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ARTICLE

Genome-Wide Identification of the *MYB* Gene Family and Screening of Potential Genes Involved in Fatty Acid Biosynthesis in Walnut

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ABSTRACT

The multifaceted roles of *MYB* transcriptional regulators are pivotal in orchestrating the complex processes of secondary metabolism, stress tolerance mechanisms, and life cycle progression and development. This study extensively examined the *JrMYB* genes using whole genome and transcriptomic data, focusing on identifying putative *MYB* genes associated with fatty acid metabolism. 126 *MYB* genes were identified within the walnut genome, characterized by hydrophilic proteins spanning lengths ranging from 78 to 1890 base pairs. Analysis of cis-acting elements within the promoter regions of *MYB* genes revealed many elements linked to cell development, environmental stress, and phytohormones. Transcriptomic data was utilized to examine the role of *JrMYB* genes in the biosynthesis of fatty acids in walnuts. The results revealed diverse expression of these genes across various tissue sites, displaying varying levels and distinct expression patterns. Furthermore, by integrating the results of the phylogenetic tree with the correlation of expression levels, a total of 10 genes potentially involved in the regulation of fatty acid synthesis were screened. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was conducted on these 10 genes and further identified 4 candidate genes, and a transcription regulatory network involved in fatty acids metabolism was constructed. This study presents a systematic analysis of *JrMYB* genes, laying the groundwork for an in-depth exploration of the *JrMYB* genes family's function in regulating fatty acid synthesis.

KEYWORDS

Walnut; *JrMYB* genes; fatty acid synthesis; gene expression; bioinformatics analysis

1 Introduction

The walnut (*Juglans regia* L.), belonging to the Juglandaceae family, represents an economically significant forest species cultivated worldwide. It holds the distinction of being one of the "four nuts" globally due to its abundant and nutritious nuts, as well as its widespread distribution. Renowned for its delectable and aromatic kernel rich in protein, crude fat, 17 distinct amino acids, and Vitamins B1 and B2, walnut is a healthful dietary choice. Its benefits include anti-aging properties, blood lipid regulation, and cardiovascular disease prevention [1]. The fatty acid content of walnuts plays a pivotal role in

cellular functions and intracellular metabolic processes in humans [2]. Unsaturated fatty acids, which are necessary for human health [3], are prevalent in walnuts [4], surpassing the levels found in conventional edible oils [5,6]. Consequently, the quality of walnuts is significantly affected by their fatty acid composition.

The *MYB* gene family is one of the most functionally diverse and abundant transcription factor families in plants. The defining structural property of plant *MYB* transcription factors is the N-terminal *MYB* structural domain, which uniquely interacts with the eukaryotic gene promoter regions to regulate gene expression [6]. This domain typically consists of 50–55 bases and adopts a helix-turn-helix (HTH) structure, often featuring 1–4 incomplete repeats in the R structure. MYB transcription factors are categorized into four subclasses, namely, *1R-MYB*, *2R-MYB*, *3R-MYB*, and *4R-MYB*, based on the number of structural domains present within the MYB gene [7].

The biological functions of *MYB* genes have been revealed through studies in *Arabidopsis thaliana* and a variety of other plant species. Notably, the *MYB* transcription factor *ScMYB52-1* in sugarcane exhibits an inducible response under stressful conditions of exogenous ABA and NaCl [8]. The Arabidopsis transcription factors *AtMYB20*, *AtMYB42*, *AtMYB43*, and *AtMYB85* serve as pivotal regulators that control the synthesis of phenylalanine and the subsequent polymerization of lignin during the maturation phase of lignification and cellulose deposition in secondary walls [9]. The *PqMYB*4 gene plays a regulatory role in anthocyanin synthesis and metabolism in the leaves of *Paeonia qiui* [10], whereas *GlMYB*4 and *GlMYB*88 positively govern flavonoid synthesis in *Glycyrrhiza uralensis* [11]. Additionally, numerous studies have revealed a strong correlation between *MYB* family members and the regulation of fatty acid synthesis. For example, *AtMYB*96 induces the expression of genes associated with fatty acid elongation in seeds and leaves, leading to the accumulation of long-chain fatty acids [12,13]. Similarly, overexpression of *AtMYB*92 in tobacco leaves promotes the expression of *BCCP2* and significantly enhances fatty acid accumulation [14]. Furthermore, *MaMYB*4 can regulate the production of unsaturated fatty acids in *Musa acuminata* by controlling the expression of ω-3 fatty acid desaturases (*FADs*) [15].

Previous studies have made substantial progress in understanding the biological roles of the *MYB* gene family, particularly with the completion of whole-genome sequencing across various plant species. However, further investigation is needed to fully elucidate how *MYB* family transcription factors influence fatty acid synthesis in walnuts, as the existing literature on this subject is limited. In this study, we applied bioinformatics techniques to systematically identify members of the *JrMYB* genes within the genome. Our analysis explored their physicochemical characteristics, evolutionary relationships, conserved motifs, chromosomal locations, and other relevant features. In conjunction with the transcriptomic data and qRT-PCR analysis, our objective was to identify potential *JrMYB* genes related to fatty acid synthesis. This study provided a theoretical basis for future investigations into the roles of walnut *MYB* transcription factors in the regulation of fatty acid synthesis.

2 Materials and Methods

2.1 Identification and Protein Characterization of JrMYB Genes

The walnut genome data (No. PRJCA002070) were sourced from National Genomics Data Center (https://ngdc.cncb.ac.cn/?lang=zh, accessed on 18 June 2024), whereas the protein sequences of the all *AtMYB* genes were obtained from the TAIR database (https://www.arabidopsis.org/, accessed on 22 April 2023). To identify potential *MYB* candidate genes in the walnut genome, a multistep approach was employed. At the outset of investigation, we utilized the HMM file (PF00249), corresponding to the evolutionarily conserved structural motifs of the *MYB* transcription factor family in the Pfam database, to conduct a comprehensive screen. Subsequently, a local BLAST comparison was performed, utilizing the protein sequence of *AtMYB* genes as a query sequence to re-identify *MYB* candidate genes within the walnut genome. Following these screenings, the candidate genes obtained from both steps were further scrutinized using NCBI's Batch CDD search tool. This manual curation process aimed to eliminate duplicated sequences and prediction errors, resulting in a refined and reliable compilation of the *JrMYB* genes.

The physicochemical properties of MYB transcription factor proteins, including isoelectric point, stability, and amino acid length, were analyzed. The analysis was conducted using Prot Param, an online tool available through ExPASy (https://web.expasy.org/protparam, accessed on 26 June 2023). Additionally, the subcellular localization and signal peptides of the members of the *JrMYB* genes were predicted. These predictions were made using Wolf Psort (https://web.expasy.org/protparam, accessed on 26 June 2023) and SignalIP-5.0 (https://web.expasy.org/protparam, accessed on 17 July 2023) online tools.

2.2 Collinearity Analysis and Chromosomal Localization of JrMYB Genes

The genome sequences along withand annotation files for Arabidopsis (*Arabidopsis thaliana*) and pecan (*Carya illinoinensis*) were obtained from the TAIR (https://www.arabidopsis.org/, accessed on 22 April 2023) and Ensemble plant databases (http://plants.ensembl.org/index.html, accessed on 22 April 2023). For the purpose of quantifying the collinearity within and between the walnut, Arabidopsis, and pecan genomes, we utilized the bioinformatics tool MCScanX, followed by graphical representation of the findings using TBtools. Using TBtools software, we extracted the genomic positions of *JrMYB* genes and identified covariance gene pairs spanning the entire walnut genome. These covariance associations were further depicted and visualized using the Advanced Circos software.

2.3 Genetic Structures, Conserved Motifs, and Characteristic Conserved Domains Analysis of JrMYB Genes

We conducted a thorough examination of the conserved protein motifs present across the 126-member *JrMYB* genes by employing the MEME suite's online analytical tool (https://meme-suite.org/meme/, accessed on 19 July 2023). Following the motif discovery, TBtools was utilized to map and visualize these motifs, with default parameters configured to recognize a maximum of 10 conserved motifs. For the prediction of conserved structural domains within the JrMYB protein, both the NCBI's Batch CDD-Search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 24 April 2023) and the InterPro resource (https://www.ebi.ac.uk/interpro/, accessed on 25 April 2023) were engaged. Moreover, the exon-intron structure of the *JrMYB* genes was subjected to a detailed analysis and graphically displayed using TBtools software.

2.4 Phylogenetic Analysis of JrMYB Genes

The phylogenetic relationships were elucidated by the neighbor-joining method in MEGA-X software, using a bootstrap of 1000 replications, the p-distance model for sequence evolution, and pairwise deletion to accommodate missing data points. The protein sequences of *AtMYB* genes were extracted from the TAIR database (https://www.arabidopsis.org/, accessed on 27 April 2023), whereas the MYB protein sequences from walnut were subjected to multiple sequence comparisons. The organization and visualization of the phylogenetic trees were facilitated using the EvolView online tool (https://www.evolgenius.info/evolview-v2, accessed on 12 May 2023).

2.5 Cis-Acting Components Analysis of JrMYB Genes

To delineate the cis-acting regulatory elements in the proximal 2000 base pairs of the promoter sequences preceding the *JrMYB* genes, the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 06 May 2023) served as a predictive platform. Subsequently,the data were subjected to visualization and analysis using the heat map tool available in TBTools.

