regia*, *P. trichocarpa*, and *A. thaliana*) were used in the evolutionary tree analysis.

4.3. Identification of Cis-Elements and Collinearity Analysis

The PlantCARE online program (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 10 May 2023) was utilized to predict cis-acting elements in the promoter region (2000 bp) of the *JrCBF* genes. The cis-acting elements were then categorized via function.

We downloaded the GFF3 files containing the location information of all genes from the annotated databases of *A. thaliana*, *P. trichocarpa*, and *J. regia*, and inputted them into the Multiple Collinearity Scan Toolkit [55], which was used to determine the repetitive

patterns in the *CBF* gene family. The specific definitions of different repetitive patterns were consistent with the methods described earlier [56].

4.4. Transcriptional Expression Analysis of Walnut JrCBF Genes

We selected the transcriptome data of walnut at different time points under low-temperature treatment to study the expression pattern of the *JrCBF* genes under cold stress and compared these results with our qRT-PCR results. These walnut transcriptome data were downloaded from the NCBI SRA database (PRJNA942426).

4.5. Expression Pattern of JrCBF Genes under Different Abiotic Stresses

Walnut saplings, six months old, were subjected to temperature stress by placing them in constant-temperature chambers at 4 °C and 38 °C. For drought stress treatment, the saplings' roots were irrigated with 20% PEG. Each treatment group consisted of 10 plants. At 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, and 48 h after treatment, the leaves of the seedlings were quickly cut off with scissors and quickly frozen in liquid nitrogen, and all the collected samples were kept in the ultra-low-temperature refrigerator at −80 °C for the extraction of total RNA.

The total RNA was extracted using the RN38-EASYspin RNA Plant Mini Kit (Aidlab Biotechnologies Co., Ltd., Beijing, China), following the manufacturer's instructions. This kit is equipped with a genomic DNA removal column that efficiently eliminates DNA residue. When performing nucleic acid electrophoresis and spectrophotometric tests to verify the quality of the extracted RNA, it is necessary to ensure that it meets the following requirements: the three bands are recognizable, the ratio of A260/A280 is between 1.8–2.0, and the ratio of A260/A230 is greater than 2. The amount of total RNA for each sample should be no less than 2000 ng to ensure the needs of the experiment. We used the PrimeScriptTM RT kit (TaKaRa, Kyoto, Japan) to synthesize the first-strand cDNA, and carried out quantitative real-time PCR analysis according to previous methods [57]. This experiment was repeated with three organisms for each sample. *JrGAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *Jrβ-actin* were used as housekeeping genes. The relative expression value was calculated by comparing the CT ($2^{-\Delta\Delta CT}$) method and then analyzed with IBM SPSS Statistics 26 software to determine any statistically significant expression differences. The results were then visualized using GraphPad Prism 9.0 software to display the expression levels of target genes in each sample. The primer sequences used for qRT-PCR are listed in Table S2.

4.6. Subcellular Localisation Analyses

We fused the coding sequences (CDSs) of *JrCBF1* and *JrCBF2* with green fluorescent protein (GFP) to construct the JrCBF1-GFP and JrCBF2-GFP vectors. Subsequently, the recombinant vectors were introduced into tobacco (strain GV3101) carrying the pSoup-p19 plasmid and co-expressed in tobacco leaves with the nuclear marker FIB2-mCherry [58]. Subcellular localization of *JrCBF1* and *JrCBF2* was then observed using confocal laser scanning microscopy (Leica, TCS-SP8, Wetzlar, Germany).

5. Conclusions

The *CBF* transcription factors have a significant positive impact on cold adaptation and are widely conserved across higher plants. In this study, we found that eight *JrCBF* genes exist in walnut, characterized by comparable genetic structures and sequences. The *JrCBF* genes showed different expression patterns under various stress conditions, suggesting that they can function in different stress responses, but with varying degrees of responsiveness to different stresses. Subcellular localization studies were conducted for the significantly expressed *JrCBF1* and *JrCBF2* genes, both of which are located in the nucleus. These findings provide support for further exploring the molecular role of the *JrCBF* gene family in the abiotic stress response.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14112274/s1, Table S1: Gene sequences of JrCBF genes. Table S2: Primer sequences used for qRT-PCR. Figure S1: Conserved motifs of JrCBF proteins derived from the MEME Suite.

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