Comprehensive analysis of the MYB transcription factor gene family in *Morus alba*

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**Abstract**

**Background:** The V-myb myeloblastosis viral oncogene homolog (MYB) family of proteins is large, containing functionally diverse transcription factors. However, MYBs in *Morus* are still poorly annotated and a comprehensive functional analysis of these transcription factors is lacking.

**Results:** In the present study, a genome-wide identification of MYBs in *Morus alba* was performed. In total 166 *MaMYB*s were identified, including 103 R2R3-MYBs and four 3R-MaMYBs. Comprehensive analyses, including the phylogenetic analysis with putative functional annotation, motif and structure analysis, gene structure organization, promoter analysis, chromosomal localization, and syntenic relationships of R2R3-MaMYBs and 3R-MaMYBs, provided primary characterization for these MaMYBs. R2R3-MaMYBs covered the subgroups reported for R2R3-MYBs in *Arabidopsis* and *Populus*, and had two *Morus*-specific subgroups, indicating the high retention of MYBs in *Morus*. Motif analysis revealed high conservative residues at the start and end of each helix and residues consisting of the third helix in R2 and R3 repeats. Thirteen intron/exon patterns (a–m) were summarized, and the intron/exon pattern of two introns with phase numbers of 0 and 2 was the prevalent pattern for R2R3-MaMYBs. Various cis-elements in promoter regions were identified, and were mainly related to light response, development, phytohormone response, and abiotic and biotic stress response and secondary metabolite production. Expression patterns of R2R3-MaMYBs in different organs showed that MaMYBs involved in secondary cell wall components and stress responsiveness were preferentially expressed in roots or stems. R2R3-MaMYBs involved in flavonoid biosynthesis and anthocyanin accumulation were identified and characterized based on functional annotation and correlation of their expression levels with anthocyanin contents.

**Conclusion:** Based on a comprehensive analysis, this work provided functional annotation for R2R3-MYBs and an informative reference for further functional dissection of MYBs in *Morus*.

**Keywords:** Anthocyanin, Fruit ripening, Functional annotation, *Morus alba*, MYB, Phenylpropanoid

**Background**

V-myb myeloblastosis viral oncogene homolog (MYB) proteins comprise one of the largest families of transcriptional regulators in plants [1]. The MYB transcription factors have been identified as important regulators that work as activators or repressors in diverse processes, including development, stress responses, and metabolism. The "oncogene" v-MYb derived from the avian myeloblastosis virus was the first MYB gene identified and further study showed that three genes related to v-MYb—c-Myb, A-Myb and B-Myb were distributed in many vertebrates [2]. Similar genes have also been widely reported in fungi, plants, and insects [3]. The first plant MYB transcription factor the c1 gene was reported in maize
(Zea mays) [4]. Currently, many MYB genes have been reported in diverse species [5].

MYB proteins are characterized by sharing a highly conserved DNA-binding domain (DBD) in their N-terminus. This domain is generally composed of up to four imperfect repeats [1, 6]. These repeat units are named R1, R2, and R3 according to their sequence similarity with the c-MYB protein [6]. Each repeat comprises approximately 50–53 amino acids with a similar folding architecture containing three well-defined α-helices. When bound to specific promoter sequences, portions of the second and third helices form a helix-turn-helix (HTH) structure [6, 7]. The third helices of R2 and R3 play a recognition role in binding to a specific DNA sequence, while R1 is not essential for specific DNA recognition [6, 7]. In addition, each repeat contains regularly spaced tryptophan residues (WX$_{18-19}$WX$_{18-19}$W), forming a tryptophan cluster in the three-dimensional (3D) HTH structure. The C terminus, which is responsible for the protein's regulatory activity, is more variable [1, 8]. MYB transcription factors have been divided into four different classes depending on the number of adjacent repeats: 1R (R1/2, R3-MYB), 2R (R2R3-MYB), 3R (R1R2R3-MYB), and 4R, which harbor four R1/R2-like repeats. MYBs with two repeats R2R3-MYBs which is the largest class of MYB factors in plants [9].

R2R3-MYBs have undergone extensive expansion and are thought to perform diverse functions in plant-specific processes [5]. Comprehensive analysis of the MYB gene family have been widely reported in many plants that have available genome information. To date, 126 2R-MYB genes are reported in Arabidopsis thaliana [1], 198 in Populus trichocarpa [10], 108 in Vitis vinifera [11], 157 in Z. mays [12], and 185 in Pyrus bretschneideri [13]. R2R3-MYBs have been classified into 28 (S1–S28) subgroups in Arabidopsis and 45 subgroups (C1–C45) in Populus trichocarpa [1, 10]. Most R2R3-MYBs in Arabidopsis have been well functionally characterized and their roles in diverse biological processes have been identified. The functions of R2R3-MYBs in the regulation of the phenylpropanoid, flavonoid, and lignin biosynthesis pathways are particularly well studied [9, 14]. The first plant MYB transcription factor, the c1 was found to regulate anthocyanin biosynthesis in the aleurone of maize kernels [4]. Arabidopsis R2R3-MYBs in subgroups 6 and 7 were also reported to control the flavonoid biosynthesis pathway, with differences in tissue preference [1]. AtMYB75/PAP1, AtMYB90/PAP2, AtMYB113 and AtMYB114 in subgroup 6 control anthocyanin biosynthesis in vegetative tissues and AtMYB11/PFG1, AtMYB12/PFG1 and AtMYB111/PFG3 in subgroup 7 control flavanol biosynthesis in all tissues [15]. AtMYB123/TT2 in subgroup 2 controls the biosynthesis of proanthocyanidins (PAs) and regulates tannin biosynthesis [1]. In addition, some R2R3-MYBs also act as repressors for controlling the lignin and flavonoids biosynthesis pathway. AtMYB3 and 4 in subgroup 4 suppress the cinnamate 4-hydroxylase (C4H) gene, and AtMYB4 represses the synthesis of sinapoyl malate [14]. AtMYB32 represses lignin and flavonoid biosynthesis in pollen. AtMYB7 is responsible for reducing flavonol by regulating flavonol synthase (FLS) [14]. PtMYB165, 181, 182, and 194 in C18 were also reported as repressors of flavonoid biosynthesis [14]. In addition, R2R3-MYBs also serve as key factors in the control of plant development and cell fate, as well as various abiotic stresses and defense responses in plants [1]. AtMYB0 and AtMYB66 are involved in epidermal cell patterning and AtMYB125 is involved in the regulation of male gametophyte development by controlling the cell cycle [16, 17]. AtMYB1, AtMYB2, AtMYB30, AtMYB41, and AtMYB44 were reported to respond to different stresses [18–21].