2.6 Screening of JrMYB Genes Involved in Fatty Acid Synthesis and Their Expression Analysis

Expression data for 126 *JrMYB* genes in the roots, stems, and leaves of walnut were compiled from previously collected transcriptomic data [16]. Subsequently, data processing and correlation analyses were

conducted using Excel 2019. For visualization, a heat map tool within TBtools was used. In addition, *JrMYB* genes were compared to previously reported genes associated with fatty acid synthesis. To construct the phylogenetic tree, *AtMYB*92 (AT5G62470) [14], *CaMYB*340 (XP_016563145.2) [17], *JcMYB*1 (AEI73171.1) [18] were used as reference points.

2.7 Quantitative Real-Time PCR and Determination of Fatty Acid

Total RNA was extracted from the roots, stems, and leaves of the walnut using MiniBEST Plant RNA Extraction Kit (Beijing, China), and cDNA templates were synthesized following the manufacturer's instructions provided by HiScript cDNA Synthesis Kit (Nanjing, China) for use in subsequent quantitative real-time PCR (qRT-PCR) assays. For normalization purposes in our walnut gene expression assays, the GAPDH gene was selected as a housekeeping gene to provide an internal reference for normalization [19]. The relative quantification of target gene transcripts was performed using the $2^{-\Delta\Delta Ct}$ formula [20], whichaccounts for variations in amplification efficiency, and the data were visualized with Origin software. Each sample was processed with three biological replicates and three technical replicates. Quantitative primers were designed using the Primer Premier 6 software, and all primers used in this study are listed in Supplementary Table S5. The fatty acid content in the roots, stems, and leaves was measured using gas chromatography-mass spectrometry (GC-MS) [21].

2.8 Construction of the Regulatory Network for Enzyme Genes and Transcription Factors Involved in Fatty Acid Biosynthesis

The Plant TF Motifs Shift plugin of TBtools was utilized, with Arabidopsis as a reference, to identify the binding motifs for *JrMYB* genes. Enzyme genes involved in the fatty acid biosynthesis pathway [16] were selected, and their promoter region sequences were extracted. A gene regulatory network was constructed using Fimo Search and visualized with Cytoscape software.

3 Results

3.1 Identification of JrMYB Genes and Analysis of Their Physicochemical Properties

Examination of the walnut genome led to the discovery of 126 *JrMYB* genes, denoted as *JrMYB*001–*JrMYB*126 (Table S1). Notably, the proteins encoded by these family members exhibit significant variations in the size and quantity of amino acids. The amino acid sequence lengths ranged from 78 amino acids (*JrMYB*015) to 1890 amino acids (*JrMYB*003), with corresponding relative molecular weights ranging from 8.936 to 207.542 kDa.

Regarding the isoelectric point (pI) of the 126 walnut MYB family proteins, it ranged from 4.34 (*JrMYB*036) to 10.06 (*JrMYB*079). Interestingly, all MYB proteins exhibited negative Gravy values, which indicated their hydrophilic nature. According to ExPasy's stability prediction software, 11 JrMYB proteins were found to be stable (with an instability coefficient <40), whereas the remaining 115 proteins were classified as unstable (with an instability coefficient >40). In terms of subcellular localization, JrMYB proteins were primarily located in the nucleus, with a minority of members also being localized in other subcellular regions such as the mitochondria and cytoplasm, as predicted for the subcellular localization of *JrMYB* genes. Notably, only *JrMYB*003, *JrMYB*076, and *JrMYB*081 were found to possess signal peptides according to signal peptide prediction, whereas the other genes lacked this feature.

3.2 Chromosomal Localization Analysis of JrMYB Genes

The distribution of 126 *JrMYB* genes across the walnut genome revealed an uneven spread, with genes dispersed across 16 chromosomes. The gene count on the individual chromosomes ranged from 4 to 13. The highest number of genes was observed on chromosomes 1 and 9, each hosting 13 genes, whereas chromosomes 8 and 12 had the lowest gene count, with only four *MYB* genes each.

Specifically, chromosome 8 housed four genes, namely *JrMYB*033, *JrMYB*030, *JrMYB*118, and *JrMYB*036, whereas chromosome 12 accommodated another set of four genes, consisting of *JrMYB*116, *JrMYB*084, *JrMYB*043, and *JrMYB*123. Chromosomes 2, 6, 7, and 10 (Fig. 1) also exhibited a relatively high number of genes distributed across them. Notably, the majority of genes were located toward the proximal or distal ends of the chromosomes, with fewer genes found in the central regions.

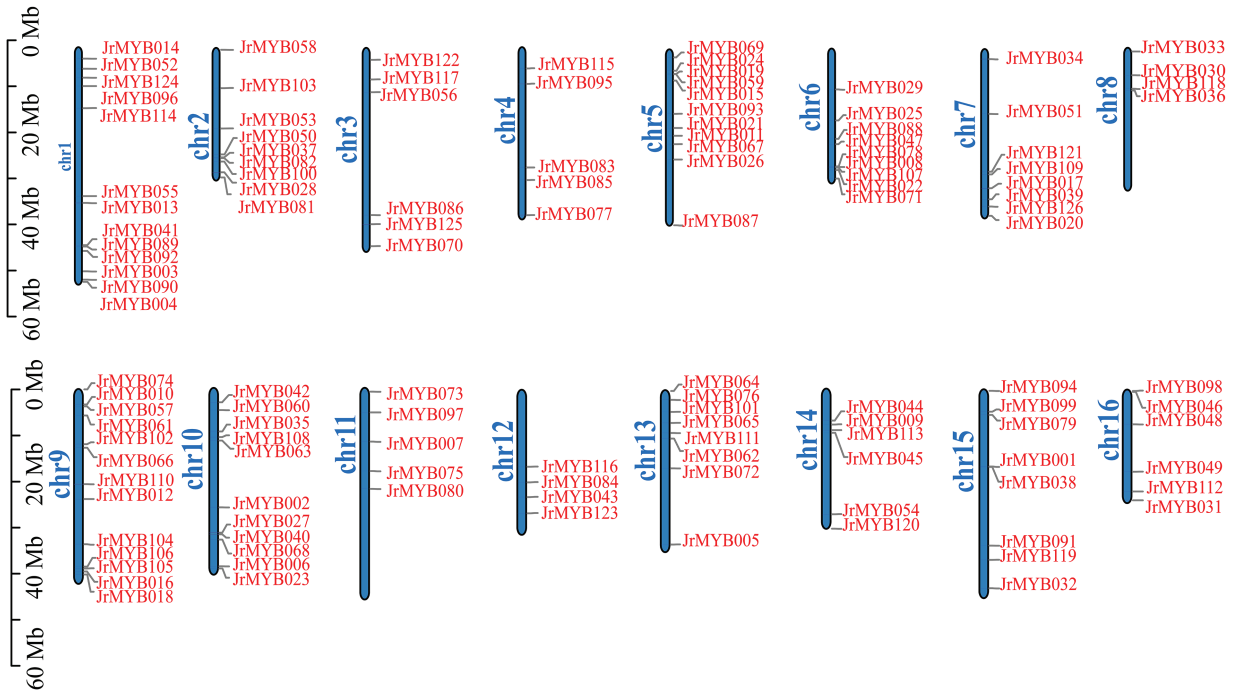

Figure 1: The distribution of *JrMYB* genes on chromosomes

3.3 Intraspecific and Interspecific Covariance Analysis of JrMYB Genes

Analysis of covariance included 50 gene family members within the *JrMYB* genes, and their distribution was observed across 11 chromosomes. Notably, chromosome 1 harbored the highest number of genes, totaling 11 (22% of the total), followed by chromosome 10, with seven genes (14%). The remaining chromosomes hosted varying numbers of *JrMYB* genes, ranging from 2 to 6 (Fig. 2). Concurrently, there exists a pronounced collinearity among segments across various chromosomes, which strongly suggests that a whole-genome duplication event has transpired within the species of walnut [22].

In the covariance analysis, 89 genes exhibited covariance between walnut and both Arabidopsis and pecan (Table S2), while 121 genes displayed covariance solely between walnut and pecan (Table S3). This observation suggests a close evolutionary relationship between the genomes of walnut and pecan (Fig. 3).

3.4 Analysis of Gene Evolution and Classification of JrMYB Genes

The *JrMYB* genes has been categorized into four groups, discerned through structural characterization of distinctive conserved domains within MYB proteins [7] and determination of the number of structural domains in InterPro. These groupings were as follows: 1R-*MYB*/*MYB*-related (102 members), R2R3-*MYB* (19 members), 3R-*MYB* (4 members), and 4R-*MYB* (1 member) (Fig. 4 and Table S4).

