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Genome-Wide Identification and Expression Analysis of the R2R3-MYB Genes Family in *Rosa chinensis*

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Abstract *Rosa chinensis* is a commercially important ornamental plant cultivated worldwide with high ornamental and economic value. The objectives of this study were to analyze the structure, conserved domain and evolution of R2R3-MYB by bioinformatics, besides, it was also to study the expression specificity of family members in different tissues and investigate the biological function of R2R3-MYB genes family in *Rosa chinensis*. According to the reported R2R3-MYB genes of *Arabidopsis thaliana*, one hundred and twenty members of R2R3-MYB genes in *Rosa chinensis* 'Old blush' were identified by BLAST and Hmmer tool. Phylogenetic tree indicated that these genes were divided into 34 subgroups. The N-terminal of the amino acid sequence of R2R3-MYB family members contains 2 unequal repeat domains R2 and R3. The results of gene structure analysis showed that the R2R3-MYB family genes contain 1 to 11 exons; 120 genes were located on 7 chromosomes of rose, and 8 tandem repeat events occurred by chromosome mapping analysis. The results of analysis of genome correlation, a total of 16 pairs of R2R3-MYB gene clusters were found. Based on the analysis of gene expression patterns, the results showed that 120 genes were wildly expressed throughout the development of the rose. What's more, they could be divided into 6 subgroups, and there were different patterns of expression between each subgroup.

Keywords *Rosa chinensis*; R2R3-MYB genes family; Bioinformatics; Expression model

Rosa chinensis is one of the four cut flowers in the world with important ornamental and economic value. Flower color, as one of the important ornamental characters of *Rosa chinensis*, has very important research significance, and is mainly regulated by MYB transcription factors. MYB gene family widely exists in eukaryotic cells and is one of the largest gene families in plants (Dubos et al., 2010). The MYB gene family has a highly conserved DNA binding domain near the N-terminal of the sequence, which is composed of 1~4 incomplete repeats in series, and each repeat contains about 52 amino acid residues. The second repeat and the third repeat constitute the HTH (helix-turn-helix) structure, which contains 3 tryptophan and is separated by 18~19 amino acid residues, forming a secondary structure that can interact with the target gene. At the C-terminal of the sequence, there are different conserved domains, which make the MYB family have different regulatory functions. According to the number of incomplete repeats, MYB genes can be divided into four different types. According to the number of incomplete repeats, that is, 2R (R2R3-MYB), 3R (R1R2R3-MYB), 4R (R1R2R2R1/2-MYB) and 1R-MYB (MYB-related proteins), in which R2R3-MYB genes account for more (Ma and Constabel, 2019).

R2R3-MYB is the main type of MYB family, which acts on the secondary metabolism of plants such as benzene, phenylpropanoid, terpenoids and glucosinolates (Zhang et al., 2017). In the metabolic pathways of anthocyanin and proanthocyanidins (PAs), the activated transcription factors related to anthocyanin synthesis, such as *AtMYB75* (*PAP1*), *AtMYB90* (*PAP2*), *AtMYB113* and *AtMYB114* in Arabidopsis, can regulate anthocyanin accumulation. Similar results were also found in *VvMYBA1* and *VvMYBA2* (Walker et al., 2007) in grapes, *MdMYB10* and *MdMYB110a* in apples (Chagne et al., 2013). And the activated transcription factors related to the synthesis of proanthocyanidins (PAs), such as *AtMYB123* (*TT2*) and *AtMYB5* (*PA1*) in Arabidopsis (Schaart et al., 2013), *VvMYBPA1*, *VvMYBPA2*, *VvMYB5a* and *VvMYBPAR* in grape (Schaart et al., 2013), *PpMYBPA1* and

