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The MYB Transcription Factor Family in *Eucommia ulmoides*: Genome-Wide Identification, Characterization, and Network Analysis in Relation to the Rubber Biosynthetic Genes

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Abstract: The MYB transcription factor family is one of the largest families of plant transcription factors (TFs), and it plays a vital role in the entire process of a plant's growth and development. Well known in China, *Eucommia ulmoides* (*E. ulmoides*) produces a form of natural rubber called *Eucommia ulmoides* gum (EUG). Nevertheless, there is little research on the evolutionary history and expression patterns of its MYBs, as well as on the regulation of EUG by MYB TFs. This research provides a comprehensive description, classification, and potential functional analysis of the EuMYB gene family. A total of 119 MYB members of *E. ulmoides* were identified based on the whole genome sequencing data, and their gene structure, phylogenetics, chromosome location, conserved motifs, etc., were analyzed. Based on the phylogenetic tree results, EuMYBs could be divided into 35 subgroups. In addition, chromosomal localization and collinearity analysis revealed the heterogeneous distribution of the MYB family in the *E. ulmoides*' genome, indicating the expansion of its gene family. Moreover, promoter cis-acting elements showed that the promoter contained abundant light-responsive elements, anaerobic-induction-responsive elements, and abscisic-acid-responsive elements. A co-expression regulatory network between the EUG biosynthesis genes and the EuMYBs was built. Meanwhile, regarding the six EuMYBs with high expression in the gum-forming tissues selected that correlated with the farnesyl diphosphate synthase (*FPS1*) structural gene, RT-qPCR experiments showed a possible regulatory relationship between EuMYBs and *FPS1*, which played an important role in EUG biosynthesis. In conclusion, this paper defines a research gap and lays a foundation for further studies on the biological functions of EuMYBs.

Keywords: *Eucommia ulmoides* (*E. ulmoides*); MYB transcription factors (MYB TF); gene expression; *Eucommia ulmoides* gum (EUG); bioinformatics

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1. Introduction

The plant transcription factor MYB (v-myb avian myeloblastosis viral oncogene homolog), being one of the largest families of plant TFs, is associated with the regulation of a plant's growth, development, and physiological metabolic processes [1]. MYB TFs are characterized by the presence of a highly conserved DNA-binding structural domain (MYB DNA binding) at the N-terminus, which contains 1–4 semi-conserved motifs (R), each consisting of approximately 50 to 53 amino acid residues and spacer sequences. These amino acid residues allow the MYB's structural domain to fold into a helix-turn-helix (HTH) structure [2]. Based on the number of R residues in the structural domain, MYB TFs can be divided into four subfamilies: 1R-MYB, R2R3-MYB, 3R-MYB, and 4R-MYB [3].

The first MYB gene in plants was cloned from *Zea mays* by Paz–Ares in 1987, when the researchers found that the gene was associated with pigment synthesis [1]. Since then, a large number of MYB TFs have been isolated and identified in plants. Studies have shown that members of the R2R3-MYB TF family anchored by the endoplasmic reticulum

in *Arabidopsis* are closely associated with root hair development [4–6]. *AtMYB16*, *AtMYB17*, and *AtMYB106* are, respectively, involved in the regulation of trichome branching, petal epidermal cell morphogenesis, and early inflorescence development [7–9]. A group of researchers transformed the *Arabidopsis* MYB TF *pap1* into a petunia and found that it resulted in darker flowers, a stronger fragrance, and significantly higher content of various volatile hydrocarbon complexes [10]. The *MYB7* gene in kiwifruit regulates carotenoid and chlorophyll biosynthesis [11]. The over-expression of *AtMYB75* in *Arabidopsis* increases secondary metabolites, such as anthocyanins and flavonols, to protect against pests [12]. The *HblMYB44* within the rubber tree is involved in the regulation of the natural rubber biosynthesis process [13]. The MYB TFs in *E. ulmoides* leaves regulate the accumulation of secondary metabolites such as chlorogenic acid [14]. They are also involved in the expression of genes related to biosynthesis, such as anthocyanins and flavonoids [15]. Based on the findings above, the MYB TF family is widely involved in plants' biological development processes, hormone signaling, and primary and secondary metabolism [16–19].

Eucommia ulmoides Oliv. is a well-known traditional Chinese medicine species, as well as an important rubber source, with *Eucommia ulmoides* gum (EUG) contained in its peel and leaves [20–22]. As a new polymer material, EUG has excellent thermoelasticity, low-temperature plasticity, and functions of wear and corrosion resistance [23,24]. EUG is known to be synthesized from isoprenyl diphosphate through two pathways, namely the mevalonate (MVA) pathway and the methylerythritol-phosphate (MEP) pathway [25–28]. Candidate genes involved in the EUG biosynthesis pathway were identified in the genome by researchers, including 13 genes involved in six reactions of the MVA pathway, 11 genes involved in seven reactions of the MEP pathway, and 12 genes involved in the initial reactions for the production of initiators or precursors, which include geranyl diphosphate synthases (GPSs), geranylgeranyl diphosphate synthases (GGPSs), farnesyl diphosphate synthases (FPSs), and so on [29]. Among them, *FPS1* is the key rate-limiting enzyme for rubber biosynthesis [29]. This was also confirmed by studies conducted on Brazilian rubber trees [30]. Researchers found that the MYB transcription factor regulates the expression of key enzymes in rubber synthesis [31]. Studies on *Hevea brasiliensis* suggested that the MYB transcription factors may have important regulatory roles in the response to trauma, as well as to ethylene and jasmonic acid, further affecting the process of rubber production [32]. However, to date, systematic studies on the MYB gene family of *E. ulmoides* have not been reported, and studies on the possible potential regulatory role of EuMYB in the biosynthesis of EUG have not been seen.

The completion of the whole genome sequencing of *E. ulmoides* has laid a strong foundation for the systematic study of the role of the MYB TF family in *E. ulmoides* [33]. In this work, we carried out the identification of MYB gene family members and bioinformatics analysis based on the whole genome sequencing of *E. ulmoides* and the transcriptome sequencing data of different tissues. The results suggest that MYB members may have a wide range of regulatory potential for *E. ulmoides*. Meanwhile, we established a co-expression network of EuMYB members with EUG biosynthesis genes, and we screened six EuMYB members with potential regulatory functions for *FPS1*, which is the key rate-limiting enzyme for gum formation. Combined with RT-qPCR experiments, we hypothesize that MYB TFs may have regulatory potential for EUG biosynthesis. This study lays the foundation for further studies on the biological functions of the EuMYB family and the genetic improvement of *Eucommia ulmoides* plants.

2. Materials and Methods

2.1. Plant Materials

Various 10-year-old healthy pest-free tissue samples (leaf, xylem, seed, and peel) of the diploid *E. ulmoides* were selected from the forest tree seed breeding base in Wei County, Hebei Province, China.

2.2. Data Source

The genome sequence, protein sequences, and annotation files of *Eucommia ulmoides* were downloaded from the Genome Warehouse (https://ngdc.cncb.ac.cn/gwh/Assembly/25206/show, accessed on 1 March 2023). The RNA-seq data of different tissues have been reported, as described in detail in our previous work, and they can be accessed with accession number PRJNA599775 (uploaded 15 January 2021) [29].

Different tissues, including the mature leaf, peel, xylem, and seed, of the 10-year-old diploid *E. ulmoides* trees were used for the extraction of RNA to perform RNA-seq experiments. Three biological replicates were used for each tissue. A total of 3 µg RNA per sample was used as input material for the RNA sample preparation. Sequencing libraries were generated using the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (Illumina, San Diego, CA, USA) following the manufacturer's recommendations, and indexing sequences were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After the cluster generation, the library preparations were sequenced on an Illumina HiSeq platform, and 125 bp/150 bp paired-end reads were generated. Raw data in fastq format were first processed through in-house Perl scripts. In this step, clean reads were obtained by removing reads containing adapter sequences, reads containing poly-N sequences, and low-quality reads from the raw data. Additionally, the Q20, Q30, and GC content and the clean data were calculated. All downstream analyses were based on clean data with high quality. The index of the reference genome was built using Bowtie [34], and paired-end clean reads were aligned to the reference genome using TopHat (http://ccb.jhu.edu/software/tophat/index.shtml, accessed on 1 November 2020). HTSeq v0.6.1 was used to count the read numbers mapped to each gene [35]. Then, the FPKM of each gene was calculated based on the length of the gene, and the read count mapped to this gene. RNA-seq data derived from different tissue types were assembled with Trinity [36], and the assembled sequences were aligned against the *E. ulmoides* genome by PASA [37].