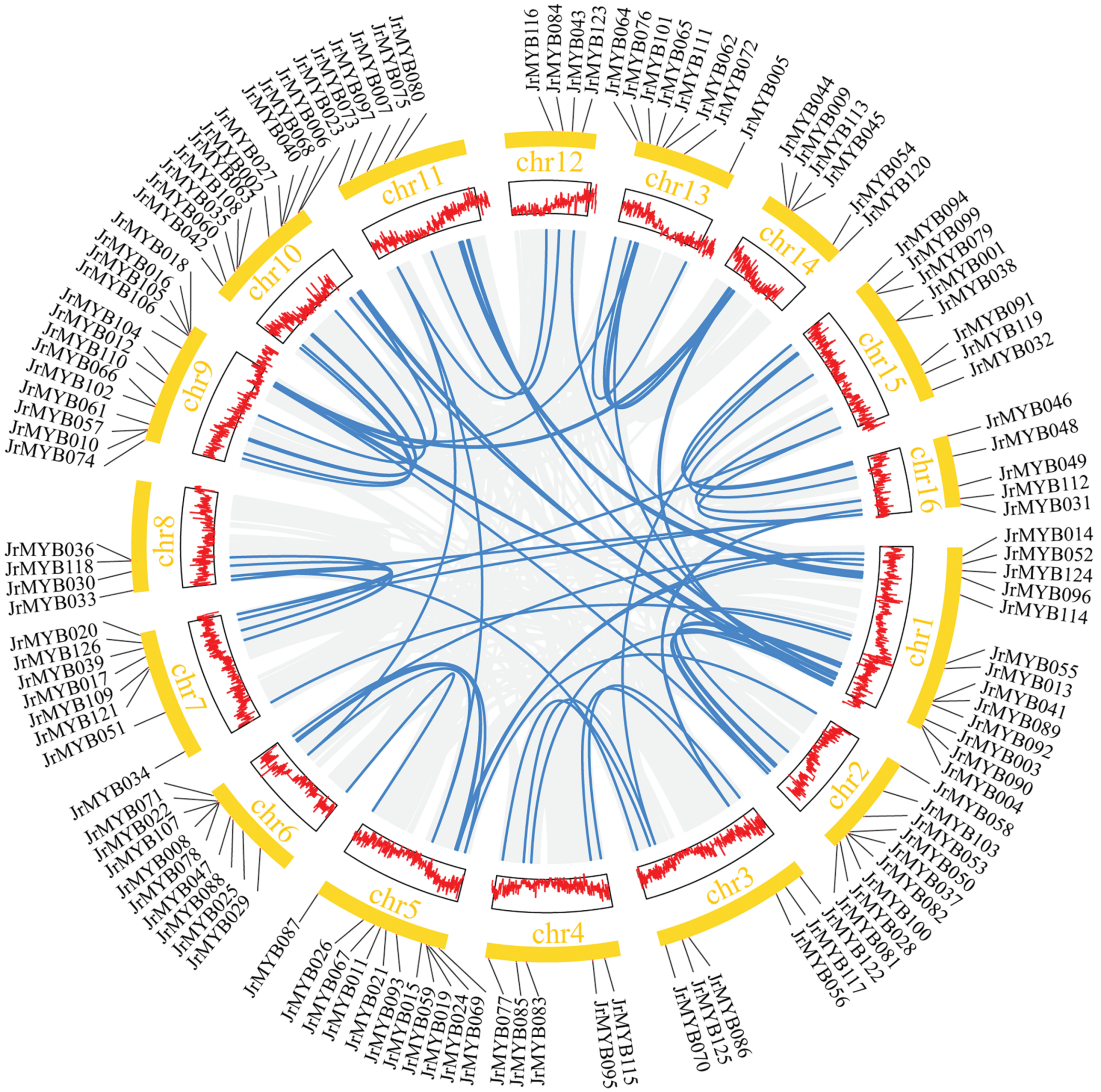

Figure 2: Collinearity analysis of *JrMYB* genes. The gray lines within the circular representation delineate the regions of collinear blocks, while the blue lines specifically indicate the pairs of collinear *JrMYB* genes. The gene density is visually depicted in the heatmap enclosed within the centralsquare, with the surrounding orange square illustrating the chromosomal organization

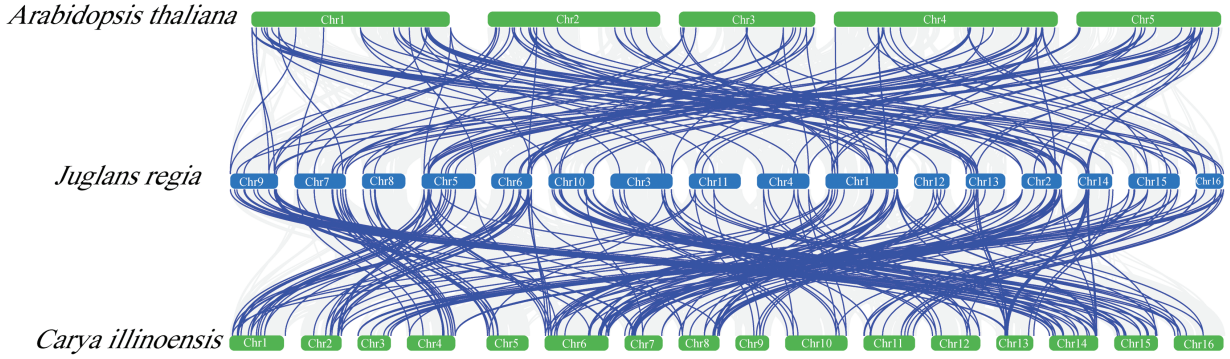

Figure 3: Collinear relationship between *A.thaliana*, *Juglans regia* L. and *Carya illinoinensis*

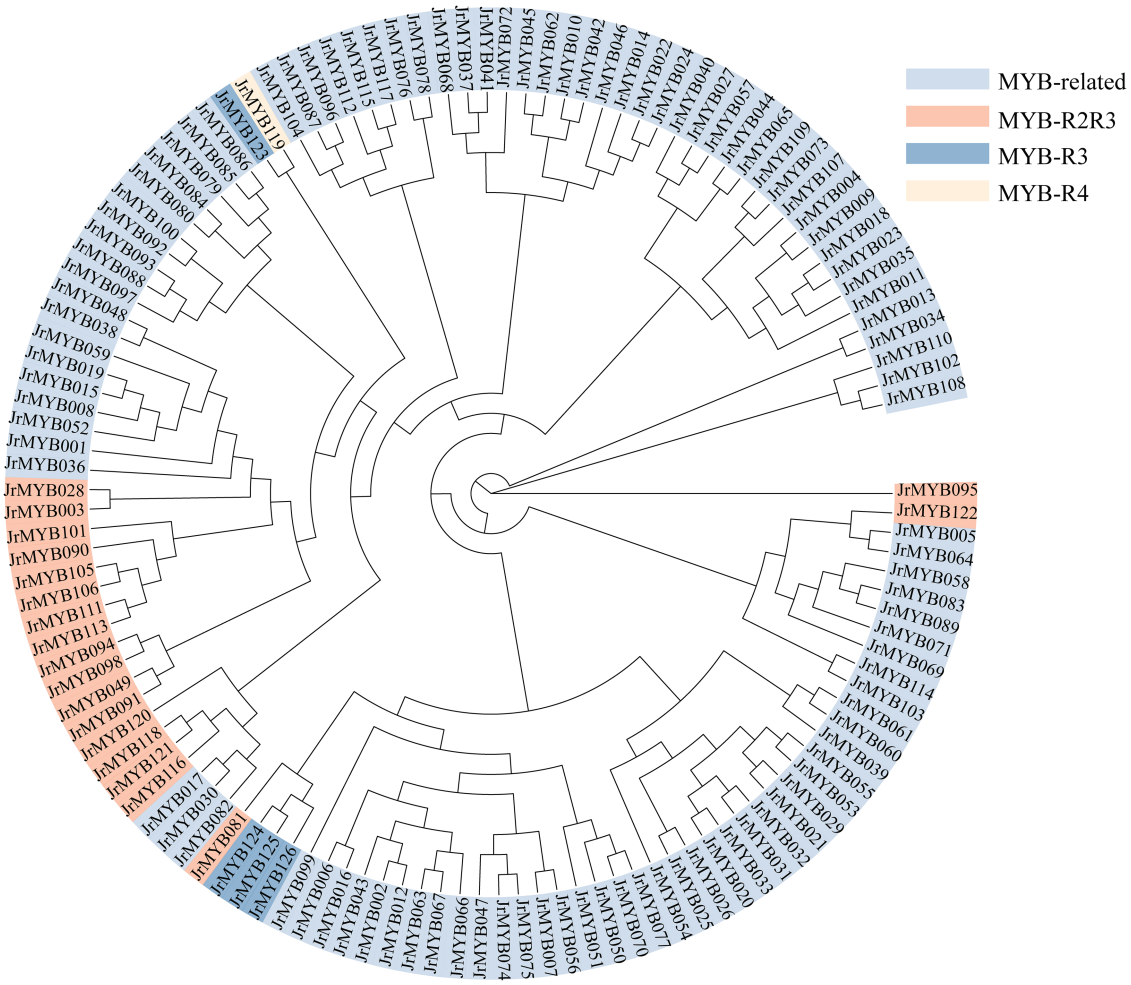

Figure 4: Phylogenetic analysis of *JrMYB* genes

To investigate the evolutionary relationships among *JrMYB* genes, phylogenetic trees were constructed using MEGA-X. These trees included 126 JrMYB gene family protein sequences and 126 AtMYB gene family protein sequences of known subgroups (Fig. 5).