Mulberry (Morus, Moraceae) is an economically important plant with nutritional, medicinal, and ecological value. A draft genome of Morus notabilis was released by He et al. (2013) [22], after which genome-wide analyses of several gene families, including the R2R3-MYB gene family, were performed [23]. The functions of four R2R3-MYBs in flavonoid biosynthesis were also characterized [24]. However, MYBs in Morus are still poorly annotated. In the present study, we performed a comprehensive analysis of R2R3 and 3R-MYBs using the latest chromosome-level genome assembly of Morus alba (Ma), which has a basic chromosome number of 14 and total genome assembly size of 338.27 Mb [25]. A strict workflow was adopted to perform a genome-wide identification of MYBs in M. alba. Comprehensive analyses, including phylogenetic analysis, putative functional annotation, gene structure, motif and promoter analysis, chromosomal localization and the syntenic relationships of R2R3 and 3R-MaMYBs, were performed. The expression patterns of R2R3 and 3R-MaMYBs in different organs and fruit development stages were analyzed using RNA-seq data. Real-time quantitative PCR (RT-qPCR) was used to validate selected differentially expressed R2R3-MYBs in different fruit development stages. This study contributed basic functional annotation of R2R3-MaMYBs in Morus and provided candidate MYB regulators as targets for genetic modification.

Materials and methods

Plant materials

Roots, stems and leaves were collected from one-year-old M. alba plants. Fruits at four different development stages (S0, inflorescence; S1, green fruits; S2, reddish fruits; S3, purple fruits) were collected from Zhongsang 5801 plants. All of the above samples were collected from
the plantation of National Mulberry Genebank (NMGB), Zhenjiang, China, and immediately stored at −80°C. Three biological replicates growing nearby were considered. At least six mulberry fruits were collected from each plant.

**Data collection and workflow for searching MYBs in M. alba**

Genome sequences were collected from different databases. The *Arabidopsis* genome sequences (.fasta) and annotation file (.gff) were downloaded from The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/), and the *Populus* genome information was obtained from Phytozone v13 (https://phytozone-next.jgi.doe.gov/). The *M. alba* genome sequences (.fasta) and annotation file (.gff) were generously provided by Professor Jiao, who released this genome information. The MYBs in *Arabidopsis* and *Populus* were extracted using Tbtools v1.098665 [26] based on the information provided in previous reports [1, 8, 10].

To search for MYBs in mulberry, firstly, HMMER search was performed using a hidden Markov model (HMM) profile of the MYB binding domain (PF00249) from the Pfam database (http://pfam.xfam.org/). The hits with E values < 1 were screened, and the short open reading frames (length < 100) were filtered out and confirmed with manual speculation. The filtered sequences were screened as primary candidate MYBs. Secondly, the putative protein sequences of primary candidate MYBs were submitted to a simple MEME wrapper assembled in Tbtools v1.098665 using the following parameters: number of motifs to find 3, minimum motif width 6, and maximum motif width 55. MEME motifs were extracted and the MYB DNA binding domains were further validated manually. The primary candidate MYBs with MYB DNA binding domain were selected as MYBs in mulberry.

**Sequence alignment, motif analysis and homologous modeling**

*Morus alba* (Ma) R2R3-MYBs and 3R-MYBs were aligned using clustal W assembled in MEGA7.0. The alignment result was exported and manually speculated for scanning the R1, R2 and R3 repeats. Seqlogos of this domain were generated using TBtools v1.098665. Protein sequence of DBD domain of R2R3-MYB, MaMYB29, was submitted to SWISS-MODEL (https://swissmodel.expasy.org/) to obtain the 3-D structure model using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002).

**Phylogenetic analysis and functional annotation of R2R3 and 3R-MYBs**

A neighbor-joining (NJ) phylogenetic tree was constructed using full-length R2R3-MYB protein sequences from *A. thaliana*, *Populus* and *Morus* using MEGA7.0 [28] with JTT+G model and bootstrap test with 1000 replicates. The other NJ tree was also constructed using only R2R3-MaMYB proteins and 3R-MaMYBs with the same parameters.

**Chromosomal location and synteny analysis of R2R3 and 3R-MYBs**

Chromosome location information of R2R3 and 3R-MYBs was extracted from *Morus alba* genome annotation file. BLASTP and Multiple Collinearity Scan toolkit (MCScanX) assembled in Tbtools v1.098665 were used to identify syntenic blocks, tandem duplication, and distinct duplication events using default parameters [29, 30]. R2R3 and 3R-MaMYBs were mapped to *Morus* chromosomes and displayed using Tbtools v1.098665 and both the tandem duplication and block duplication gene pairs were marked.