PpMYB7 in peach (Zhou et al., 2015a), and *MdMYB9* and *MdMYB11* in apple (An et al., 2015), can act on the synthesis of proanthocyanidins in plants. Moreover, R2R3-MYB can respond to biotic and abiotic stresses. In *Rosa rugosa*, external stress such as wound and oxidation can induce the high expression responses of PA1-type and TT2-type R2R3-MYB transcription factors *RrMYB5* and *RrMYB10*, thereby promoting the expression of flavonoid structural genes. The synthesis of anthocyanin and proanthocyanidins further enhanced the tolerance of plants to trauma and oxidative stress (Shen et al., 2019). Light could activate the expression of *HY5* gene in *Citrus sinensis* by binding to the G-box response element in the promoter region of R2R3-MYB gene *CsRuby1* (Huang et al., 2019), so that the flesh color changed from white to purple. In addition, studies have reported that some peculiar flower patterns in plants are also related to R2R3-MYB genes. The petal blotch formation of *Paeonia suffruticosa* is since R2R3-MYB gene *PsMYB12* can specifically activate the expression of *PsCHS* gene in the blotch, resulting in the accumulation of anthocyanin in the blotch to form the blotch-type flowers (Gu et al., 2019). R2R3-MYB gene has always been a hotspot in plant color research, but the comprehensive identification and analysis of R2R3-MYB gene family in *Rosa chinensis* are rarely reported. The publication of the rosa genome (Raymond et al., 2018) has greatly promoted the study of important traits of the *Rosa chinensis* and laid the foundation for the comprehensive identification of the R2R3-MYB gene family. In this study, the R2R3-MYB gene family of *Rosa chinensis* was identified based on the rosa genome, and the structure, conserved domain and tissue-specific expression of these genes were studied. According to the transcriptome data of Rosa chinensis *Rosa chinensis* 'Old blush', the expression pattern of R2R3-MYB gene was analyzed, which provides a theoretical basis for the future analysis of R2R3-MYB involved in the growth and development of *Rosa chinensis* and the cultivation of new varieties of *Rosa chinensis*.

1 Results and Analysis

1.1 Analysis of the R2R3-MYB genes family in *Rosa chinensis*

To study the gene structure and phylogenetic relationship of the R2R3-MYB family in *Rosa chinensis*, BLAST and Hmmer were used for searching, and the sequences without MYB-domain or incomplete reading region were removed by combining Pfam and SMART analysis. Finally, 120 R2R3-MYB genes were obtained. It was found that these genes had two MYB conserved domains at the N-terminal of the sequence. Cluster analysis of these 120 R2R3-MYB genes and *Arabidopsis thaliana* R2R3-MYB genes showed that R2R3-MYB genes were divided into 34 subgroups (Figure 1), of which 39 protein sequences could not be clustered. The conserved motifs of R2R3-MYB family proteins were analyzed by MEME software, and 20 conserved motifs were identified (Figure 2). Each protein sequence contained motif-1, motif-2 and motif-3 at the N-terminal of the sequence. There were conserved tryptophan residues in these three motifs, which were related to R2 and R3 domains. However, the conserved motifs at the C-terminal of the sequence were quite different. Motif-5 exists in the subfamilies S4, S9, S10, S11 and S24, belonging to the C2 structural box (EAR motif), which is a conserved domain with negative regulation ability. Motif-11 to motif-17 were unknown boxes, mainly concentrated in S21, S24, S25, S26 subfamilies.

1.2 Conservative domain analysis of R2R3-MYB gene in *Rosa chinensis*

MUSCLE multiple alignment was performed on the 120 identified R2R3-MYB sequences in *Rosa chinensis*, and the sequences were pruned with G-block to identify 103 highly conserved amino acids. The N-terminal of these amino acid sequences formed two special domains R2- and R3- (Figure 3), which were composed of 51 and 52 amino acid residues, respectively. Each domain forms three helixes, separated by periodic tryptophan residues (W, Trp), forming HTH structure, which is used as a marker of MYB domain. Further analysis of the two domains showed that most of the first tryptophan in R3- was replaced by hydrophobic amino acids. In addition, in domain R2-, E (Glu)-8, D (Asp)-9, R (Arg)-35\43, N (Asn)-46, L (Leu)-48 were located at the junction of R2- and R3-. In domain R3-, E (Glu)-10, G (Gly)-22, R (Arg)-35, T (Thr)-36, and K (Lys)-41 were highly conserved in the R2R3-MYB gene family. Among the three helical structures, the third helix is obviously more conservative than the other two helical structures. These structural features in R2R3-MYB are similar to previous studies (Kaneiishii et al., 1990).