JrMYB proteins were further divided into 17 subgroups, denoted as S1–S17, following the criteria used for classifying the Arabidopsis *MYB* gene family [7]. Among these subgroups, seven (S3, S4, S5, S7, S9, S15, and S16) included *JrMYB* genes that clustered with *AtMYB* genes. The remaining four subgroups, designated S1, S6, S11, and S12, contained *JrMYB* genes that were individually clustered. Notably, S1 contained only one gene, *JrMYB*095.

3.5 Genetic Structures, Conserved Motifs, and Characteristic Conserved Domains Analysis of JrMYB Genes

In this study, MEME software was used to scrutinize the conserved motifs present in the protein sequences encoded by the *JrMYB* genes, with the aim of gaining deeper insights into the structural characteristics of the 126 *JrMYB* genes. Ten conserved motifs, designated as motifs 1 through 10, were identified (Fig. 6). Among these, motif 1 was found in all 126 *JrMYB* genes, indicating that it was the most highly conserved motif within the family. Motifs 1 and 5 were detected in 73 *JrMYB* genes,

highlighting their significance in shaping MYB protein sequences. The graphical representation of the data highlighted that protein subclasses exhibiting comparable affinities for their targets were more conserved at the sequence level and displayed common structural elements. For instance, 17 family members situated in the same branch, characterized by motifs 1, 6, and 7, were likely to have similar structural functions. Likewise, 10 family members possessing motifs 1, 6, 8, 9, and 10 may share a common structural function. In contrast, the number and type of motifs varied among the different evolutionary branches.

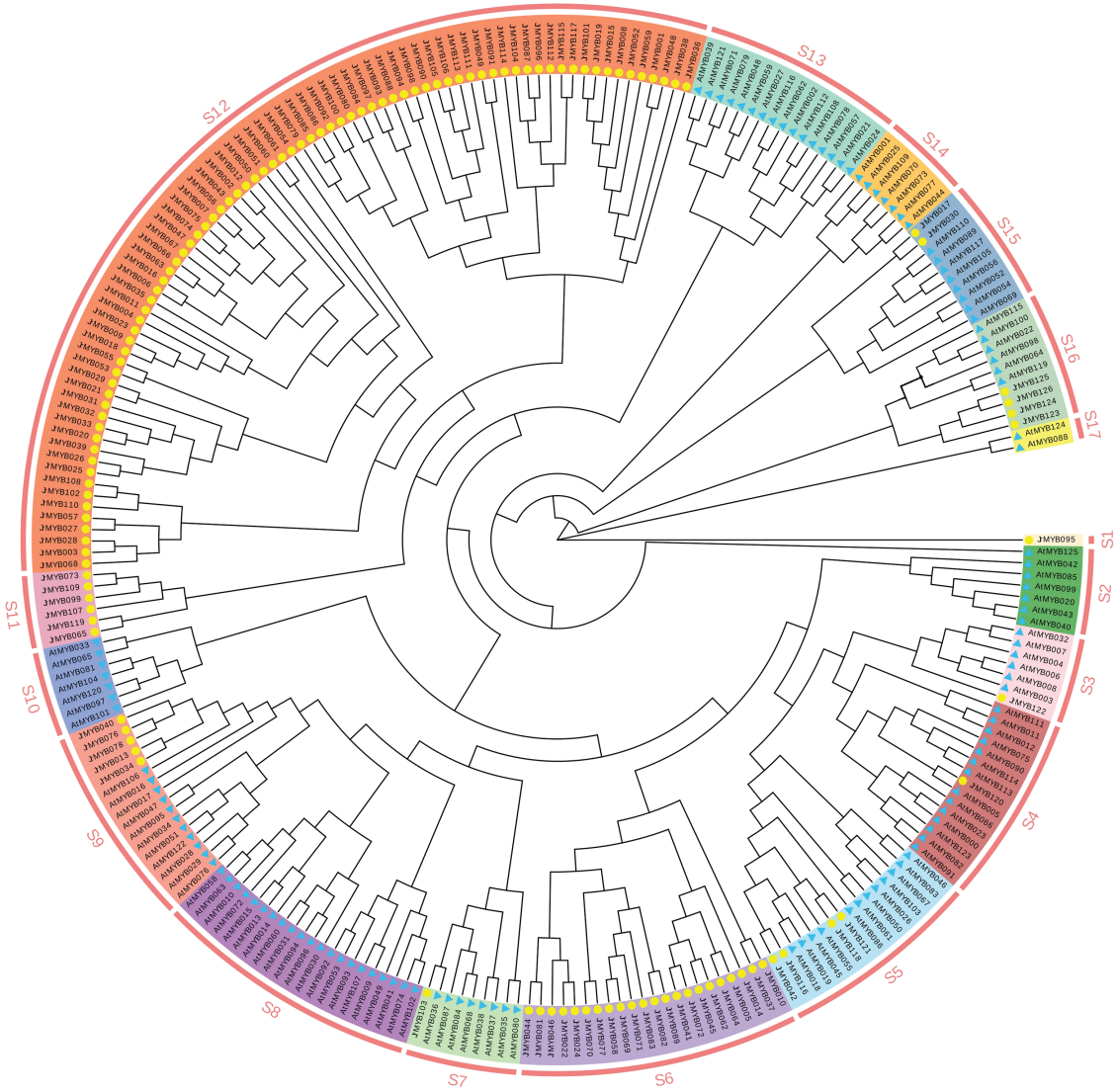

Figure 5: Phylogenetic relationship between *Juglans regia* L. and *A. thaliana MYB* families. The yellow circle represents *Juglans regia* L., blue triangle represents *A. thaliana*

Analysis of the walnut MYB protein sequence revealed the presence of ten structural domains, including *MYB*_DNA-binding, SANT, and *MYB*_CC-LHEQLE (Fig. 7). A comprehensive examination of 126 amino acid sequences using the CDD-search tool in NCBI and InterPro indicated that various members of the sequence possessed varying numbers and types of structural domains. The SANT and *MYB*_SHAOKYF domains were among the most frequently observed.

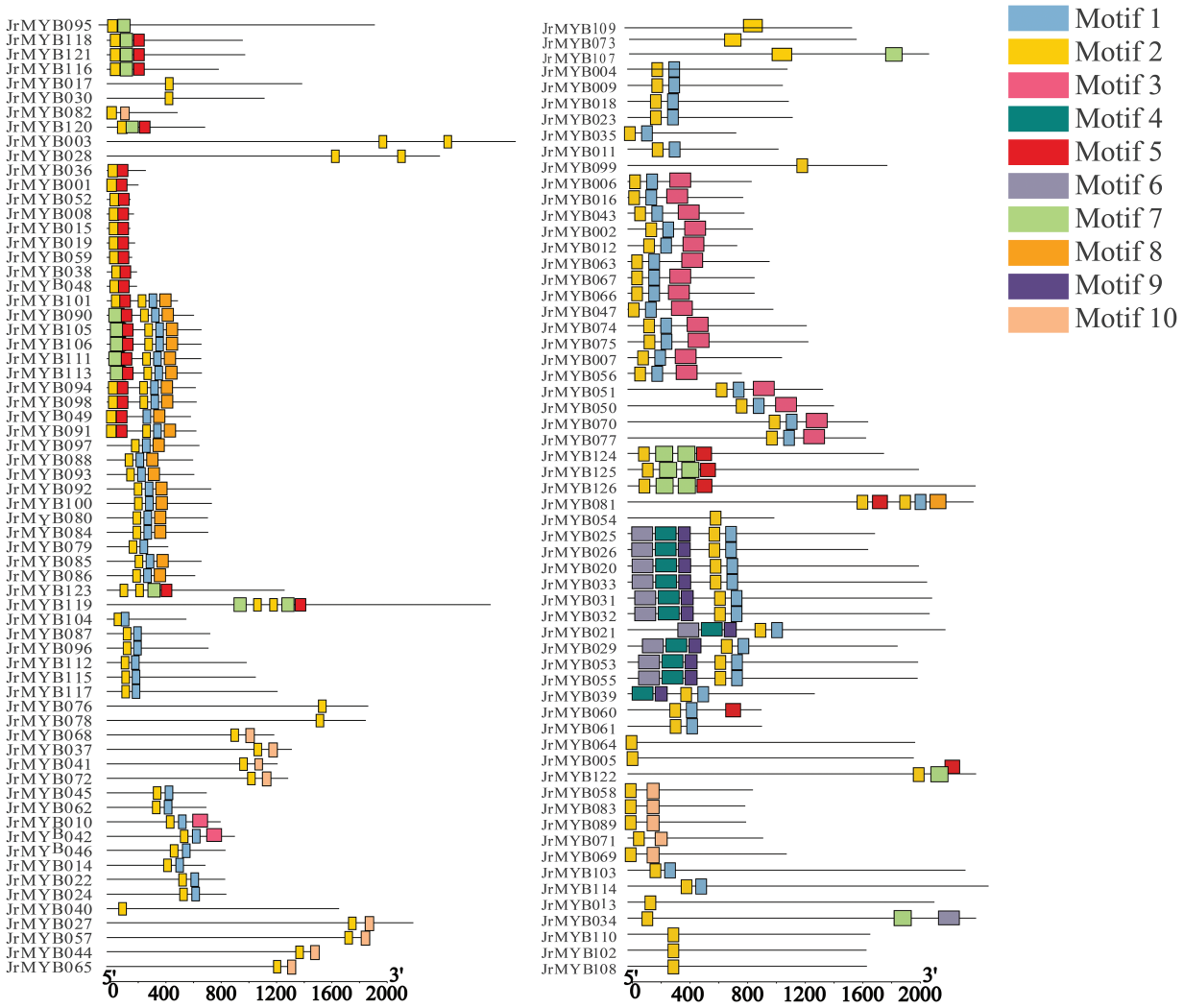

Figure 6: Conserved motifs of 126 *JrMYB* genes. Different colors signify different motiftypes

Furthermore, detailed gene structure analysis was conducted of all *JrMYB* genes (Fig. 8). The results revealed substantial variations in the number, distribution, and length of coding sequences among members, ranging from 1 to 20 in number and exhibiting diverse lengths. For instance, *JrMYB*013 and *JrMYB*034 featured 20 coding sequence regions, whereas *JrMYB*052 and *JrMYB*008 contained only one region. This variation underscores distinct evolutionary trajectories within the *JrMYB* genes, leading to diverse gene structures.