**Transcriptome based expression profile analysis**

Our previous reported RAN-seq data (Accession number: PRJNA660559) was used to obtain the expression profiles of R2R3 and 3R-MaMYBs in roots, stems and leaves [31]. Expression profile of R2R3 and 3R-MaMYBs in different fruit development stages was obtained using RNA-seq data with accession number: CRA006074 in national genomics data center (NGDC). Both above RNA-seq datasets were reanalyzed. Chromosome-level *M. alba* genome was used as reference genome for alignment using bowtie2 [32]. The genome annotation file was used for calculating the expression matrix using String-Tie v2.15 [33]. Differential expressed genes (DEGs) were obtained using DEseq2 by comparing expression levels of each two stages [34]. Organ expression preference genes were identified while ABS (log2EXPi - log2EXPj) > = 1 (i, j indicating different organs) were obtained.
RNA extraction and RT-qPCR analysis

RNA extraction and cDNA synthesis were performed as our previous report using Plant RN52 Kit (Aidlab, Beijing, China) and PC54-TRUEscript RT kit (Aidlab, Beijing, China) according to the manual [35]. qRT-PCR (quantitative real-time PCR) was performed to validate the expression patterns of R2R3-MaMYBs in different fruit development stages using ABI StepOnePlus™ Real-Time PCR System (USA). The primers are available in Additional file 1. Actin was used as reference gene [36]. Graphpad Prism8.0 was used to visualize the qRT-PCR results. SPSS19.0 was used to perform T-test and ANOVA, p <0.05 was marked as significance. Three biological replicates were firstly mixed and three technical replicates respectively were performed for qRT-PCR.

Measurement of anthocyanins content in mulberry fruits

Extraction and measurement of anthocyanins content were performed according to our previous study [35]. The anthocyanins content was given in cyanidin-3-glucoside equivalents.

Results

Identification of MYBs in *M. alba*

Based on our workflow, 166 genes encoding MaMYBs were identified in *M. alba*. Among 166 MYB genes, there were 59 1R-MYBs (named MaMYBR1–59), 103 R2R3-MYBs (named MaMYB1–103) and 4 3R-MYBs (named MaMYB3R1–4); and 4R-MYB was not found in *M. alba* (Table 1 and Additional file 2). Some MYB genes had alternative splicing and generated more than one transcript. The alternative splicing occurred more frequently for 1R-MYBs in *M. alba*. Similar alternative splicing events were also reported in *Arabidopsis* and *Populus* MYBs. A previous study identified 124 MYB-related genes, 116 R2R3-MYBs, and 4 3R-MYBs using a de novo transcriptome of *M. alba* [23]. This study used a chromosome-level genome and provided stricter and accurate recognition of MYBs in *M. alba*. Both R2R3-MYBs and 3R-MYBs contained DNA-specific recognition helices, and R2R3-MYBs were the largest class of MYBs in plants, playing diverse roles in plant specific processes. Therefore, we mainly focused on the investigation of 103 R2R3-MYB and 4 3R-MYB family members in this study.

Characterization of R2R3 and 3R-MaMYBs in *M. alba*

Alignment was performed using 115 R2R3 and 3R-MYBs, including the alternative splicing products. The DBD domain was recognized and analyzed based on the alignment result. It was evident that all of these MaMYBs contained DBD domains and two or three repeats could be identified easily (Additional file 2). The DBD 3D structure of MaMYB29 was constructed using AtMYB66 (PDB accession number: 6kks) as a template to identify the helix regions (Fig. 1A). Helices 1, 2, and 3 are shown in Fig. 1. The highly conserved spaced W residues formed the WX19WX19W motif in R2 repeats (Fig. 1B, Additional file 2). Several R2R3-MYBs showed minor differences in the number of spaced amino acids. MaMYB5, 32, 63, 64, 70, and100 had additional G, forming WX18GXWX19W, and MaMYB13 had additional P and L residues, forming WX19WX19PLX10W. The additional amino acids were beyond the helix structures according to the helix regions identified (Fig. 1 and Additional file 2). The spaced W residues in R3 repeats were variable especially at the first W (site 54 in Fig. 1C), although W is still dominant amino acid. The (W/F/I/M)X18WX19W was recognized in MaMYB R3 repeats (Fig. 1C). The replacement of the first W with F, (less frequently) I, or M in R3 was also reported in *Populus*, *Z. may*, *Arabidopsis*, and *Vitis* [1, 10–12]. MaMYB35 and 37 had replacement of the third W with Y and F, respectively. More conservative amino acids were observed in the third helix region of R2 and R3 repeats than those consisting of the first and second helices (Fig. 1B, C). In addition to the conserved W, there were also several highly conserved amino acids including the amino acids at the start and the end of each helix and amino acids consisting of the third helix in R2 and R3 repeats. In addition, the important MYB function related residues K35, L39, N86, K89, and 90, indicated by red boxes, were also highly conserved. Therefore, we could further modify the three helix regions to W(T/S)X2EDX10GX3WX5(G/L/I)X2RX (G/S)K(S/Q)CR(L/E)WXN(Y/Q/H) for R2 and (W/F/I/M)(T/S)X2E(D/E)X10GX3WX5(W/S/A)X2AX3RTDNX(I/V)KYN(Y/H/F)W(N/H/R) (T/S/V) for R3 (Fig. 1B, C). Interestingly, it was clear that the patterns, W(T/S)X2EDX10G for R2 and (W/F/I/M)(T/S)X2E(D/E)X10GX3WX5GW(S/A)X2AX3RTDNX(I/V)KN(Y/H/F)W(N/H/R) (T/S/V) for R3, formed the first helix were almost same for both R2 and R3 repeats. These patterns were annotated as InterPro MYB domain signature patterns (PS00037). In contrast, the patterns, (G/S)K(S/Q)CRWXN(Y/Q/H) for R2 and TDNX(I/V)KN(Y/H/F)W(N/H/R) (T/S/V) for R3, formed the third helices and were quite different for R2 and R3 repeats.