2.3. Identification and Characterization of the EuMYB Family

The hidden Markov model profile of MYBs with accession number PF00249 was downloaded from the Pfam database [38]. The file produced was used to build the hidden Markov model profile of the MYB domains by HMMER (http://hmmer.org/, accessed on 4 March 2023). Using the MYB domain and protein sequence of *Arabidopsis thaliana* as templates, the protein database derived from genome sequencing data was subjected to Blastp comparison (TBtools v1.09876, E-value 1×10^{-5}) [39], and the assumed EuMYB proteins were preliminarily screened out. Using SMART (http://smart.embl-heidelberg.de/, accessed on 8 March 2023) to identify the conserved sequence of these proteins [40], we deleted the members that did not contain the MYB protein characteristic domain or had low confidence in the characteristic domain, checked the redundancy of candidate proteins, and named them according to the NCBI Blast result (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 14 March 2023). The ExPASY server was used to analyze the characteristics and basic physical and chemical properties of EuMYB family proteins (https://web.expasy.org/protparam/, accessed on 20 March 2023) [41].

2.4. Phylogenetic Analysis of the EuMYB Family

To study the phylogenetic relationship of the EuMYB gene family, based on the selected MYB protein sequences of *E. ulmoides* and *Arabidopsis thaliana*, we aligned all protein sequences using ClustalW with the default parameters. Then, a phylogenetic tree (1000 bootstrap replicates) was constructed by using the maximum likelihood method (ML) of the MEGA-X software (v10.0.5, https://www.megasoftware.net/, accessed on 26 March 2023) [42]. The parameters were all defaulted, and the phylogenetic tree was visualized using iTOL (https://itol.embl.de/tree/125755724220760163383959 6, accessed on 18 April 2023) [43].

2.5. Chromosome Location and Collinearity Analysis of the EuMYB Family

Map Gene 2 Chromosome (MG2C, v2.1, http://MG2C.iask.in/MG2C_v2.1/, accessed on 28 March 2023) was used to map the chromosome distribution of the MYB genes in *E. ulmoides* [44]. Files relevant to the *E. ulmoides* genome were downloaded from the Genome Warehouse (https://ngdc.cncb.ac.cn/gwh/Assembly/25206/show, accessed on 1 March 2023), and TBtools was used to perform self-alignment of the *E. ulmoides* genome sequence and copy analysis and display of the EuMYB gene family [39]. The genome-relevant files of *Arabidopsis thaliana*, *Vitis vinifera*, *Sorghum bicolor*, and *Coffea canephora* were downloaded from the Plant TFDB database (http://planttfdb.gao-lab.org/, accessed on 22 April 2023), and the whole genome sequence of *E. ulmoides* was compared with that of *Arabidopsis thaliana* (model plant), *Vitis vinifera* (dicotyledon), *Sorghum bicolor* (monocotyledon), and *Coffea canephora* (dicotyledon), using TBtools (v1.09876) to analyze the collinearity of genes and display the results [39].

2.6. Analysis of Conserved Motifs, Gene Structures, and Domains of the EuMYB Family

Multiple Expectation Maximization for Motif Elicitation (MEME, v5.5.1, http://meme-suite.org/tools/meme, accessed on 1 May 2023) was used to analyze the conserved motifs of the MYB protein family in *E. ulmoides* [45]. The motif parameter was set to 10. The motif width was between 6 and 50 (inclusive), which further clustered the obtained results. Finally, the conserved motifs, gene structures, and domains obtained were combined with phylogenetic trees for display.

2.7. Analysis of Cis-Acting Elements of EuMYB Family Promoters

The 2000-bp sequence upstream of the initiation codon of each EuMYB family gene was extracted, and cis-acting element prediction analysis of the promoter was conducted on the PlantCARE website (http://BioInformatics.psb.ugent.be/webtools/PlantCare/html/, accessed on 15 May 2023) [46]. The results were displayed visually by TBtools (v1.09876) [39].

2.8. Gene Expression of the EuMYB Family in Different Tissues

To survey the expression patterns of EuMYB genes in different tissues, the transcriptome data of *E. ulmoides* in various tissues (leaf, xylem, seed, and peel) were obtained from the NCBI sequence read archive (SRX7525252-54, SRX7532003-05, SRX7531725-27, and SRX7533248-50) [29]. The transcript abundance of *E. ulmoides* genes was calculated as fragments per kilobase of the exon model per million mapped reads (FPKM). The expression values of all EuMYB family members (FPKM values) were selected (Supplementary Table S5), the values were logarithmically (Log2) analyzed statistically, and the EuMYB members were clustered to reflect EuMYB gene expression.

2.9. EuMYB and EUG Biosynthesis Gene Co-Expression Network Construction

It has been reported that at least 52 structural genes in *E. ulmoides* are involved in the biosynthetic pathway of EUG [29]. To study the relationship between EuMYB genes and EUG biosynthesis structural genes in *E. ulmoides*, we used the FPKM of these genes (Supplementary Table S6) to construct the network in OmicStudio (https://www.omicstudio.cn/tool/62, accessed on 18 May 2023). Genes with a Pearson's correlation coefficient within the appropriate range ($|r| \geq 0.60$ and $p < 1$) were selected to generate a co-expression network using Cytoscape (v3.9.0). The connectivity degree of genes was calculated using the Cytoscape software.

2.10. EUG Synthesis-Related EuMYB Expression Analysis by RT-qPCR

An RT-qPCR experiment was designed for EuMYBs related to *FPS1* in gum-containing (leaf, peel) and non-gum-containing tissue (xylem, seed). Total RNA extracted from different *E. ulmoides* tissues was reverse-transcribed into cDNA using a cDNA Synthesis Kit (Tiangen, Beijing, China, Cat KR106). Quantitative real-time polymerase chain reaction (RT-qPCR) assays were performed using TransStart Top Green qPCR SuperMix (TRANSGEN,

Beijing, China, cat AQ132-22) on the Applied Biosystems 7500 real-time PCR system according to the manufacturer's manual. Three biological replicates were performed for each tissue sample. *UBC E2* was used as a reference gene [47]. All primers used in this study were designed by Primer3plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi, accessed on 18 July 2023). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression levels.

3. Results

3.1. EuMYB Family Identification and Characterization

By using the MYB domain and the MYB protein sequence of *Arabidopsis thaliana* as templates, the protein database of *E. ulmoides* was compared by Blast, and 137 EuMYB family proteins were preliminarily identified. The screened proteins were identified using SMART to identify their conserved sequences, and sequences without the characteristic structural domain of MYB proteins and those with low confidence in the characteristic structural domain were deleted to obtain 119 final EuMYB family gene sequences, which were renamed *EuMYB1–EuMYB119* (Supplementary Table S1). Then, we analyzed the chromosome number, amino acid number (aa), molecular weight (MW), theoretical isoelectric point (pI), instability index, aliphatic index, and grand average of hydropathicity (GRAVY) of each family member based on the protein sequences.

Our study found that the number of amino acids in EuMYB family proteins ranges from 76 (*EuMYB56*) to 1641 (*EuMYB76*), with an average length of 428.4 and a large variation interval. The molecular weight ranges from 8.99 KDa (*EuMYB56*) to 178.92 KDa (*EuMYB76*), the theoretical isoelectric point ranges from 5.01 (*EuMYB39*) to 10.75 (*EuMYB56*), the instability index ranges from 20.81 (*EuMYB43*) to 78.70 (*EuMYB85*), and the aliphatic index ranges from 48.63 (*EuMYB97*) to 82.45 (*EuMYB69*). In addition, combining the theoretical PI and instability index, we found that the EuMYB family contains three acidic stable proteins (2.5%), seven acidic unstable proteins (64.77%), 38 basic unstable proteins (31.93%), and one neutral unstable protein (*EuMYB3*, 0.8%).