3.6 Cis-Acting Components Analysis of Promoters of JrMYB Genes

The findings revealed that the *JrMYB* genes predominantly featured four types of cis-acting components associated with cell development, light responsiveness, environmental stress responses, and phytohormone regulation (Fig. 9). Specifically, the four principal cis-acting components linked to cell development included the RY element, MSA-like, CAT-box, and GCN4_motif. These components were distributed across 66 genes, with CAT-box being the most prevalent, present in 56 genes.

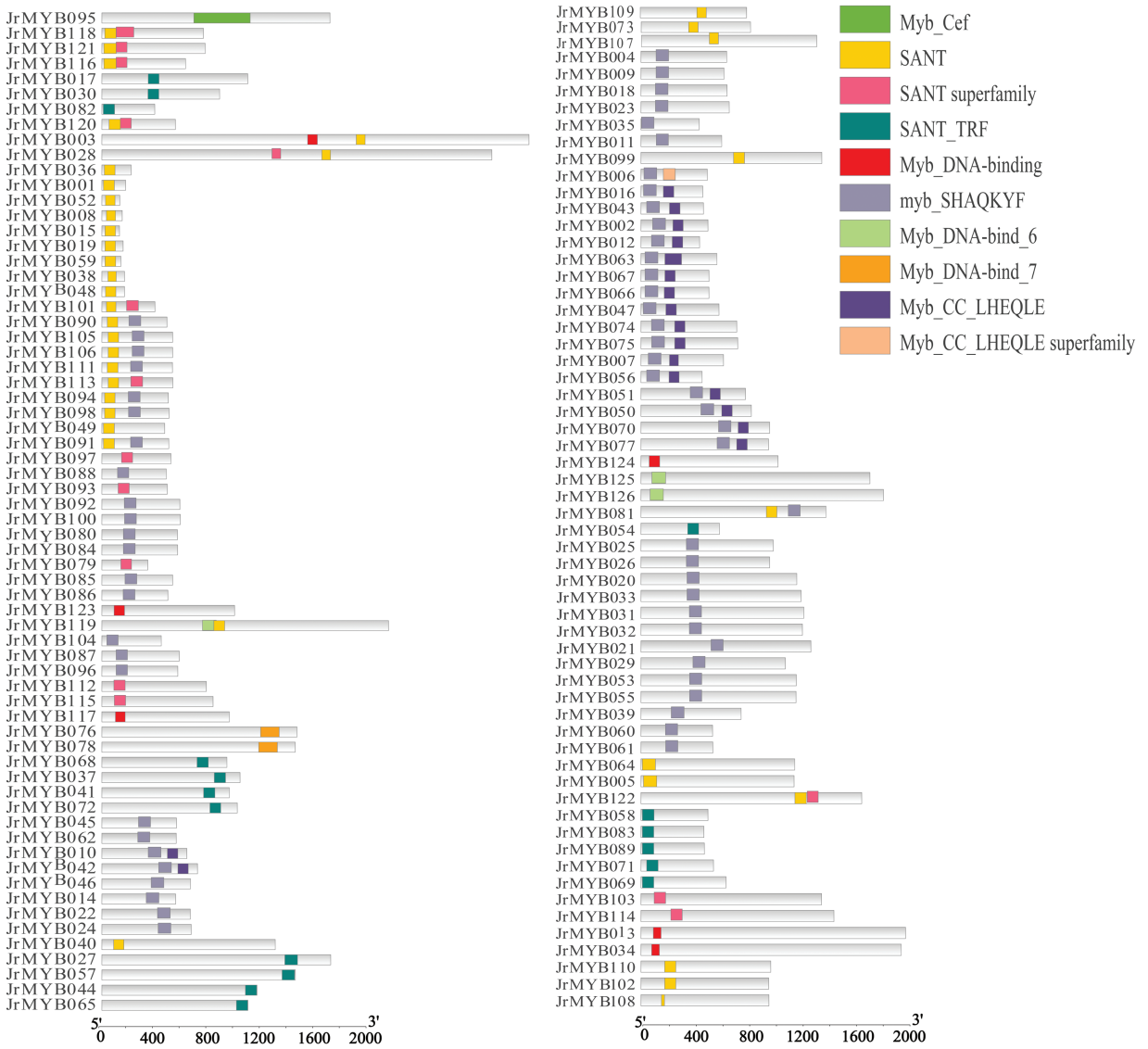

Figure 7: Characteristic conserved domains of 126 *JrMYB* genes

For light responsiveness, seven cis-acting components were used, including ACE, AE-box, AT1-motif, ATCT-motif, chs-CMA2a, Sp1, and G-box. Except for *JrMYB*019, all genes exhibited various light-responsive components. Notably, the analysis identified nine ATCT motifs in three genes (*JrMYB*023, *JrMYB*065, and *JrMYB*100) and *JrMYB*080 and *JrMYB*100 contained nine chs-CMA2a elements.

Six components, namely ARE, circadian rhythm, GC-motif, LTR, TC-rich repeats, and MBS, were associated with external defense and stress responses. Stress-related elements were identified in 120 genes, with ARE being the most prevalent and found in 102 genes. In the context of hormone response, six cis-acting components were observed: TCA element, TGA element, GARE motif, CGTCA motif, AuxRR core, and abscisic acid response element (ABRE). These components were primarily linked to responses involving abscisic acid (ABA), growth hormones (indole-3-acetic acid, IAA), methyl jasmonate (MeJA), and gibberellin (GA). Only nine *JrMYB* genes contained the AuxRR core element related to the growth hormone response, whereas 100 *JrMYB* genes featured the cis-acting component ABRE associated with the abscisic acid response.

Figure 8: Genetic structures of 126 *JrMYB* genes

3.7 Screening of JrMYB Genes Involved in Fatty Acid Synthesis

Using GC-MS to analyze the fatty acid content in the roots, stems, and leaves of walnuts, the data showed that the fatty acid content in the walnut roots was the lowest, at 32.43 mg/g, followed by the leaves at 36.39 mg/g, while the stems had the highest fatty acid content, at 61.73 mg/g. And the content of fatty acid Transcriptomic data [16] were used to explore the potential role of *JrMYB* genes in walnut fatty acid synthesis. The expression patterns of 103 *JrMYB* genes were analyzed across the major tissues of the roots, stems, and leaves, following the removal of 23 genes with low expression levels. A clustered heat map was employed to visually represent the results, successfully delineating the unique expression patterns observed, as shown in Fig. 10.

The *JrMYB* genes were categorized into three branches based on their expression patterns: Branch I: Comprising 28 genes that exhibited high expression in stems and low expression in leaves. Branch II: Encompassing 35 genes with high expression primarily in roots and low expression primarily in stems.

Branch III: Consisting of 40 genes that were highly expressed mainly in leaves, with exceptions like *JrMYB*073 and *JrMYB*058. The other 38 genes displayed little to no expression in the stems, and no gene displayed high expression in all three tissues, reflecting the distinct tissue-specific expression patterns within the *JrMYB* genes. Heat map analysis further revealed that 16 genes exhibited opposite expression patterns in roots, stems, and leaves, with a "high-middle-low" sequential decrease in expression. Conversely, 26 genes displayed similar expression patterns across these tissues, demonstrating a "low-middle-high" increase in expression. In the root-stem-leaf context, 16 genes exhibited the opposite expression pattern, transitioning from "high-middle-low." Among these *JrMYB* genes, 44 genes were found negative correlations with the content of fatty acid, 59 genes showed positive correlations. Especially, 23 *JrMYB* genes were found highly correlated with fatty acid at the level of $|r| \geq 0.9$. For example, *JrMYB*084 had a correlation of 0.9999, and *JrMYB*066 had a correlation of 0.9996 (Table S6). Additionally, we further constructed a phylogenetic tree of the *JrMYB* genes involved in fatty acid metabolism in other plants, showing that 24 *JrMYB* genes cluster with *MYB* genes of known functions, suggesting that these *JrMYB* genes may be involved in fatty acid metabolism. Based on the intersection of the results from both analyses, we have selected 10 candidate *JrMYB* genes that may be involved in the fatty acid metabolism of walnut (Fig. 11).