Table 1 MYBs in different plants

| Types | Arabidopsis | Populus | Vitis | Morus |
|-------|-------------|---------|-------|-------|
| 1R    | 64          | 152     | 46    | 59(88) |
| 2R(R2R3) | 126      | 196     | 108   | 103(108) |
| 3R    | 5           | 5       | 5     | 4(7)   |
| 4R    | 1           | 1       | 1     | 0      |
| Ref   | [1]         | [10]    | [11, 37] | This study |

The number in brackets indicates the number of MYB transcripts in Morus alba.
The above results indicated that the conservation of the third helix in R2 or R3 preserved the DNA binding function and the difference in the third helix between R2 and R3 guaranteed recognition for specific DNA sequences. The WX₁₉W₁₈W box was detected for MaMYB3Rs and formed the R1 repeat.

**Phylogenetic analysis and functional classification of R2R3 and 3R-MaMYBs**

Phylogenetic analysis of R2R3 and 3R-MYBs in Arabidopsis, Populus, and Morus showed that MaMYBs clustered well with orthologs in Arabidopsis or Populus. Previous studies have divided R2R3-AtMYBs into 28 subgroups (S1–S28) and R2R3-PtrMYBs into 45 subgroups (C1–C48). R2R3-MaMYBs covered all 28 R2R3-AtMYB subgroups and 44 R2R3-PtrMYB subgroups, except for C13 (Table 2, Additional file 3). In addition, MaMYB65 and 101 in addition to MaMYB53 and 54 (TT2L3) were classified as two Morus specific subgroups. MaMYB54, previously named as TT2L3, was reported to show a similar expression pattern with flavan-3-ol specific gene LAR encoding leucoanthocyanidin reductase during fruit...
**Table 2** Classification of MaMYBs based on phylogenetic analysis

| Subgroups | Arabidopsis | Populus | Morus | Function annotation | Reference |
|-----------|-------------|---------|-------|---------------------|-----------|
| C1(S11)   | AtMYB41, 49, 74 and 102 | PtrMYB017, 043, 047, 226 | MaMYB14 and 48 | drought response and suberin biosynthesis | [38–40] |
| C2(S9)    | AtMYB16, 106 | PtrMYB038, 138 and 186 | MaMYB11, 38 and 94 | cuticular wax biosynthesis | [41] |
| C3(S10)   | AtMYB9, 39 and 107 | PtrMYB059, 130 and 149 | MaMYB19 | suberin biosynthesis and transport | [42] |
| C4(S24)   | AtMYB53, 92 and 93 | PtrMYB027, 030, 032 and 109 | MaMYB28, 34 and 60 | suberin biosynthesis | [43] |
| C5        | AtMYB40 | PtrMYB139 and 188 | MaMYB6 | arsenic resistance | [44] |
| C6        | AtMYB20 and 43 | PtrMYB018 and 152 | MaMYB18 | secondary cell wall formation and lignification | [45–47] |
| C7        | AtMYB42 and 85 | PtrMYB075, 92, 125, 199 | MaMYB22 and 31 | cell wall production | [48] |
| C8(S9)    | AtMYB17 | PtrMYB077, 146, 187and 209 | MaMYB20 and 97 | meristem identity transition from vegetative growth to flowering | [49] |
| C9(S1)    | AtMYB30, 31 and 60 | PtrMYB053, 81, 155 and 225 | MaMYB10, 44 and 90 | cell death during the hypersensitive response upon pathogen attack | [50, 51] |
| C10(S2)   | AtMYB13, 14 and 15 | PtrMYB185, 190 and 220 | MaMYB43 and 77 | cold or wound stress | [52, 53] |
| C11(S3)   | AtMYB10, 58, 63 and 72 | PtrMYB028 and 129 | MaMYB86 | lignin biosynthesis | [54] |
| C12       | NA | PtrMYB048, 049, 063, 064 and 065 | MaMYB15 and 16 | – | – |
| C13       | NA | PtrMYB016, 44, 45, 46 and 230 | NA | – | – |
| C14       | AtMYB35 | NA | MaMYB9 | sex determination | [55, 56] |
| C15       | AtMYB8 | PtrMYB005 and 094 | MaMYB1 | – | – |
| C16(S14)  | AtMYB36, 37, 38, 68, 84 and 87 | PtrMYB025, 079, 085, 088, 099100, 102,112, 133, 184 | MaMYB5, 32,46,63, 64, 70 and 100 | transition from proliferation to differentiation | [57, 58] |
| C17(S4)   | AtMYB3, 4, 6, 7, 8 and 32 | PtrMYB057, 093, 156, 168, 180 and 221 | MaMYB59, 73 and 76 | General phenylpropanoid and lignin R2R3-MYB repres-sors | [14] |
| C18       | NA | PtrMYB165, 181, 182, 194 and 203 | MaMYB39(MYB4) | Flavonoid R2R3-MYB repres-sors | [14] |
| C19(S7)   | AtMYB11, 12 and 111 | PtrMYB035, 056 and 111 | MaMYB42, 61 and 75(MYBF) | flavonoids biosynthesis | [9, 24] |
| C20       | AtMYB5 | PtrMYB006, 050, 060, 061, 107 and 126 | MaMYB83 | flavonoids biosynthesis | [9] |
| C21       | NA | PtrMYB009, 115, 123, 153 and 201 | MaMYB98 | proanthocyanidin biosyn-thesis and enhances fungal resistance | [9] |
| C22       | AtMYB82 | PtrMYB084 and 135 | MaMYB4 | response to low oxygen, trichome development and anthocyanin production and differentiation of trichome cells | [59] |
| C23(S15)  | AtMYB0, 23 and 66 | PtrMYB071, 072 and 143 | MaMYB36 and 89 | anthocyanin production and differentiation of trichome cells | [16] |
| C24       | NA | PtrMYB097 and 101 | MaMYB52(TTL2) | – | – |
| C25(S5)   | AtMYB123 | PtrMYB086, 087 and 134 | MaMYB50, 81, 82 and 99(TT2L1) | anthocyanin biosynthesis | [9, 24] |
| C26       | NA | PtrMYB011, 129, 159, 171 and 178 and 183 | MaMYB51 and 66 | – | – |
| C27(S6)   | AtMYB75, 90, 113 and 114 | PtrMYB116, 117, 118, 119 and 120 | MaMYB58(MYBA) | anthocyanin biosynthesis in vegetative tissues | [60, 61] |
| C28       | AtMYB46 and 83 | PtrMYB002, 003, 020 and 021 | MaMYB72 and 85 | triggers the expression of secondary cell wall related MYBs | [62] |
| C29       | AtMYB26, 67 and 103 | PtrMYB008, 010, 040, 103, 108, 127, 128 and 137 | MaMYB26, 29, 30, 47 and 49 | stamen endothecium lignification and tapetum and trichome development | [63–65] |
| C30       | AtMYB18, 19 and 45 | PtrMYB145 | MaMYB21 | response to infection with *Pseudomonas syringae*. | [18] |
ripening in HG2 [24]. MaMYB59, 73, and 76 belonging to C17 (S4) and MaMYB39 (MYB4) belonging to C18 were annotated as repressors of the phenylpropanoid pathway. AtMYB3, 4, and 32 in S4 were reported to be involved in suppressing lignin or flavonoid biosynthesis [14]. PtrMYB165, 182, and 194 in C18 were characterized as repressors for anthocyanin and proanthocyanidin biosynthesis [14]. MaMYB39, previously named MYB4, can negatively affect both anthocyanin and proanthocyanidin accumulation [24]. MaMYB42, 61, and 75 belonging to C19 (S7), MaMYB83 belonging to C12, MaMYB50, 81, 82, and 99 belonging to C25 (S5), and MaMYB58 belonging to C27 (S6) are annotated as flavonoid related genes based on the functions of their orthologs in *Arabidopsis* and *Populus* (Table 2). Other R2R3-MaMYBs with different annotated functions such as responsiveness to abiotic and biotic stresses are also displayed in Table 2.