3.2. Phylogenetic Analysis of the EuMYB Family

To investigate the phylogenetic relationships of the EuMYB gene family, a phylogenetic tree was generated based on the EuMYB protein sequences (Supplementary Table S2) and Arabidopsis MYB protein sequences (Supplementary Table S3). Based on the classification principles of the MYB subfamilies and the model plant *Arabidopsis thaliana*, they could be divided into 35 subfamilies (Figure 1). Among them, the MYB family members of *E. ulmoides* were distributed in 34 of the 35 subfamilies in the evolutionary tree, with no EuMYB family members in the S12 subfamily. The S6, S10, S17, S19, S27, and S29 subfamilies contained only one EuMYB member, while the S24 subfamily contained nine EuMYB members. These findings indicated that the MYB genes of *E. ulmoides* and *Arabidopsis thaliana* had undergone specific expansion and differentiation during evolution.

3.3. Chromosome Location and Collinearity Analysis of the EuMYB Family

Analysis of MYB gene locations on the *E. ulmoides* chromosomes revealed that 115 EuMYB genes were unevenly distributed on the 17 chromosomes of *E. ulmoides*, while four genes (*EuMYB41*, *EuMYB46*, *EuMYB50*, and *EuMYB80*) were localized to unattributed scaffolds (Figure 2). Among them, eleven EuMYB members (*EuMYB18*, *EuMYB22*, *EuMYB23*, *EuMYB25*, *EuMYB55*, *EuMYB59*, *EuMYB67*, *EuMYB69*, *EuMYB75*, *EuMYB113*, and *EuMYB115*) were on chromosome 14, which contained the most EuMYB members, followed by chromosome 10 containing ten EuMYB members, and, in contrast, chromosome 7 contained four EuMYB members. In addition, EuMYB genes belonging to the same subfamily in the phylogenetic tree were distributed on different chromosomes or scattered at different positions on the same chromosome. For example, the eight EuMYB members of S14 were scattered on chromosomes 8, 11, 15, and 16. Among them, *EuMYB36* and *EuMYB111* were at different positions on chromosome 11.

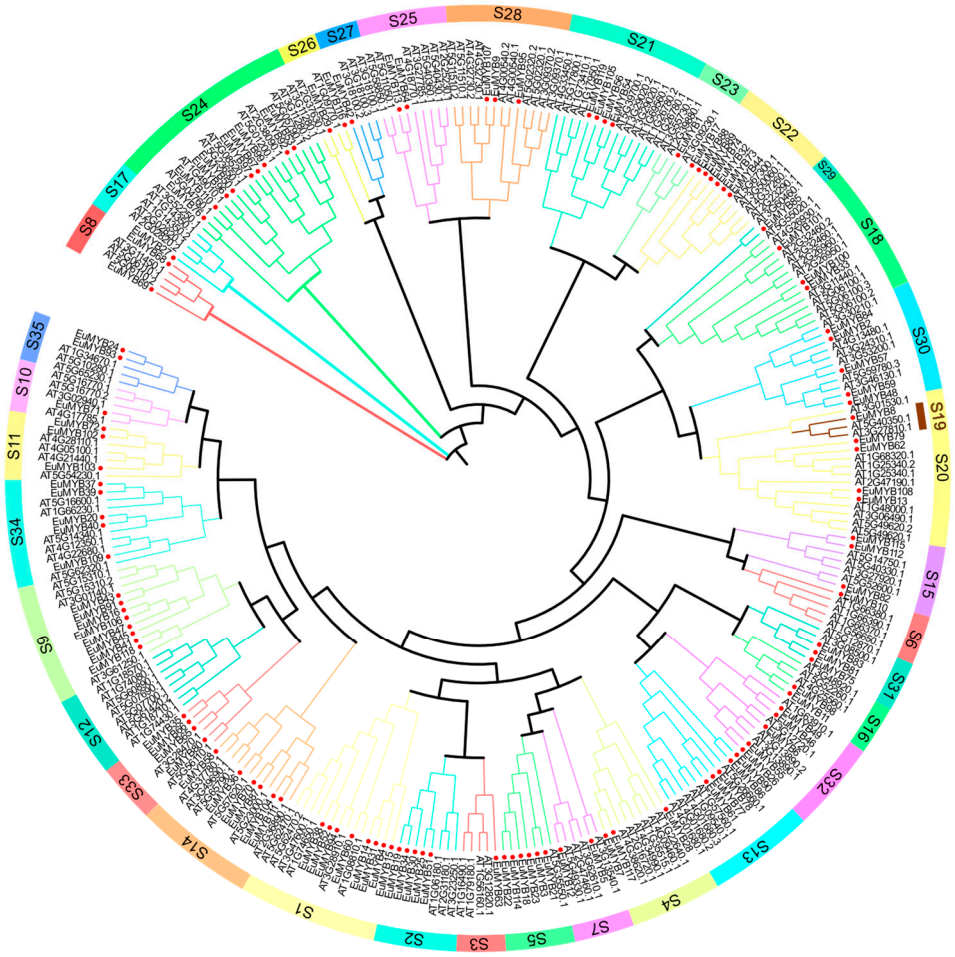

Figure 1. Phylogenetic tree of the *E. ulmoides* and *Arabidopsis thaliana* MYB gene family (different colors represent different subfamilies).

To reveal the evolutionary mechanisms of the EuMYB gene family, tandem repeats and fragment repeats were further analyzed (Figure 2). Altogether, three pairs of tandem repeat genes were identified in the EuMYB family, including one pair of tandem repeat genes in each of chromosome 2 (*EuMYB4–EuMYB31*), chromosome 10 (*EuMYB3–EuMYB26*), and chromosome 13 (*EuMYB45–EuMYB47*). In addition to the tandem repeat genes, a total of 12 pairs of fragment duplication events that occurred in the EuMYB gene family within the *E. ulmoides* genome were identified (Figure 3). Among them, three fragment duplication genes were located on chromosomes 3 and 5, which were the chromosomes containing the most fragment duplication genes. To further analyze the phylogenetic relations of the MYB gene family in *E. ulmoides*, a collinearity map was constructed between *E. ulmoides* and the model plant *Arabidopsis*, *Vitis vinifera* (dicotyledon), *Sorghum bicolor* (monocotyledon), and *Coffea canephora* (dicotyledon). Among the homologous genes of EuMYBs in other species, the most homologous genes were found in *Vitis vinifera* (89 pairs), followed by *Arabidopsis* (82 pairs), *Coffea canephora* (75 pairs), and *Sorghum bicolor* (30 pairs), which had the fewest homologous genes (Figure 4).

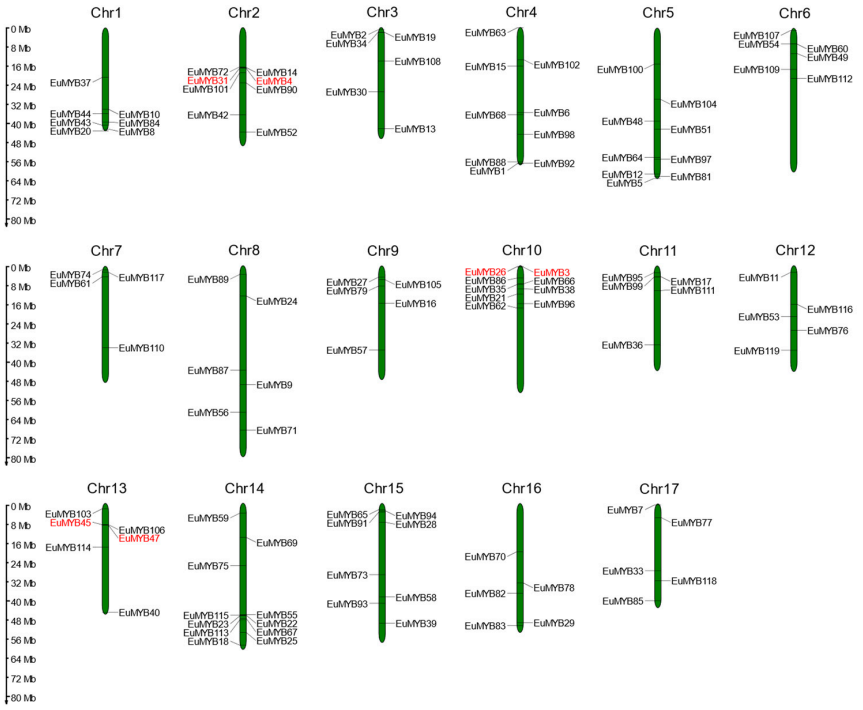

Figure 2. Chromosomal location of EuMYB genes. The chromosomal position was mapped according to the *E. ulmoides* genome. Only 115 EuMYB genes (not including *EuMYB41*, *EuMYB46*, *EuMYB50*, and *EuMYB80*) were mapped to the 17 chromosomes of *E. ulmoides*. Three pairs of tandem repeat genes are marked in red in the figure.