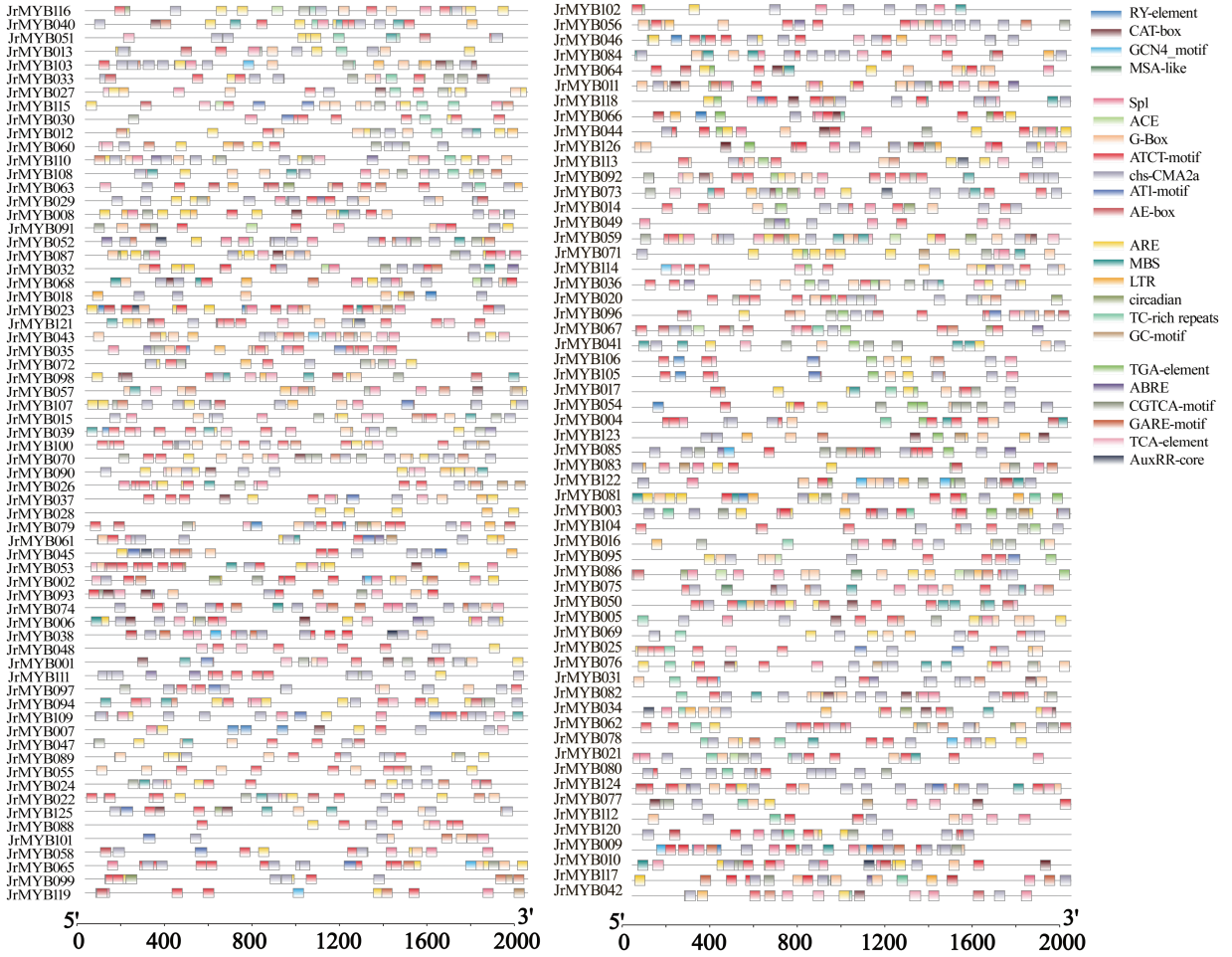

Figure 9: Analysis of cis-acting components of *JrMYB* genes

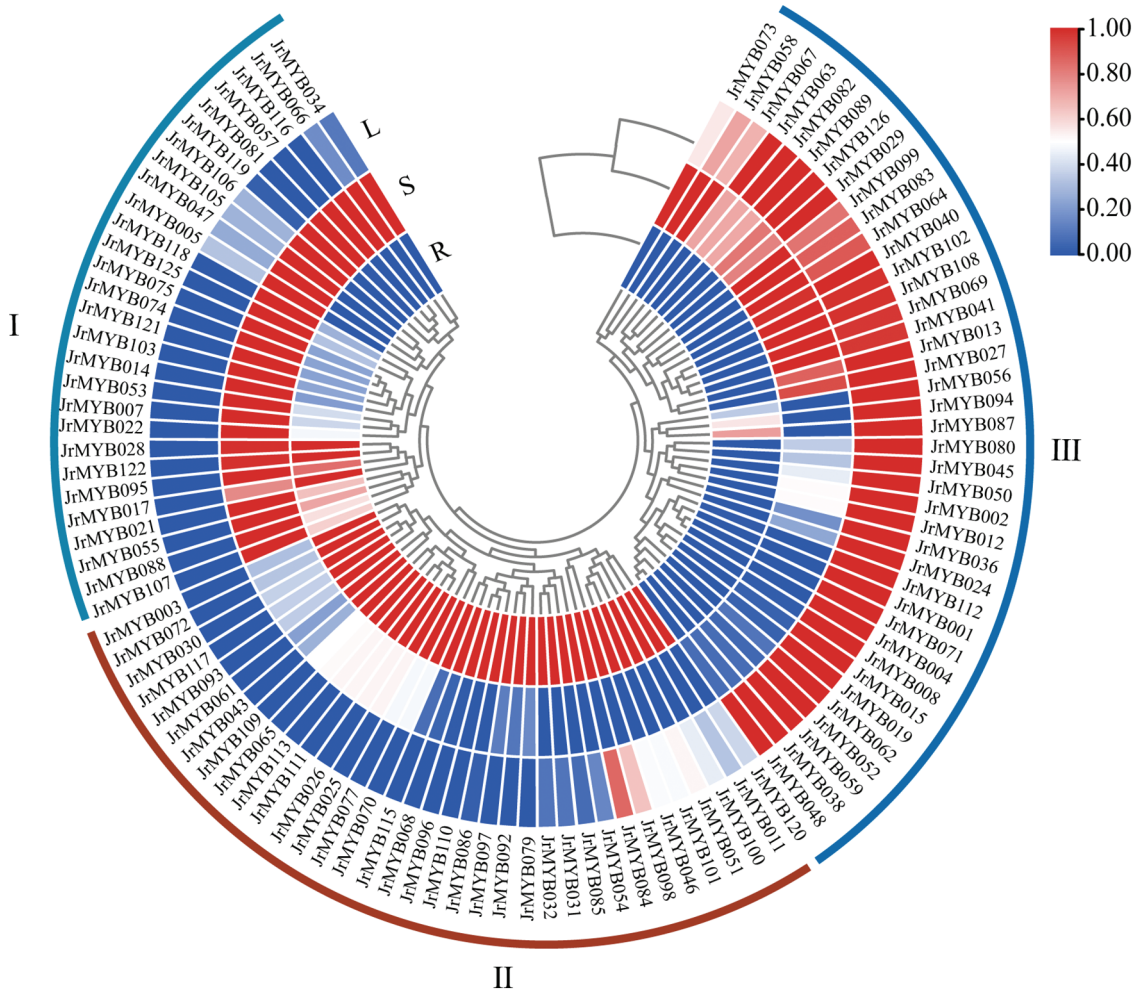

Figure 10: Fatty acid expression profile of *JrMYB* genes in different tissues. R: root; S: stem; L: leaf. Red areas indicate regions of high gene expression, and blue areas indicate regions of low expression in the corresponding tissues. The color coding for expression levels is shown on the right. The color scale, indicating expression levels, is provided on the the right of the heatmap

3.8 qRT-PCR Verification and Construction of the Transcription Regulatory Network

To further study the expression of walnut *JrMYB* genes in various tissues, we performed qRT-PCR on ten genes selected from transcriptomic data [16] and phylogenetic analysis, assessing their expression in walnut roots, stems, and leaves. As shown in the Fig. 12A, a correlation analysis between qRT-PCR data and transcriptome FPKM values yielded 3 genes with high correlation coefficients ($R^2 > 0.7$), which are considered key candidate genes for enhancing the fatty acid content in walnuts. To further investigate the regulatory mechanisms of *JrMYB* genes on fatty acid synthesis in walnuts, we projected the regulatory network Based on the *MYB* binding motifs (Table S7) found in the promoter regions of enzyme genes, we have identified three potential target genes that may be regulated by *JrMYB* genes (Fig. 12B,C).