### Table 2 (continued)

| Subgroups | Arabidopsis | *Populus* | *Morus* | Function annotation | Reference |
|-----------|-------------|-----------|---------|---------------------|-----------|
| C31(S13)  | AtMYB50, 55, 61 and 86 | PtrMYB042, 055, 074, 121, 148, 170 and 216 | MaMYB67 and 87 | stomatal aperture | [66, 67] |
| C32(S20)  | AtMYB2, 62, 78, 108, 112 and 116 | PtrMYB034, 036, 066, 142, 164, 202 and 210 | MaMYB40, 41, 74 and 95 | response to fungal attack | [61, 68, 69] |
| C33(S19)  | AtMYB21, 24 and 57 | PtrMYB204 and 207 | MaMYB88 | flower-specific transcription factor, is regulated by COP1 | [70, 71] |
| C34(S18)  | AtMYB33, 65, 81, 97, 101, 104 and 120 | PtrMYB007, 012, 024, 110, 124 and PtrMYB3R03 | MaMYB2, 17 and 25 | anther and pollen development. Response to ABA, auxin and cold stress | [1] |
| C35       | AtMYB71 and 79 | PtrMYB014, 015,098, 195, 214 and 229 | MaMYB12 | stress response | [72] |
| C36       | AtMYB27 | PtrMYB103 | MaMYB45 | Phenylpropanoid pathway | [73] |
| C37       | AtMYB48, 59, | PtrMYB023 and 206 | MaMYB69 | Stress response | [74–76] |
| C38       | AtMYB125 | PtrMYB208 and 218 | MaMYB84 | male gametophyte development in | [17] |
| C39(S25)  | AtMYB22, 98, 100, 115 and 118 | PtrMYB001, 004, 022, 073, 147 and 151 | MaMYB7, 68 and 88 | Induction of green root and petal development | [8] |
| C40(S22)  | AtMYB44, 70, 73 and 77 | PtrMYB019, 029, 033, 105, 122, 140, 173, 176 and 177 | MaMYB23, 33, 56, 57, 62, 78, 79 and 80 | stress responses, and leaf senescence | [77–79] |
| C41(S23)  | AtMYB1, 25 and 109 | PtrMYB041 and 163 | MaMYB24 and 27 | stress resistance-related transcription. | |
| C42(S21)  | AtMYB52, 54, 56 69, 105, 110 and 117 | PtrMYB026, 031, 039, 052, 062, 082, 090, 136, 158, 161, 167, 175 and 189 | MaMYB35, 55, 96, 102 and 103 | lignin, xylan and cellulose biosynthesis, | [9, 80] |
| C43/44    | AtMYB91 | PtrMYB091, 154 and 221 | MaMYB13 | cell differentiation | [8] |
| C45       | AtMYB88, 89 and 124 | PtrMYB196 and 213 | MaMYB37 | cell proliferation in stomatal lineage and cold hardness | [8, 81] |
| S12       | AtMYB28, 29, 34, 51, 76 and 122 | NA | MaMYB91 | Seed development and aliphatic glucosinolate biosynthesis | [82] |
| Ma-1      | NA | NA | MaMYB65 and 101 | – | – |
| Ma-2      | NA | NA | MaMYB53 and 54(TT2L3) | – | – |
| 3R-related | AtMYB3R1, 2, 3, 4 and 5 | PtrMYB3R01, 02, 04 and 05 and PtrMYB131, 231 and 232 | MaMYB3R1, 2, 3 and 4 | coordinate cell proliferation with differentiation in shoot and root | [83] |