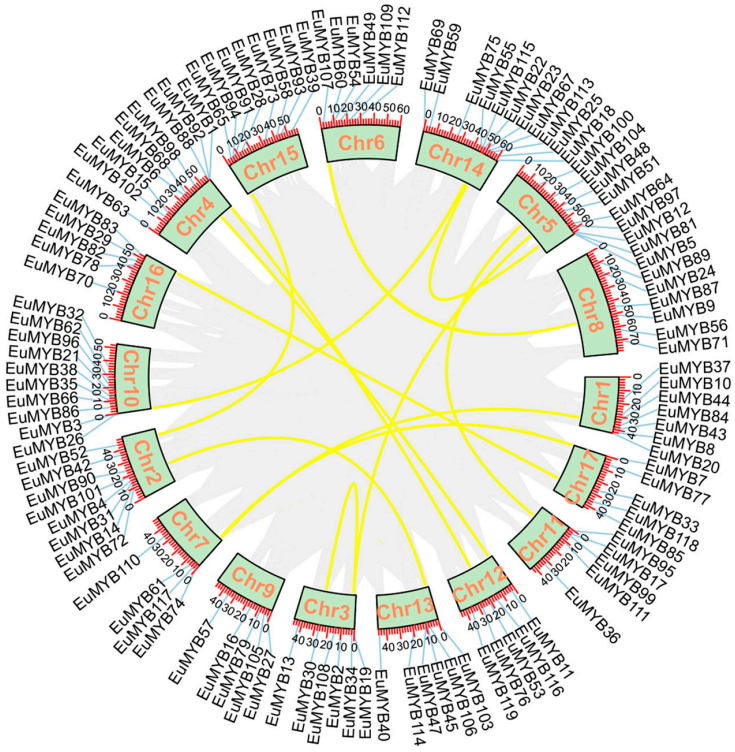

Figure 3. Collinearity analyses of the MYB gene family in *E. ulmoides*. The yellow line represents EuMYB fragment repeat members.

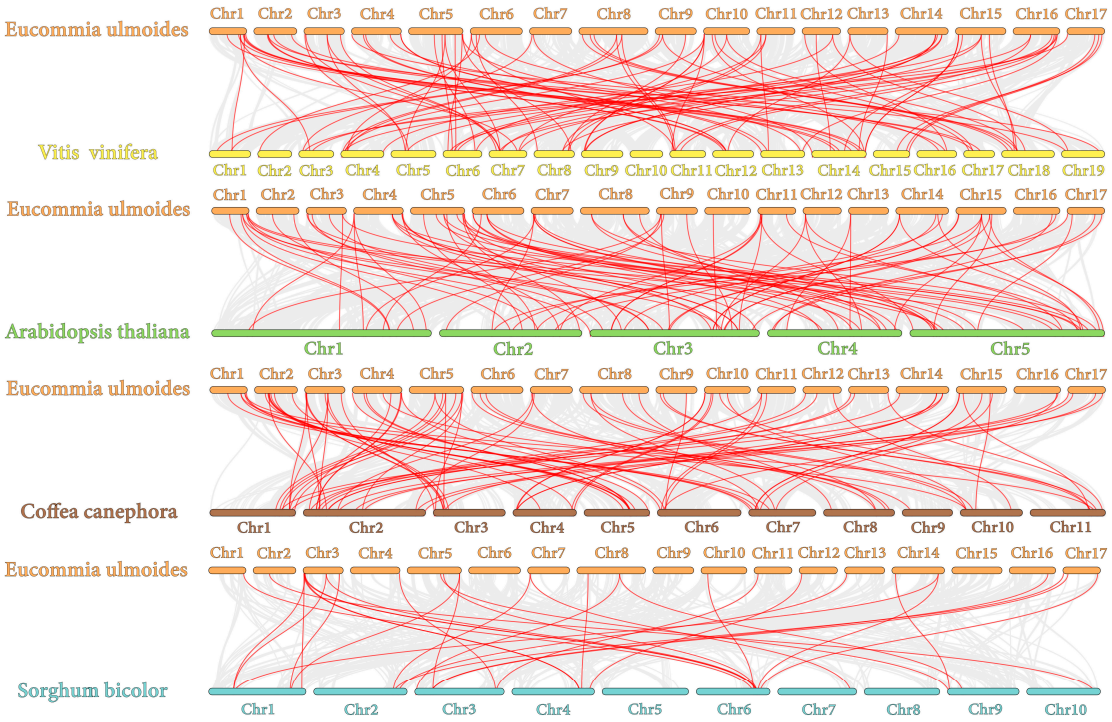

Figure 4. Collinearity analyses of the MYB gene family between *E. ulmoides* and four other species. From top to bottom, the species collinearity analysis of *E. ulmoides*–*Vitis vinifera* (yellow), *E. ulmoides*–*Arabidopsis thaliana* (green), *E. ulmoides*–*Coffea canephora* (brown), *E. ulmoides*–*Sorghum bicolor* (blue). Gray lines in the background indicate the collinear blocks within *E. ulmoides* and different plant genomes, whereas red lines highlight syntenic MYB gene pairs.

3.4. Analysis of Conserved Motifs, Gene Structures, and Domains of the EuMYB Family

Members of the same gene family usually have a conserved motif composition. To analyze the structural diversity and evolutionary relationships of EuMYB family genes, the 119 conserved structural domains of the EuMYB family proteins were analyzed (Figure 5). EuMYB family proteins consisted of 1–10 motifs (Table 1), which varied widely in length from 8 to 50 amino acids. There were differences in the motif compositions of different subfamilies of EuMYB, and members within each subfamily of EuMYB were composed of similar numbers and types of motifs. Most MYB proteins contained motif 3, motif 5, motif 1, and motif 2. The EuMYB56 protein only contained two conserved motifs, while five EuMYB family proteins, EuMYB42, EuMYB45, EuMYB47, EuMYB106, and EuMYB91, each contained a total of eight conserved motifs. Some motifs were only present in specific subfamilies, which may be related to the different functions of different subfamilies.

Analysis of the EuMYB gene structure revealed that the number of CDS (exons) of EuMYBs ranged from 1 to 11, while six genes did not contain introns. The EuMYB family genes in the same subfamily had similar structures, with a similar size and distribution of exon segments, while EuMYB family genes in different subfamilies had significantly different exon and intron structures. Additionally, all EuMYB families contained MYB conserved structural domains.