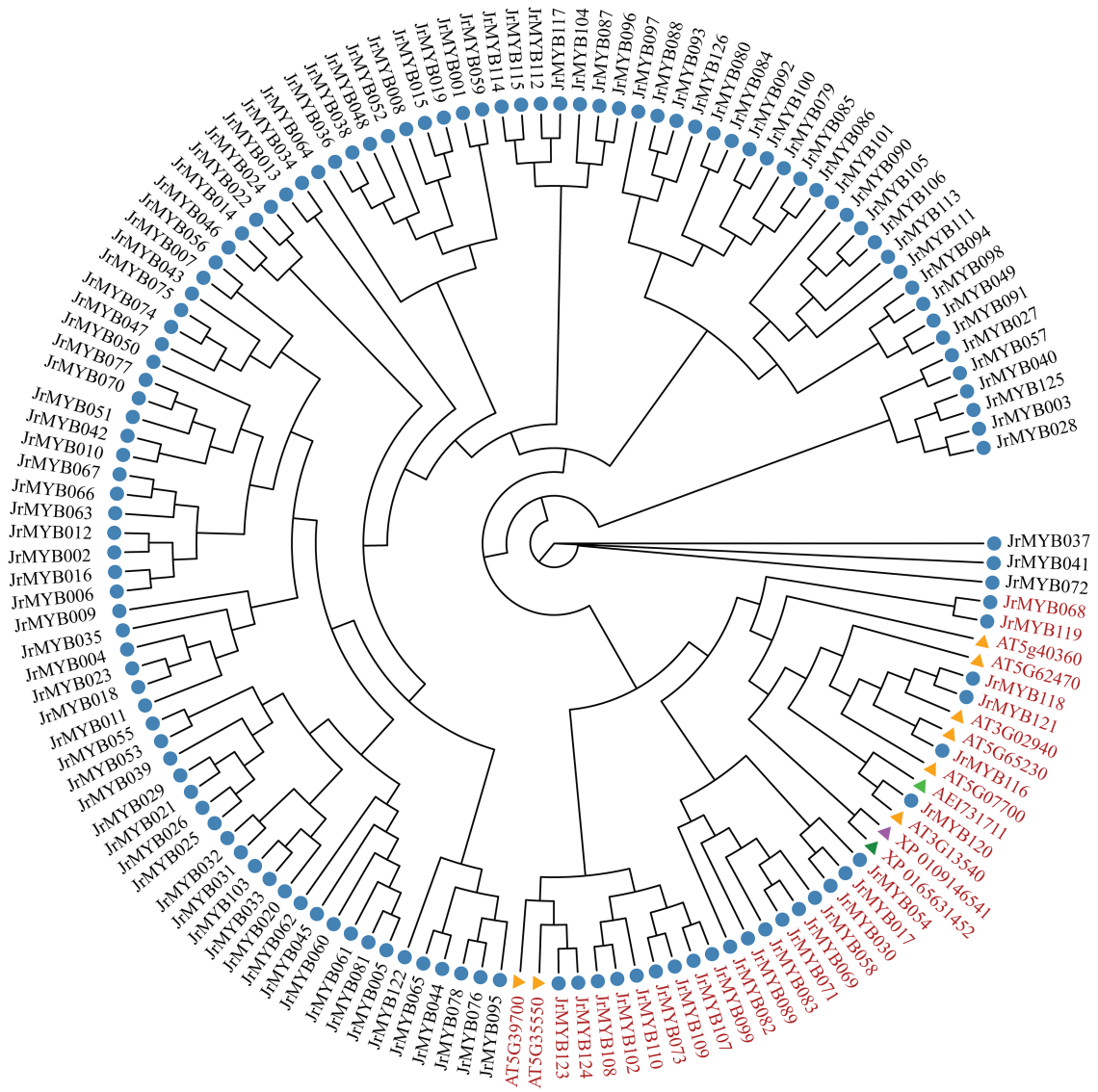

Figure 11: Phylogenetic relationship between *JrMYB* genes and multispecies fatty acid synthesis related *MYB* genes. Genes marked in red are related genes that may be involved in fatty acid synthesis. Blue circles represent *JrMYB* genes, yellow triangles represent *AtMYB* genes (*A. thaliana*), green triangles represents Jc*MYB* (*Jatropha curcas*), purple triangles represents Eg*MYB* (*Elaeis guineensis* Jacq.), and blue triangles represents Ca*MYB* (*Capsicum annuum* L.)

The results indicated that *JrMYB*120 has a targeting relationship with the promoter regions of 20 enzyme genes, *JrMYB*121 and *JrMYB*118 have targeting relationships with the promoter regions of 17 enzyme genes. The targeted enzyme genes are predominantly concentrated in *ACA1* (acetyl-CoA acyltransferase 1), *FADS6* (omega-6 fatty acid desaturase), and *ELOVL5* (elongation of very long chain fatty acids protein 5), among others.

4 Discussion

MYB gene family is a pivotal group of transcription factors in plants that plays a significant role in the regulation of plant biology. Although *MYB* genes have been extensively studied in various species, including

Arabidopsis [7], soybean [23], and citrus [24], their investigation in walnut has been relatively limited. In this study, we identified 126 members of the *MYB* family from the walnut genome. Through bioinformatics, we conducted a comprehensive analysis of the physicochemical properties, chromosomal localization, collinearity relationships, evolutionary relationships, gene structures, and cis-acting elements of these genes. By integrating transcriptomic data with qRT-PCR results, we ultimately selected 3 *JrMYB* genes that are likely involved in the synthesis of fatty acids in walnuts and predicted the transcription regulatory network involved in fatty acid biosynthesis. These results provide genetic resources for the selection of superior walnut strains with high fatty acid content, identifying candidate genes for the regulatory network of fatty acid production in walnut, and providing a theoretical foundation for further in-depth investigations in this area.

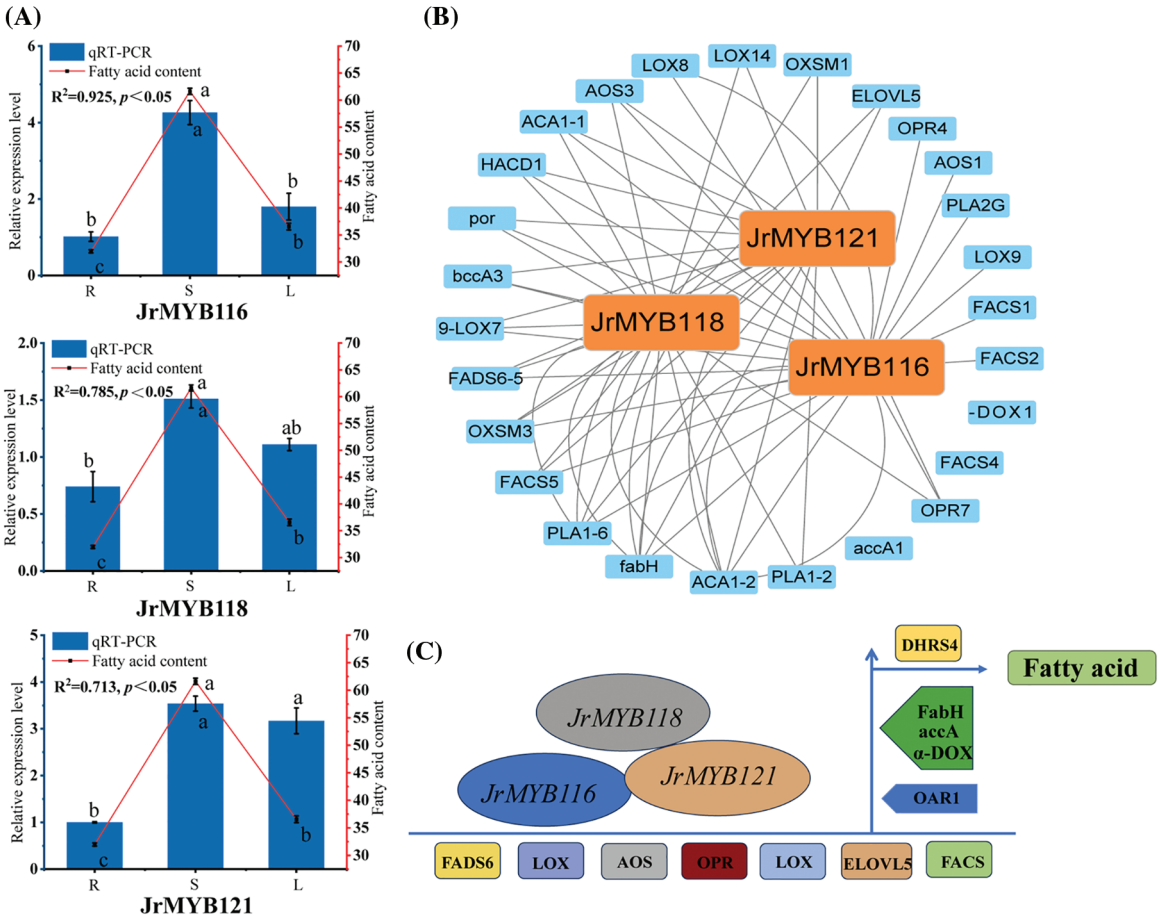

Figure 12: (A). Relative expression level of *JrMYB* genes in the roots, stems, and leaves of the walnut. (B). The transcription regulatory network of *JrMYB* genes involving in fatty acid biosynthesis. (C). Model for *JrMYB* genes and enzyme genes regulating fatty acid biosynthesis

4.1 The Abundant Walnut MYB Genes Exhibit Diverse Functions

The number of *JrMYB* genes identified in walnut was similar to that in *Arabidopsis thaliana* (168) and *Jatropha curcas* (115). However, there were significant differences when compared with the number of *MYB* family members in peanut (*Arachis hypogaea*, 31) and torch pine (*Pinus taeda*, 35), where the number of *MYB* family members displayed considerable variation. These differences could potentially be attributed

to chromosome rearrangements, fusions, and other genomic modifications as well as genome-wide duplication events [25]. Notably, all *MYB* genes in the walnut family are characterized as hydrophilic proteins. Assessment of instability coefficients indicated that the majority of walnut *MYB* family members were categorized as unstable proteins, with the exception of 11 stable genes. These findings align with similar observations made for *MYB* genes in other species such as *Lonicera macranthoides* [26], and *Lysimachia christinae* [27], suggesting that the *MYB* gene family has maintained a relatively conserved nature throughout evolution.