Promoter analysis and gene structures of R2R3 and 3R-MYBs

Promoter regions (2000 bp upstream) of R2R3 and 3R-MaMYBs were extracted and the cis-elements were predicted. Light-response related cis-elements made up the greatest proportion (48%) of detected cis-elements (Additional file 4). Thirty-one classes of cis-elements were identified and the hormone related cis-elements were widely detected in the promoter regions of *MaMYBs* (Fig. 2). *R2R3-MaMYBs* with annotated functions of stress-responsiveness (blue boxes) had more abscisic acid (ABA), salicylic (SA) acid or methyl jasmonate (MeJA) related cis-elements. In particular, *MaMYB78*, 79, and 80, which were annotated as *MYBs* involved in stress responses and leaf senescence, had responsive cis-elements for all ABA, SA, MeJA, auxin, and gibberellin. *MaMYBs* with putative functions of
Fig. 2  Phylogenetic relationships and cis-elements in promoter regions of R2R3 and 3R-MaMYBs. A  Phylogenetic tree using 107 R2R3 and 3R-MaMYBs. B  Cis-element distribution in the promoter regions of R2R3 and 3R-MaMYBs
regulating phenylpropanoid pathway were indicated by reddish shadows and blue shadows (for repressors). These R2R3-MaMYBs also possessed MeJA, ABA and stresses related cis-elements. The intron/exon organizations of R2R3 and 3R-MaMYBs were detected, and the exon phases were also indicated (Fig. 3). Thirteen intron/exon patterns (a–m) were summarized, among which, an “a” pattern (two introns with phase numbers of 0 and 2) was the prevalent pattern for MaMYBs at 63.5%. All 3R-MaMYBs belonged to “m” pattern and possessed more than five introns.

**Chromosomal distribution and synteny analyses of R2R3 and 3R-MaMYBs**

All 107 R2R3 and 3R-MaMYBs were mapped to 14 chromosomes based on the genome information. Each chromosome contained several MaMYBs with nonuniform distribution (Fig. 4). Chromosomes 1 and 2, the top two longest chromosomes occupying 30.37% of the whole genome, contain only five and three MaMYBs, respectively (7.17% of total R2R3 and 3R-MaMYBs). Chromosomes 5, 8, and 10 held 13, 14 and 14 members each, respectively, and had the densest distribution of MaMYBs. This nonuniform distribution of MYBs was also observed in other plants including *Z. may* and *Populus* [10, 12]. Whole genome duplication (WGD) is the main force driving gene family duplication and is important for neo-function gene occurrence. The duplication events for MaMYBs were detected. Duplicated gene pairs resulting from intra-chromosome duplication and inter-chromosome block duplication were revealed (Additional file 5). Six tandem events in 16 MaMYBs and six block duplication gene pairs in 12 MaMYBs were found for R2R3-MaMYBs (Figs. 4 and 5). Compared with 145 R2R3-MYB in *Populus*, fewer duplication events for MaMYBs occurred because the *M. alba* lineage underwent no WGDs after its separation from the Euroid I clade, while *Populus* underwent a recent WGD event [25, 84].

**Expression profiles of R2R3 and 3R-MaMYBs**

A spatial expression profile based on RNA-seq data of roots, stems and leaves showed that 66 of 107 R2R3 and 3R-MaMYBs exhibited organ preference in expression. Among these organ-preferential differentially expressed genes, only 14 MaMYBs (marked with blue colors) showed higher expression levels in leaves (Fig. 6A and Additional file 6). These MaMYBs may be responsible for specific metabolite accumulation in leaves. For example, MaMYB11, 38 and 94 (C2/S9) were annotated as MYBs involved in cuticular wax biosynthesis and showed significant preference in leaves. Flavonoid biosynthesis related MYBs, including MaMYB36, 42, 61, and 102 had the highest expression levels in leaves rather than in stems and roots. MaMYB91, which was annotated as an aliphatic glucosinolate biosynthesis related MYB, was also preferentially expressed in leaves. Most R2R3-MaMYBs with higher expression levels in roots or stems were MYBs involved in secondary cell wall components and stress responsiveness. For example, cell wall component biosynthesis related MYBs, including MaMYB35, 55, and 103 (C42/S21) as well as MaMYB22 and 31 (C7) and stress responsiveness related MYBs, including MaMYB33, 35, 57, 62, 78, 79, and 80 (C40/S22) and MaMYB4 (C22), showed higher expression levels in roots or stems (Fig. 6A). It was obvious that R2R3-MaMYBs with similar annotated functions always maintained similar expression patterns in different organs.