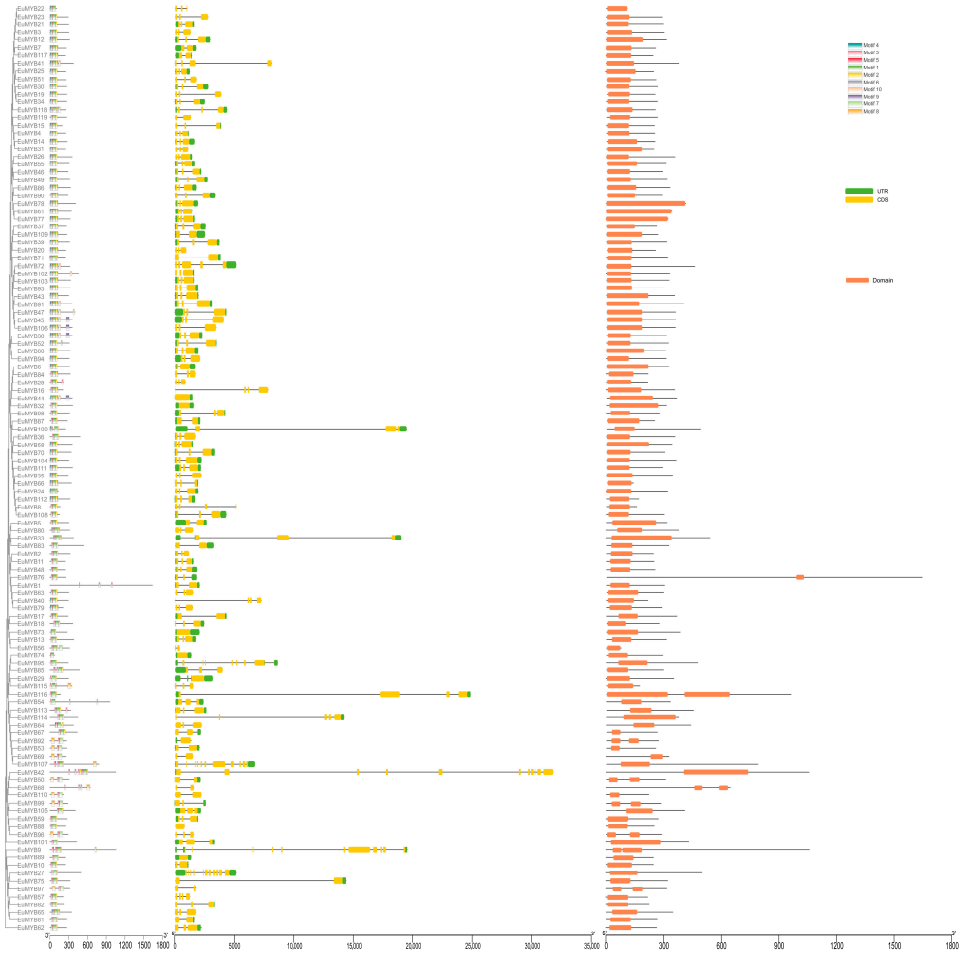

Figure 5. Conserved motifs, gene structures, and domains of the MYB gene family in *E. ulmoides*. Left: Different colored boxes represent different motifs and their positions in each EuMYB protein sequence. Middle: The green boxes represent UTR, black lines represent the intron regions, and yellow boxes represent CDS (exons). Right: The orange-red boxes represent domains.

Table 1. Conserved motif distribution of EuMYB proteins (quantity from largest to smallest).

Name	E Value	Sites	Width	Best Match
Motif 3	2.9×10^{-1218}	116	21	KKGPWTPEEDQKLLAYIEEHG
Motif 5	1.1×10^{-237}	118	8	GCWSSVPK
Motif 1	2.9×10^{-3712}	104	50	AGLLRCGKSCRLRWINYLRPDIKRGNFTQEEEDTIIKLHAILGNRWSAIA
Motif 2	2.2×10^{-1592}	103	21	HLPGRTDNEIKNYWNTHLRKK
Motif 4	6.2×10^{-341}	60	11	MGRSPCCDKVG
Motif 6	1.5×10^{-201}	33	16	LTKMGIDPVTHKPKSS
Motif 7	7.8×10^{-155}	8	50	NFVITRTPTQVASHAQKYFIRQLSGGKDKRRASIHDITTVNLNDNQTPSP
Motif 10	5.2×10^{-102}	8	30	ANLSHMAQWESARLEAEARLVRESKLLSNA
Motif 9	1.2×10^{-109}	4	50	QRFINNVSIKAHNYDVENPMQFRDVAYPIDPTLNMEPWKLPNFVEGFTDV
Motif 8	1.3×10^{-151}	9	50	WTAEENKLFENALAMIDKDMPDRWQRVAAMVPGKTVMDVIKQYKELEDDV

3.5. Analysis of Cis-Acting Elements of EuMYB Family Promoters

To explore the functions and regulation patterns of EuMYB family genes, cis-acting elements were analyzed in the 2000 bp upstream of the EuMYB family start codon.

A total of 24 cis-regulatory elements were identified in the promoter region of the EuMYB gene (Supplementary Table S4), and these elements can be classified into five categories, including cell development, phytohormones, environmental stress, physiological regulatory elements, and MYB gene-binding site elements (Figure 6).

Figure 6. Cis-acting elements of the MYB gene family in *E. ulmoides*. The name of each regulatory element is shown on the right with different colors. The relative location of each element on each promoter can be estimated by the scale at the bottom.

Among the 24 cis-regulatory elements identified, there were four cis-acting elements related to phytohormones: abscisic-acid-responsive elements, gibberellin-responsive elements, MeJA-responsive elements, and auxin-responsive elements. Six cis-acting elements

were associated with environmental stresses: light-responsive elements, low-temperature-responsive elements, wound-responsive elements, defense- and stress-responsive elements, enhancer-like elements involved in anoxic-specific inducibility, and anaerobic-induction-responsive elements. Four cis-acting elements were related to MYB binding sites: the MYBHv1 binding site, the MYB binding site involved in flavonoid biosynthetic gene regulation, the MYB binding site involved in drought inducibility, and the MYB binding site involved in light responsiveness. Seven cis-acting elements were related to cell development: the cell cycle regulation element, the circadian control element, the differentiation of the palisade mesophyll cells element, the endosperm expression element, the meristem expression element, the root-specific element, and the seed-specific regulation element. In addition, it also contained the binding site of AT-rich DNA-binding protein (ATBP-1), the element for maximal elicitor-mediated activation and zein metabolism regulation.

Further studies revealed that light-responsive elements were the most common in the EuMYB gene promoter, followed by abscisic-acid-responsive elements and anaerobic-sensing elements. Among the cis-acting elements associated with MYB binding sites, 52 EuMYBs contained MYB binding sites involved in drought inducibility elements, 44 EuMYBs contained MYBHv1 binding site elements, 44 EuMYBs contained MYBHv1 binding site elements, and 9 EuMYBs contained MYB binding site elements involved in flavonoid biosynthetic gene regulation. These results suggested that the MYB genes were widely involved in a variety of life activities, such as plant growth and development and stress responses.

3.6. Analysis of Gene Expression of EuMYB in Different Tissues

The analysis of gene expression patterns can provide clues for the study of gene functions. Based on the RNA-seq database, the expression of all the EuMYB genes in the leaf, xylem, peel, and seed was extracted (Supplementary Table S5). EuMYB family expression showed that 113 EuMYB members were expressed in all four tissues. More than 50% of EuMYB members had extremely low expression (0 < FPKM < 1) in all four tissues of *E. ulmoides*, and some of these members were not expressed (Figure 7).

Further analysis found that there were 93 EuMYB members expressed in *E. ulmoides* leaves. Among them, 43 members had extremely low expression (FPKM < 1). The highest expression, that of *EuMYB96*, was 53.46. The expression of *EuMYB29*, *EuMYB43*, *EuMYB60*, and *EuMYB91* was high only in leaves, while it was low or not expressed in other tissues. *EuMYB18* was expressed only in leaves, but it was expressed at low levels.

There were 87 EuMYB family members expressed in the *E. ulmoides* xylem. Among them, 35 members had extremely low expression (FPKM < 1). The highest expression, that of *EuMYB109*, was 313.44. *EuMYB49*, *EuMYB109*, and *EuMYB119* were less expressed or not expressed in other tissues. *EuMYB39*, *EuMYB48*, *EuMYB83*, *EuMYB86*, and *EuMYB117* members were highly expressed in the xylem only, and their expression was low or not detected in other tissues. Two EuMYB family members were expressed in the xylem only. These included *EuMYB23* and *EuMYB49*, but the expression of *EuMYB23* was extremely low.

There were 94 EuMYB family members expressed in the *E. ulmoides* peel. Among them, 41 members had extremely low expression (FPKM < 1). The highest expression, that of *EuMYB28*, was 40.85. The *EuMYB36* was expressed only in the peel, but the expression was extremely low.

There were 78 EuMYB family members expressed in *E. ulmoides* seeds. Among them, 38 members had extremely low expression (FPKM < 1). The highest expression, that of *EuMYB73*, was 55.77. There were three EuMYB family members expressed only in the seeds, including *EuMYB19*, *EuMYB88*, and *EuMYB113*, but their expression was extremely low.