Furthermore, subcellular localization predictions revealed [28] that approximately 80% of *MYB* transcription factors were likely localized to the nucleus. This prediction aligns with the known functions of transcription factors, which play a pivotal role in controlling the expression of downstream genes [29]. Analysis of the conserved motifs and gene structures of *JrMYB* genes indicated that members within the same evolutionary tree branch of walnut shared similar conserved structural domains and motif compositions [30]. This observation lent credibility to the phylogenetic tree constructed in this study and, to some extent, laid the foundation for predicting gene function [31]. Additionally, the distribution of the majority of genes at both ends of the 16 chromosomes in walnut aligns with the chromosome distribution patterns observed in *MYB* family members of other plants such as potato [32], bell cherry [33], and cocoa [34].

4.2 Phylogenetic Relationship Analysis and Functional Exploration of JrMYB Genes

In general, genes that share evolutionary relationships are expected to have functional similarities [35], and phylogenetic analysis serves as a valuable reference for family level investigations. Based on the results of phylogenetic analysis, certain *JrMYB* genes exhibited clustering patterns with specific *AtMYB* genes, suggesting potential functional similarities.

*JrMYB*017 and *JrMYB*030 were found in the same branch as *AtMYB*052, a gene known to control the expression of ABA-responsive genes, and consequently, cell wall biosynthesis [36]. This suggests that *JrMYB*017 and *JrMYB*030 may play a role in regulating cell wall biosynthesis. *JrMYB*040, *JrMYB*076, *JrMYB*017, *JrMYB*013, and *JrMYB*034, which are grouped in the same branch as *AtMYB*17, might share functions related to early inflorescence development and seed germination [37], similar to *AtMYB*17. *JrMYB*120 and *AtMYB*111, situated in the same branch, may have comparable functions in promoting genes within the phenylpropane pathway, potentially enhancing flavonol production [38]. *JrMYB*017 and *JrMYB*030 clustered together and could be associated with fatty acid synthesis, as *AtMYB*089 is involved in seed oil and major fatty acid biosynthesis [39]. Similarly, *AtMYB*076 [40], which belongs to the S9 group of the evolutionary tree, was linked to the regulation of fatty acid synthesis, suggesting that *JrMYB*040, *JrMYB*076, *JrMYB*078, *JrMYB*013, and *JrMYB*034 may play a role in fatty acid synthesis regulation.

S1, S6, S11, and S12 represent walnut species-specific groupings, which differ from the classification of the Arabidopsis *MYB* family. These distinctions may be attributed to variations or divergences that arose during evolutionary processes in response to environmental adaptations between walnut and *Arabidopsis thaliana* [41]. It is hypothesized that the functions of *MYB* family members within these branches are specific to walnut, a characteristic also observed in cherry *MYB* genes.

Furthermore, the distribution of multiple *JrMYB* genes within most subgroup branches suggests a potential functional redundancy within these *MYB* transcription factors. This redundancy implies that multiple genes in the same branch may have similar or overlapping functions, and silencing or inactivating a single gene may not significantly affect the overall function or expression of this particular group of genes [42].

4.3 Functional Exploration of Promoter Regions of JrMYB Genes

In this study, cis-acting components were analyzed by examining the 2000 bp genomic sequence upstream of the genes and classifying the identified cis-acting components into four main categories. This analysis aimed to gain insights into the functions of *JrMYB* genes, as the distribution and types of cis-acting components in the promoter region play a crucial role in determining gene expression and function [43]. Six dealt with hormone response, six with stress response, seven with light response, and four with cell development. The findings from this analysis suggest that *JrMYB* genes may play significant roles in the light and stress response mechanisms in walnut. Notably, almost all genes except *JrMYB*018 were found to have light response elements in their promoter regions. Furthermore, stress-related components were abundant and present in all 120 genes, indicating their potential involvement in the stress response pathways.

Among the hormone-related cis-acting components, ABRE was identified in 100 genes, highlighting its importance in the regulation of *JrMYB* genes expression. However, the growth hormone response-related AuxRR-core element was found in only nine genes, suggesting that these specific genes may be associated with the regulation of growth hormone metabolism. We analyzed the cis-acting elements of 10 potential genes and found that both *JrMYB*054 and *JrMYB*118 contain the RY-element (CATGCATG). Previous studies [44] have shown that the *AtLEC2* can bind to the RY-G-Box and RY elements in the upstream promoter regions of the storage protein gene *At2S3* [45], thereby regulating downstream genes and enhancing oil content in seeds. This implies that the RY-element may be a key component in the regulation of fatty acid synthesis.

Additionally, this study revealed the presence of various cis-acting components related to hormone responses, and their distribution varied considerably among the *JrMYB* genes. This diversity in response elements underscores the complexity and specificity of the regulation of *JrMYB* genes expression [46].

4.4 Mining and Identification of JrMYB Genes Related to Fatty Acid Synthesis in Walnut

In *A.thaliana*, *Brassica napus*, and *Elaeis guineensis*, the role of *MYB* in modulating fatty acid production has been extensively documented. However, limited attention has been directed towards its role in walnut. The co-regulation of *AtAAD2* and *AtAAD3* expression in *A. thaliana* by *AtMYB*118, along with its homolog *AtMYB*115, has been found to significantly enhance monounsaturated fatty acid production in the endosperm [47]. Subsequent research has indicated that *EgMYB*108 can effectively bind to the promoters of *EgLACS* and *EgKCS*, resulting in enhanced fatty acid content [48]. Similarly, overexpression of *CaMYB340* in sweet orange resulted in a reduction in fatty acid desaturation [17].

In this study, a comprehensive examination of 103 genes related to fatty acid synthesis was conducted using correlation analysis. Among these genes, 54 *JrMYB* genes exhibited a positive correlation, whereas 49 *JrMYB* genes displayed a negative correlation. The gene expression patterns revealed that 126 *JrMYB* genes lacked a common highly expressed gene in the three tissues studied, indicating tissue-specific expression heterogeneity among the *JrMYB* genes. To further elucidate the role of *JrMYB* genes in fatty acid synthesis, phylogenetic analyses were performed using the aforementioned *MYB* genes (*AtMYB92* (AT5G62470) [14], *CaMYB340* (XP_016563145.2) [17], *JcMYB1* (AEI73171.1) [18], *EgMYB108* (XP_010914654.1) [49]). This analysis allowed for the identification of 24 genes that are potentially involved in fatty acid synthesis. Among these 24 genes, ten exhibited high correlation coefficients. Notably, *JrMYB*116, *JrMYB*118, *JrMYB*119, and *JrMYB*121 displayed correlation coefficients exceeding 0.9, suggesting that they were important genes involved in fatty acid synthesis.

Following qRT-PCR verification of 10 candidate genes, we identified 3 key genes. The data show distinct differences in the expression levels of most genes in the roots, stems, and leaves of the walnut,

aligning with the transcriptomic data [16] and further indicating that *JrMYB* genes have tissue specificity [50]. The transcription regulatory network suggests that *JrMYB*116, *JrMYB*118 and *JrMYB*121 can bind to the promoter regions of enzyme genes involved in the fatty acid biosynthetic pathway, thereby influencing gene expression and enhancing the fatty acid content in walnuts. This finding is in line with this finding is in line with the research conducted by Xu et al. [47], who demonstrated that the overexpression of *EgMYB*108 can elevate lipid levels in fruit. Their study further confirmed that *EgMYB*108 is capable of binding to the promoters of *EgLACS* and *EgKCS*, which in turn enhances the content of fatty acids.

5 Conclusions

In this study, the walnut genome served as the foundation for the identification of 126 *JrMYB* genes, which underwent a comprehensive analysis of their physicochemical properties, conserved motifs, gene structure, and gene architecture using bioinformatic methodologies. To establish phylogenetic relationships, Arabidopsis *MYB* family genes were employed, resulting in the categorization of *JrMYB* genes into 17 distinct subgroups. The chromosomal distribution of these 126 *JrMYB* genes across 16 walnut chromosomes was elucidated through chromosomal localization. Results from interspecies covariance analysis revealed a close relationship between walnut and pecan, with a covariance analysis of the 126 *JrMYB* genes identifying 50 family members displaying covariance within the walnut species. Cis-acting component analysis indicated that the promoters of these 126 *JrMYB* genes primarily contained four types of cis-acting components, each of which was predicted to play various roles in walnut growth and development.

By employing correlation analysis, gene expression profiling, phylogenetic systematics, and qRT-PCR analysis, we identified 3 *JrMYB* genes that are likely involved in fatty acid synthesis and constructed a transcription regulatory network. Collectively, the findings of this study provide a solid foundation for the functional identification and mechanistic study of MYB gene regulation in fatty acid biosynthesis in walnuts, as well as for enhancing the content of unsaturated fatty acids in walnuts.

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Ethics Approval: Not applicable.

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