Mulberry fruits are rich in flavonoids and anthocyanins and are used for wine, nutrient food, and medicine. The expression patterns of R2R3-MaMYBs and 3R-MaMYBs were revealed, and 72 of 107 R2R3 and 3R-MaMYBs were identified as DEGs during mulberry ripening (Fig. 6B and Additional file 6). Two clusters of R2R3 and 3R-MaMYBs based on the expression patterns, 27 early-expression MaMYBs, MaMYBs with higher expression levels in S0 or S1 and 45 late-expression MaMYBs, MaMYBs with higher expression levels in S2 or S3, were marked using red and blue respectively. Twenty differentially expressed R2R3-MaMYBs involved in flavonoid biosynthesis, anthocyanin biosynthesis, fruit development, and stress responsiveness and with relatively high overall expression levels were selected for qRT-PCR validation (Fig. 6B, Fig. 7A and Additional files 7 and 8). The mulberry fruit ripening process occurs along with the accumulation of anthocyanin and color changes (Fig. 7B). Both RNA-seq and qRT-PCR showed that R2R3-MaMYBs with putative roles in the phenylpropanoid pathway had different expression patterns during fruit ripening. The correlation relationships of the expression levels of these MaMYBs and anthocyanin contents were also revealed (Fig. 7C). It was clear that MaMYBs with late-expression patterns were positively correlated with anthocyanin accumulation, while MaMYBs with early-expression pattern are negatively correlated with anthocyanin accumulation (Fig. 7C). R2R3-MaMYBs such as MaMYB43, 77, 83, 98, and 58, which may play direct roles in PA or anthocyanin accumulation, showed late-expression patterns. These MaMYBs are likely to positively regulate anthocyanin biosynthesis. For instance, MaMYB58 (MYBA) has been reported to interact with bHLH3 to activate the expression of anthocyanin biosynthetic genes to control anthocyanin biosynthesis. Moreover, some phenylpropanoid pathway repressors, such as MaMYB59 and MaMYB39, also showed increasing expression levels along with fruit ripening. MaMYB59 was annotated as a repressor
Fig. 3 Phylogenetic relationships and gene organization of R2R3 and 3R-MaMYBs. A Phylogenetic tree using 107 R2R3 and 3R-MaMYBs. B Exon/intron structures of *Populus* R2R3-MYBs. C Schematic of intron distribution patterns of R2R3 and 3R-MaMYBs.
of lignin biosynthesis, and MaMYB39 was annotated as a repressor of flavonoids biosynthesis. In addition, there were also early-expression pattern R2R3-MaMYBs involved in flavonoid biosynthesis or anthocyanin biosynthesis. MaMYB75 (MYBF), which was reported to be responsible for flavonol accumulation and negatively regulate anthocyanin biosynthesis, showed a decreasing expression level along the fruit ripening. MaMYB36 and 89 were annotated as having functions in anthocyanin biosynthesis and trichome cell differentiation, and also exhibited early-expression patterns, indicating that MaMYB36 and 89 may have been responsible for mulberry fruit trichome development rather than anthocyanin biosynthesis. The different expression patterns of flavonoid related R2R3-MaMYBs along with mulberry fruit ripening suggested that different R2R3-MaMYBs coordinated to maintain homeostasis and avoid the over-accumulation of anthocyanins during fruit ripening.

Discussion
Genome-wide identification of R2R3-MYB TFs is influenced by quality of genome annotation and assembly [5]. A previous study identified 116 R2R3-MYBs based on de novo transcriptome data [23]. However, in the present study, we identified 103 R2R3-MYBs in M. alba using the latest M. alba genome assembly with a stricter workflow. Sequence length and MEME motif extraction validation were considered as additional two filtering steps to further screen MYBs in this study. The numbers of different kinds of MYBs are close in different plant species, except for Populus (Table 1). Moreover, the number of R2R3-MYBs (103) identified in Morus was close to that of V. vinifera (108), but far less than that of Populus (198). Although the number of R2R3-MaMYBs in Morus was less than that of Populus, the R2R3-MaMYBs covered almost all subgroups of Populus R2R3-MYBs and Arabidopsis MYBs (Table 2). It has been suggested that WGDs and tandem duplication events have contributed to the expansion of R2R3-MYBs in land plants [85]. It was reported that no lineage-specific WGD occurred in M. alba after the yhexaploidization event [25]. Compared with duplication events identified for R2R3-MYBs in Populus which experienced a recent lineage-specific WGD [84], fewer duplication events were found in Morus for the R2R3-MYBs and only 28 MaMYBs resulting from tandem duplication and block duplication were found for the R2R3-MaMYBs (Figs. 4 and 5). The above differences further support the hypothesis that the expansion of R2R3-MYB TFs in land plants has been subfamily/clade asymmetric and lineage specific [5].

The conserved DBD domain in the N-terminal of MYBs was identified in R2R3-MaMYBs. The spaced W forming
WX\textsubscript{19}WX\textsubscript{19}W was highly conserved in R2 repeat, while the replacement of the first W with F, I, or M at position 54 for R3 was generally reported in plants [10–13]. The conserved spaced W motifs in R2 and R3 are signature sequences for the DBD domain of MYBs. In addition to the conserved W, there are several highly conserved residues in the DBD domain. These conserved residues mainly contained residues located at the start and end of helices and residues consisting of the third helix (Fig. 1). Residues located at the start and end of helices may be responsible for maintaining the helix structure. The conserved third helices in R2 and R3 are responsible for the specific base recognition in the major groove of the DNA [6]. In addition, the residues consisting of the third helices...
helix in R2 and those consisting of the third helix in R3 are greatly different which might be important for recognition of diverse DNA sequences. It has been reported that the residues including L39, N86, K89 and 90 in the third helices of R2 and R3 are important for maintaining MYB functions [7]. In addition, K35 was reported to play an important role in sensing DNA methylation at the fifth position of cytosine (5 mC) [7]. The InterPro MYB domain signature pattern (PS00037) was detected and conserved in the first helices of both R2 and R3 repeats. Given that the first helices were beyond the DNA binding regions, it was possible that the first helix functioned as structure helices.