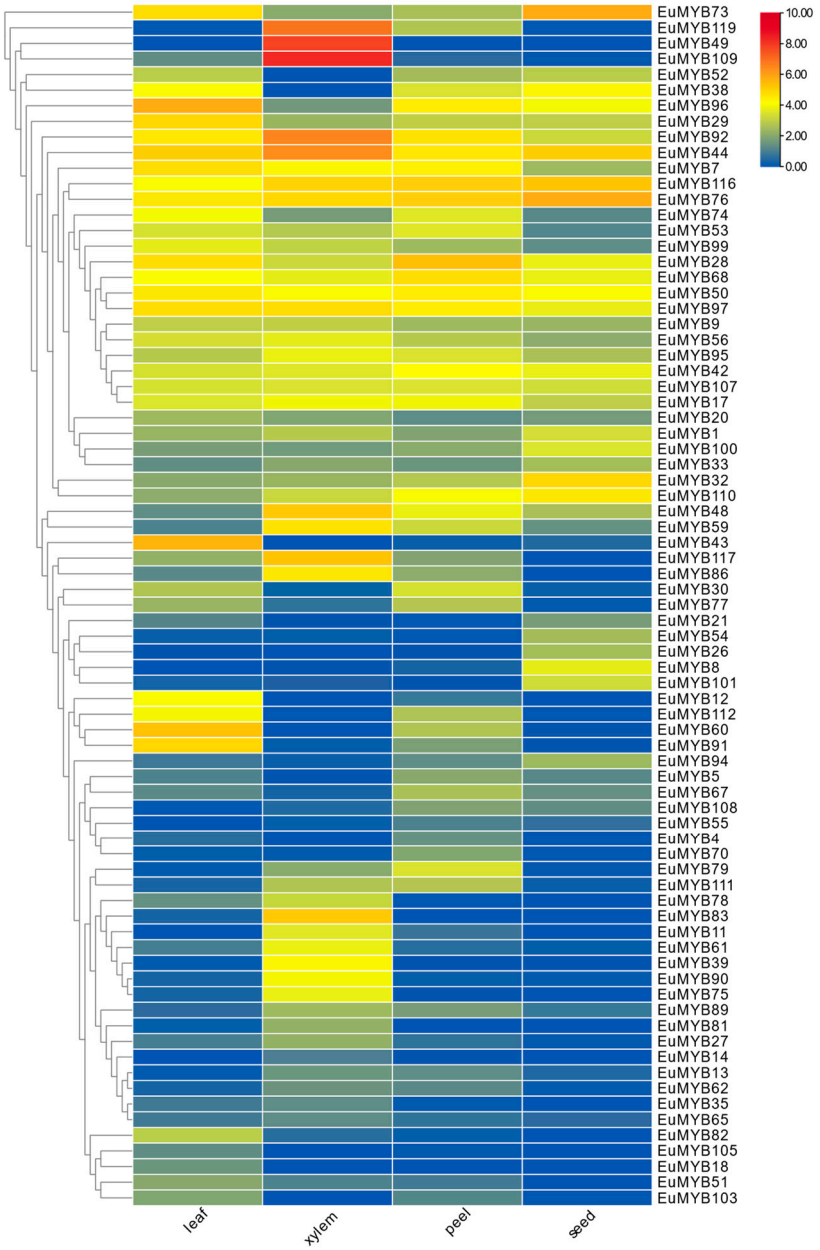

Figure 7. Expression profiling of EuMYB genes in leaf, xylem, peel, and seed. The figure only shows members with FPKM value > 1. Expression value is normalized to log2 (fragments per kilobase for a million reads, FPKM). Different colors represent different expression levels from low to high (blue–yellow–red).

3.7. EuMYBs and EUG Biosynthesis Gene Co-Expression Network

To research the possible regulating function between EuMYB and EUG biosynthesis genes, we constructed a co-expression network (Supplementary Table S7). The results showed that it had 510 pairs positively correlated between 45 EUG biosynthesis genes and 66 EuMYB genes (Figures 8 and 9). Among them, *EuMYB112* had the highest degree of connection, which was positively correlated with 20 EUG biosynthesis genes, followed by *EuMYB12* (19), *EuMYB51* (18), *EuMYB60* (18), *EuMYB77* (18), *EuMYB82* (18), and *EuMYB103* (18). At the same time, it had 198 pairs negatively correlated between 41 EUG biosynthesis genes and 57 EuMYB genes. Among them, *EuMYB110* had the highest degree of connection, which was negatively correlated with 14 EUG biosynthesis genes, followed by *EuMYB32* (13), *EuMYB33* (13), *EuMYB76* (12), *EuMYB116* (12), and *EuMYB101* (10). These genes may play a crucial role in regulating EUG biosynthesis. Additionally,

a previous study indicated that farnesyl diphosphate synthases (*FPS1*) may be the key rate-limiting enzymes for EUG synthesis. Through our analysis, we found that there were sixteen EuMYBs that were positively correlated with the *FPS1* structural gene, and two EuMYBs were negatively correlated with the *FPS1* structural gene. We predicted that these EuMYBs may have a possible regulatory relationship with the *FPS1* structural gene.

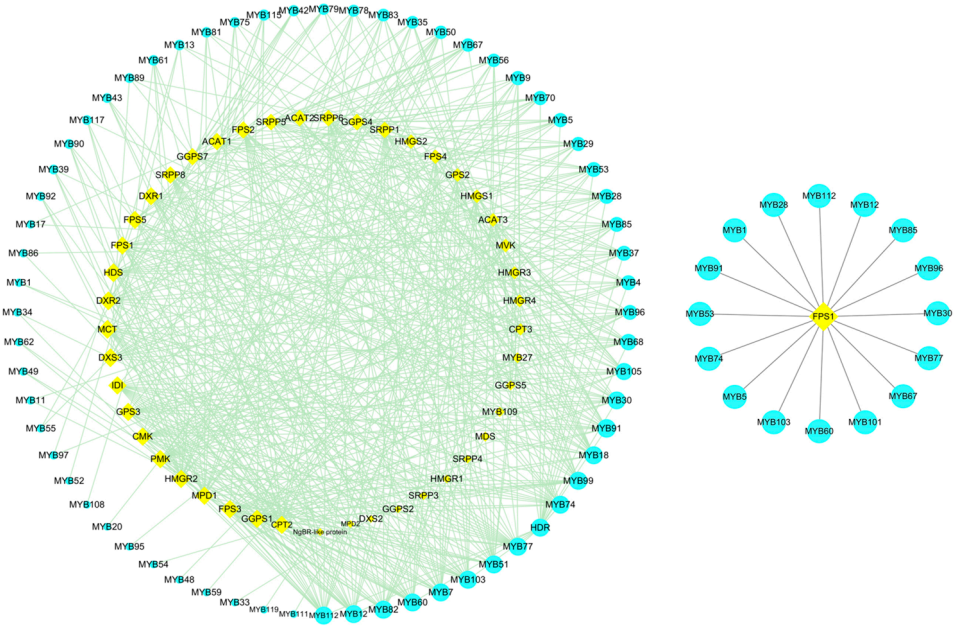

Figure 8. Positive regulation co-expression networks between EuMYBs and EUG biosynthesis genes. The node size is positively correlated with the degree of the connectivity of genes.

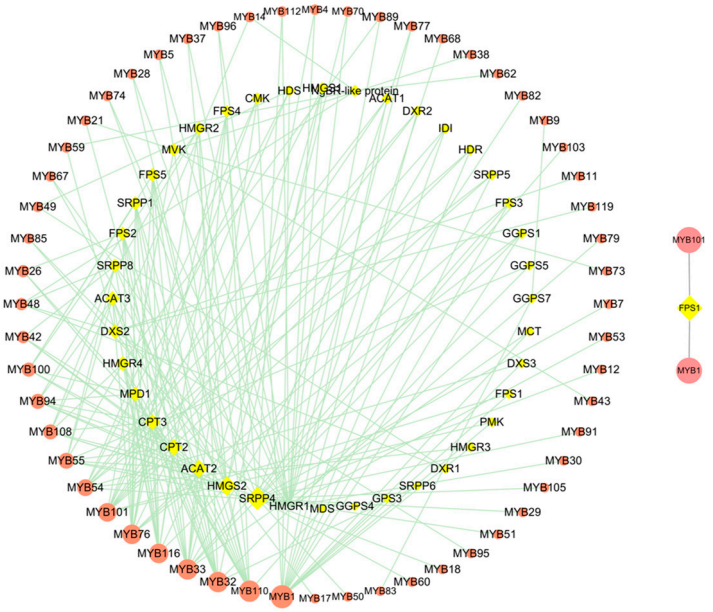

Figure 9. Negative regulation co-expression networks between EuMYBs and EUG biosynthesis genes. The node size is positively correlated with the degree of the connectivity of genes.

3.8. Expression Patterns of EuMYBs in Different Tissues by qRT-PCR

Combined with the gene expression of EuMYB in different tissues and the gene co-expression network, we conducted gene expression validation using RT-qPCR as a means to investigate the EuMYB functions of EUG biosynthesis (Figure 10). Six EuMYB members were selected that were correlated with *FPS1* and highly expressed in gum-forming tissues

(leaf and peel). We used the designed primers to experiment (Supplementary Table S8). The bar chart visually showed that all of the selected EuMYB genes exhibited expression patterns that agreed with the transcriptome expression profile (Figure 7). These genes were all expressed in gum-forming tissues (leaf and peel). Among them, the expression of *EuMYB53*, *EuMYB74*, and *EuMYB112* was significantly different between the tissues containing EUG (leaf and peel) and other tissues not containing EUG (xylem and seed), suggesting that they may have potential regulatory roles in the gum synthesis of *E. ulmoides*.

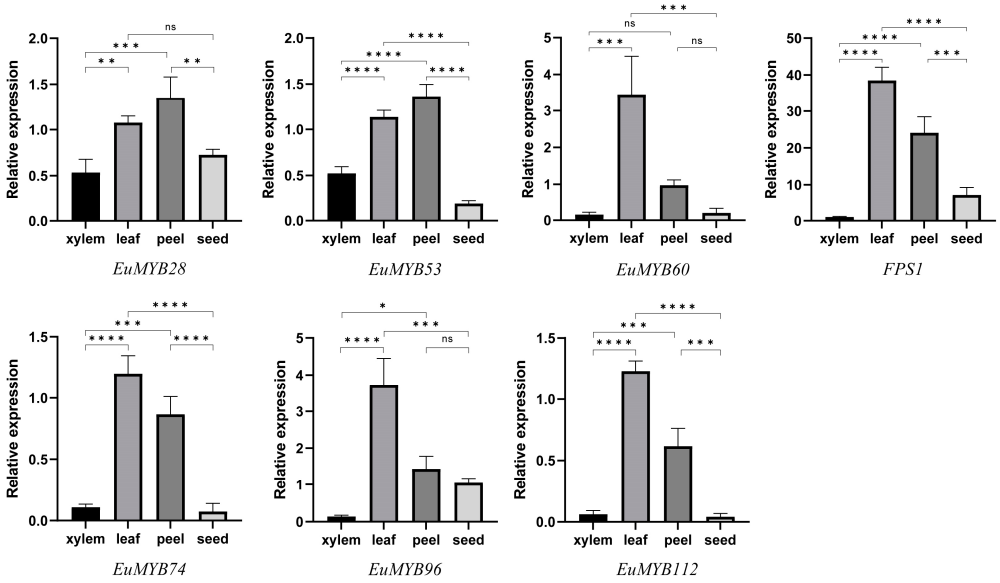

Figure 10. Expression levels of key rate-limiting enzyme *FPS1* and six EuMYB genes in different tissues, as indicated by RT-qPCR. Error bars represent the standard deviations of three biological replicates. Gum-containing tissues (leaf and peel) and non-gum-containing tissues (xylem and seed) were used for the significance analysis. Different numbers of asterisks in the bars indicate significant differences in EuMYB expression levels of different tissues (ns: no significant difference, * $0.01 < p \leq 0.05$, ** $0.001 < p \leq 0.01$, *** $0.0001 < p \leq 0.001$, **** $p \leq 0.0001$).

4. Discussion

4.1. Analysis of Basic Characteristics of EuMYB Family Members

The MYB gene family varied in number and structure in different plants. In terms of MYB family member identification and characteristics, a total of 119 EuMYB family members were identified based on the whole genome data of *E. ulmoides*, which was close to the number of *Panax notoginseng* (123 MYB), *Cymbidium ensifolium* (136 MYB), the monocotyledonous plant *Oryza sativa* (131 MYB), and Chinese pear (*Pyrus bretschneideri*, 129 MYB) [48–50]. The number was less than the 244 of the largest known plant MYB family of soybean, 235 of chili pepper (*Capsicum* spp.), 177 of sweet orange, 198 of *Arabidopsis thaliana*, and 141 of the Brazilian rubber tree (R2R3-MYB). The number of EuMYB members was only more than that of a few plants, such as *Curcuma wenyujin* (88) and *Beta vulgaris* (70), which possess a relatively small number of MYB family members [13,51–54]. The reason for the quantitative differences may be due to the genome size and replication events, which also reflect the diversity of MYB families during plant evolution.

By comparison, it was found that the characteristics of the MYB family of *E. ulmoides* were more similar to those of other plants such as rubber tree, *Cymbidium ensifolium*, chili pepper, and sweet orange [30,48,54,55]. However, some differences existed [13,54,55]. For example, the minimum molecular weight of rubber tree MYB proteins is 2.7 KDa and that of Jianlan is 5.82 KDa, while the maximum molecular weight of chili pepper is 183.5 KDa. The molecular weight range of *E. ulmoides* MYB proteins was 8.99–178.92 KDa, which was different from several other plants. The maximum theoretical pI of rubber tree MYB members was 9.44, that of chili pepper members was 9.36, and the range of *E. ulmoides*

members was from 5.01 to 10.75, similar to the range of *Cymbidium ensifolium* and other plants. These differences in the characteristics of MYB members are also the biological basis for the distinctions between *E. ulmoides* and other plants.

The phylogenetic analysis showed that EuMYB members were divided into 34 subfamilies, each containing 1–9 members, similar to the results of Brazilian rubber trees and much fewer than the 47 subfamilies of the largest plant MYB family, that of soybean, each containing 2–28 members [52,54]. The MYB members in Chinese pear MYB and *Cymbidium ensifolium* MYB, which were similar in number to the EuMYB family, were divided into 31 and 32 subfamilies, which contained 2–8 and 2–24 members, respectively [48,49]. The variability among MYB families also demonstrates the diversity of the MYB genes that have evolved in different plants.

Chromosome analysis showed that EuMYB family members were unevenly distributed on the 17 chromosomes of *E. ulmoides*, and most EuMYB members were concentrated at both ends or one end of a chromosome, which was similar to the results of MYB chromosome studies in plants such as chili pepper and soybean [52,55] The expansion of gene families and plant genomes is thought to be associated with gene duplication events [56]. Replication events are important in the expansion and evolution of gene families; gene duplication culminates in the production of proteins with sub-functionalization, neo-functionalization, or non-functionalization [57,58]. The EuMYB family contained three pairs of tandem repeat genes and 12 pairs of fragment repeat genes, indicating the phenomenon of EuMYB family expansion, which may have led to changes in the function of some evolved novel members and thus enhanced plant adaptation [59]. This result was different from the soybean MYB family, which used tandem repeats as the main driver of amplification, while the Chinese pear MYB family used whole genome repeats and fragment repeats as the main drivers of amplification [49,52]. The different amplification patterns of gene family members in different species also underlie the diversity of MYB families in plants.

Analysis of the conserved structural domains and motifs of EuMYB showed that different phylogenetic tree subclades had different structural domains and motif compositions, which may be related to the MYB TF function and regulatory mode of action. Among the EuMYB family subclades, the S5 subclade contained only one AtMYB member, and its members were mainly EuMYB, which may be due to the distant kinship between EuMYB and Arabidopsis MYB or the evolution of the EuMYB family. Similar clustering has been found in chili pepper, sweet orange, rubber tree, and Chinese pear plants [49,54,55,60]. Analysis of the gene structure of the EuMYB family revealed that, with a few exceptions, such as *EuMYB42* and *EuMYB116*, the exon and intron structures of EuMYB family genes located in the same subfamily were consistent. However, there were six EuMYB members that did not contain introns [61], which was similar to the results of studies in Chinese pear and *Cymbidium ensifolium* [48]. This finding suggested the existence of highly conserved structures within EuMYB subfamilies and a high degree of sequence diversity among different EuMYB subfamilies, in strong agreement with the results of the analysis of other plant MYB families.