A total of 103 R2R3-MaMYBs, except for MaMYB53, 54, 65, and 101, were grouped with the R2R3-MYBs from Arabidopsis and Populus, indicating the high retention of R2R3-MYBs in M. alba after paleohexaploidy [25]. MaMYB3, 54, 65, and 101 formed two Morus specific subgroups (Ma1–2). Because R2R3-MYB gene retention after duplication events is biased among angiosperm taxa, and the pervasive duplications of R2R3-MYBs in core eudicots are thought to be gamma-triplication derived [5], R2R3-MYBs in Morus seem to exhibit relatively conserved R2R3-MYBs from before the divergence of the core eudicots. Most R2R3-MYBs in Arabidopsis have been well annotated and functionally characterized [1, 9, 14]. Phylogenetic analysis based functional annotation suggested that R2R3-MaMYBs were mainly involved in responses to abiotic and biotic stresses, development and secondary metabolite biosynthesis (Table 2). AtMYB20, 41 and 44 were reported to involve in response to abiotic stresses and AtMYB44 was reported to enhance stomatal closure to confer abiotic stress tolerance [38, 45, 86, 87]. Wheat TaMYB31 was reported to involve in drought stress responses [81] MaMYB14, 48, 79 and 80 were annotated as MYBs involved in abiotic stress responses in mulberry. AtMYB30 was reported to act as positive regulator of hypersensitive cell death program in plants in response to pathogen attack [50]. MdMYB30 in apple and SimYB28 in tomato regulated plant resistance to pathogen attack [88, 89]. MaMYB10, 44 and 90 as homologs clustered with AtMYB30 may also function in response to pathogen attack. Mulberry is rich in phenylpropanoid-derived bioactive compounds, such as flavonoids and polyphenols, and mulberry fruits are known for their abundance of anthocyanins [90, 91]. Possible anthocyanin homeostasis was maintained by both cooperative and antagonistic interactions of phenylpropanoid pathway related R2R3-MaMYBs. Both the increasing expression of activators such as MaMYB43, 77, 83, 98 and 58 and decreasing expression levels of MaMYB54 (TT2L3), which was involved in flavonol biosynthesis along with fruit ripening, positively affected anthocyanin accumulation. In contrast, increasing the expression levels of

Fig. 6 Expression profiles of R2R3 and 3R-MaMYBs in Morus. A Heatmap of R2R3 and 3R-MaMYB expression levels in Morus roots, stems, and leaves. MaMYBs with higher expression levels in leaves are shown in blue, and MaMYBs with higher expression levels in roots or stems are shown in red. MaMYBs referred to in the text are also indicated by stars. B Heatmap of R2R3 and 3R-MaMYB expression levels in fruits at different developmental stages. The MaMYBs with higher expression levels in S0 or S1 are colored with blue, and the MaMYBs with higher expression levels in S2 or S3 are colored with red. Differentially expressed MaMYBs involved in the phenylpropanoid pathway are indicated by stars.
Fig. 7 Relationships between the expression levels of these MaMYBs and anthocyanin accumulation during mulberry fruit ripening. A Expression levels of selected MaMYBs during fruit ripening using qRT-PCR. B Changes in anthocyanin contents of mulberry fruits during fruit ripening. C Correlation between the expression levels of selected MaMYBs and anthocyanin accumulation. The pearson correlation coefficients were marked
repressors such as MaMYB39 (MYB4) and MaMYB59 helped to avoid the overaccumulation of anthocyanins and preserve the balance between lignin biosynthesis and flavonoid biosynthesis (Fig. 6). Both our expression profiles and a previous study on MaMYB54 (TT2L3) and MaMYB39 (MYB4) support this postulation [24]. The homeostasis between anthocyanin and lignin biosynthesis regulated by cooperation of repressor MdMYB16 and activator MdMYB1 was also reported in apples [92].

Conclusion
A comprehensive analysis of the R2R3-MYB gene family using the latest M. alba chromosome-level genome was conducted in the present study. In total, 166 MYB genes were identified and 103 R2R3-MaMYBs and four 3R-MaMYBs were further analyzed. Functional annotation for R2R3-MaMYBs based on phylogenetic analysis as well as functional annotation of orthologs from Arabidopsis and Populus suggested the possible functional subgroups of R2R3-MaMYBs. Conserved gene organization, promoter analysis, and expression profiles also provided evidence for putative functions. Moreover, R2R3-MYBs involved in the phenylpropanoid pathway were investigated, and MaMYBs involving in flavonoid biosynthesis and anthocyanin accumulation were identified based on functional annotation and the correlation of their expression levels with anthocyanin contents. These findings will be valuable for future genetic improvement of specific biological processes, such as flavonoid or anthocyanin biosynthesis, and provide a basic reference for research on the functional characterization of MaMYB genes involved in specific biological process.

Ethical statement for experimental research and field studies on plants
Experimental research and field studies on plants complies with relevant institutional, national, and international guidelines and legislation.

Supplementary Information
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