Cis-acting elements are the DNA-binding sites of TFs and are responsible for regulating target genes at the transcriptional level [62]. In response to stress, plants have promoters and TFs that regulate stress gene expression and thus initiate protective mechanisms. Therefore, the analysis of promoter cis-acting elements is important for the study of gene function [63]. In this study, we found that light-responsive elements were the most common in the EuMYB gene promoter, followed by abscisic-acid-responsive elements and anaerobic-sensing elements. The development process and morphogenesis of leaves, stems, fruits, and flowers are usually highly light-dependent [64]. This finding suggests that EuMYB family members may have potential regulatory roles in plant photosynthesis, shoot dormancy, leaf abscission, the inhibition of cell growth, and anaerobic induction. Members of subgroups S11, S18, and S35 all contained auxin-responsive elements. S16 and S35 all contained defense- and stress-responsive elements. S16, S26, and S35 all contained

gibberellin-responsive elements. The S5, S26, and S34 subgroups contained abundant zein metabolism regulation elements. This suggests that members within these subgroups may regulate the corresponding biological processes in *E. ulmoides*. Furthermore, both *EuMYB91* and *EuMYB97* showed differentiation of the palisade mesophyll cells element, and they were highly expressed in leaves. *EuMYB38*, *EuMYB44*, *EuMYB50*, *EuMYB73*, and *EuMYB76* contained light-responsive elements, and they were highly expressed in leaves and seeds. This suggests that they may have important regulatory functions. Meanwhile, some PbMYB genes were also found in Chinese pear to contain BOXP and BOXL elements, which may be involved in lignin biosynthesis [49].

4.2. Analysis of Expression Patterns of EuMYB Family Members

In this research, we analyzed the expression levels of the EuMYB family based on transcriptome data from different tissues of *E. ulmoides*, including the leaf, xylem, peel, and seed (Supplementary Table S5). We found that the EuMYB members that were specifically highly expressed in *E. ulmoides* leaves included *EuMYB29*, *EuMYB43, EuMYB60*, and *EuMYB91*, while they had extremely low or no expression in other tissues. *EuMYB18* and *EuMYB84* were only expressed in leaf tissues, but their expression was extremely low. The EuMYB members that were specifically highly expressed in the xylem of *E. ulmoides* were *EuMYB39*, *EuMYB48*, *EuMYB49*, *EuMYB83*, *EuMYB86*, *EuMYB109, EuMYB117*, and *EuMYB119*, and they had very low or no expression in other tissues. *EuMYB23* and *EuMYB49* were only expressed in the xylem, but *EuMYB23* expression was extremely low. *EuMYB19* and *EuMYB88* were only expressed in the seed, but their expression was extremely low. The EuMYB member that was specifically highly expressed in the seed of *E. ulmoides* was *EuMYB32*, and it had very low or no expression in other tissues. *EuMYB19*, *EuMYB88*, and *EuMYB113* were only expressed in the seed, but their expression levels were extremely low. Thus, the EuMYB gene family members may play a key role in the development of different tissues. Only *EuMYB44*, *EuMYB50*, and *EuMYB76* were expressed to some extent in all five different tissues of *E. ulmoides* and may play an important role as a regulatory factor in all five.

The composition of motifs in proteins reflects the evolution of function. According to the phylogenetic relationships, the 119 MYB family members were divided into 35 subgroups. The gene structures and motif arrangement of the genes within each subgroup were similar (Figure 5), and the genes within a subgroup may have similar biological activities and functions (Figure 1). Based on the functions of Arabidopsis homologues, we can predict the functions of EuMYB genes, which may also be used for further functional studies. We analyzed the related functions and the results were as follows (Table 2).

In addition, research showed that R2R3-MYBs of Arabidopsis in the subclades of S3, S4, S5, S6, and S7 regulated phenylpropanoid biosynthesis [3], which is an important synthetic pathway of chlorogenic acid in *E. ulmoides* [75]. Therefore, we speculated that *EuMYB28* that was highly expressed in leaves had similar functions. Flavonoids are proposed to act as reactive oxygen species (ROS) scavengers to maintain normal plant growth and development in response to abiotic stresses [76]. Research shows that the S7 subgroup of Arabidopsis can participate in the regulation of flavonoid synthesis [77]. At the same time, we also found that *EuMYB12* located in the S7 subgroup contained an MYB binding site involved in flavonoid biosynthetic gene regulation (Supplementary Table S4). We predicted that *EuMYB12* may have similar functions. Furthermore, the S5 subgroup plays a regulatory role in the synthesis of proanthocyanidins [78], the S6 subgroup regulates anthocyanin synthesis [79,80], and the S24 subgroup regulates suberin biosynthesis [81]. Therefore, EuMYB in the corresponding subgroup may also play a similar role. These candidate genes and their putative functions will be further validated in the future, which will provide a new insight for further studies on the functions of EuMYB genes.

EUG is an important industrial raw material in the world today. It is found that 52 key genes are involved in the biosynthesis of EUG, among which *FPS1* may be the key rate-limiting enzyme for EUG synthesis [29]. This is also confirmed by studies in

Brazilian rubber trees [30]. Some studies have reported that MYB TFs play important regulatory roles in plant natural rubber biosynthesis. For instance, it was found that *HblMYB19* and *HblMYB44* are involved in the regulation of *FDPS1*, *SRPP*, and *HRT1* in the natural rubber synthetic pathway [69]. With treatment involving MeJA induction, the expression of MYB, bHLH, and WRKY genes encoding TFs such as *HMGCR*, *FPPS*, *IDI*, and *GGPPS* in *Taraxacum koksaghyz* showed a positive correlation, which can affect the synthesis of natural rubber [82]. In view of this, to research the possible relationship between EuMYB genes and EUG biosynthesis pathway genes, we constructed a co-expression network containing EUG biosynthesis genes and EuMYBs (Figures 8 and 9). The results showed that EuMYB family members had strong correlations with structural genes of EUG synthesis. Furthermore, we selected six EuMYBs (*EuMYB28*, *EuMYB53*, *EuMYB60*, *EuMYB74*, *EuMYB96*, *EuMYB112*) associated with *FPS1*, a key rate-limiting enzyme in gum formation, for qRT-PCR experiments, and the results were consistent with the transcriptome data; all of them were highly expressed in gum-forming tissues. These results imply that EuMYBs may have an important regulatory role in EUG synthesis.

Table 2. Prediction of EuMYB functions by phylogenetic tree.

Arabidopsis	*E. ulmoides*	Regulation Function
MYB2/7	*MYB7/28*	seed germination, pollen formation, abscisic acid, drought stress [65]
MYB4	MYB7/28/117	inhibits the production of UV protective shade [66]
MYB20/80	*MYB32/39/44/73/109*	regulates drought stress and salt stress [67,68]
MYB60	*MYB38/60*	regulates guard cells, stomatal, anthocyanin biosynthesis, ABA [69,70]
MYB73/80	*MYB32/73/74*	salt stress, pollen development [71–73]
MYB85	*MYB56/119*	plant secondary cell wall biosynthesis [74]

5. Conclusions

In summary, 119 MYB family members were identified in *E. ulmoides*, which were classified into 35 subgroups based on phylogenetic relationships. The gene structure, motif composition, chromosome distribution, gene duplication, phylogenetics, cis-acting elements, and collinearity among the family members were comprehensively analyzed. The results showed that the EuMYB family members differed in gene length, molecular weight, and PI, while the gene structure and motifs were relatively conserved and also indicated the phenomenon of EuMYB family expansion. The results of promoter cis-acting elements suggested that the EuMYB family may regulate various physiological metabolic processes, such as stomatal opening, anther formation, secondary cell wall synthesis, and biotic and abiotic stresses, in *E. ulmoides*. In addition, the expression of EuMYB genes in different tissues and co-expression network analysis implied that EuMYB genes may participate in multiple physiological processes and EUG biosynthesis. These results lay the foundation for in-depth studies on the biological functions of EuMYB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14102064/s1, Supplementary Table S1: Characteristics of the MYB gene family in *Eucommia ulmoides.* Supplementary Table S2: Sequence information of EuMYB and AtMYB proteins that were used to construct the phylogenetic tree. Supplementary Table S3: *Arabidopsis thaliana* ID number. Supplementary Table S4: Analysis of cis-acting elements of promoters. Supplementary Table S5: FPKM value of EuMYBs. Supplementary Table S6: FPKM value of 52 EUG synthetic structural genes. Supplementary Table S7: The results of EuMYB and EUG biosynthesis gene co-expression. Supplementary Table S8: Primers for EuMYB RT-qPCR.

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Conflicts of Interest: This manuscript has not been submitted to any other journals for publication and the study complies with the original research results. We also confirm that all the listed co-authors participated actively in the study and have seen and approved the submitted manuscript. The authors do not have any possible conflicts of interest.

Abbreviations

Eucommia ulmoides (*E. ulmoides*); *Eucommia ulmoides* gum (EUG).

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