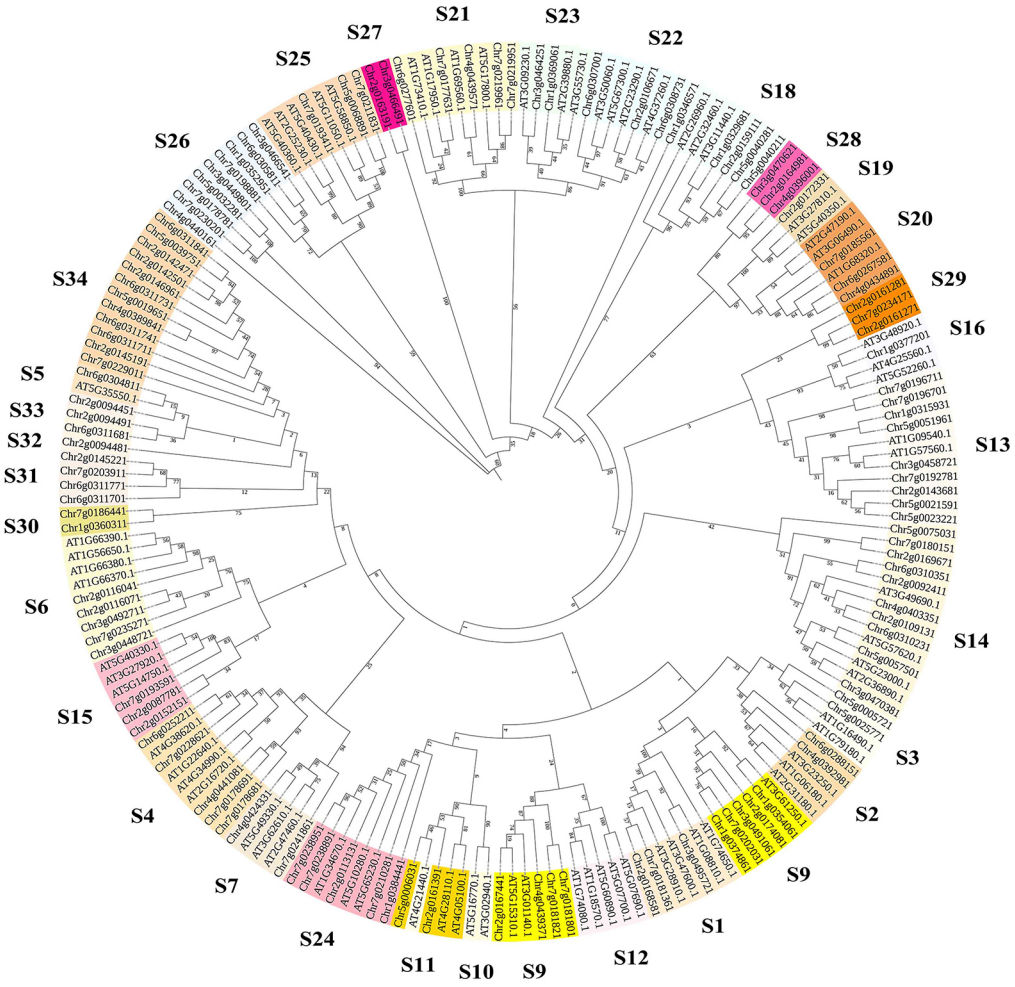

Figure 1 The phylogenetic tree analysis of R2R3-MYB family proteins in *Rosa chinensis* and *Arabidopsis thaliana*

1.3 Analysis of gene structure and chromosome location

By analyzing the position of 120 R2R3-MYB family genes on seven chromosomes (Figure 4), it was found that most of the family genes were distributed in the upstream/downstream of the chromosome. The genes were less distributed on chromosome 1, 3, 4 and 5, but mainly focused on chromosome 2, 6 and 7, and eight tandem repeat events occurred on these three chromosomes. The online software GSDS was used to analyze the structure of R2R3-MYB family genes in *Rosa chinensis* (Figure 5). The results showed that the number of introns and exons of the R2R3-MYB family genes in *Rosa chinensis of R2R3-MYB gene family* was highly different. The number of introns was 0 to 10, and the number of exons was 1 to 11. Among them, the gene structures of S1, S9 and S12 subgroups were highly consistent, and the differences of S21, S25 and S26 subgroups were extremely high and the differentiation was serious.

1.4 Correlation analysis and species evolution analysis

The correlation analysis of 120 R2R3-MYB family genes in *Rosa chinensis* showed that there were 16 repeat genes in seven chromosomes (Figure 6A), most of which were concentrated on chromosome 2. Compared with Arabidopsis and strawberry genomes (Figure 6B), the position of R2R3-MYB family genes in rose is highly variable. Rose and strawberry genome similarity is very high, R2R3-MYB family gene distribution in *Rosa chinensis* is similar to strawberry. Most of the genes on chromosomes 1, 2 and 3 correspond to strawberry chromosomes 5, 6 and 7 (Figure 6B).

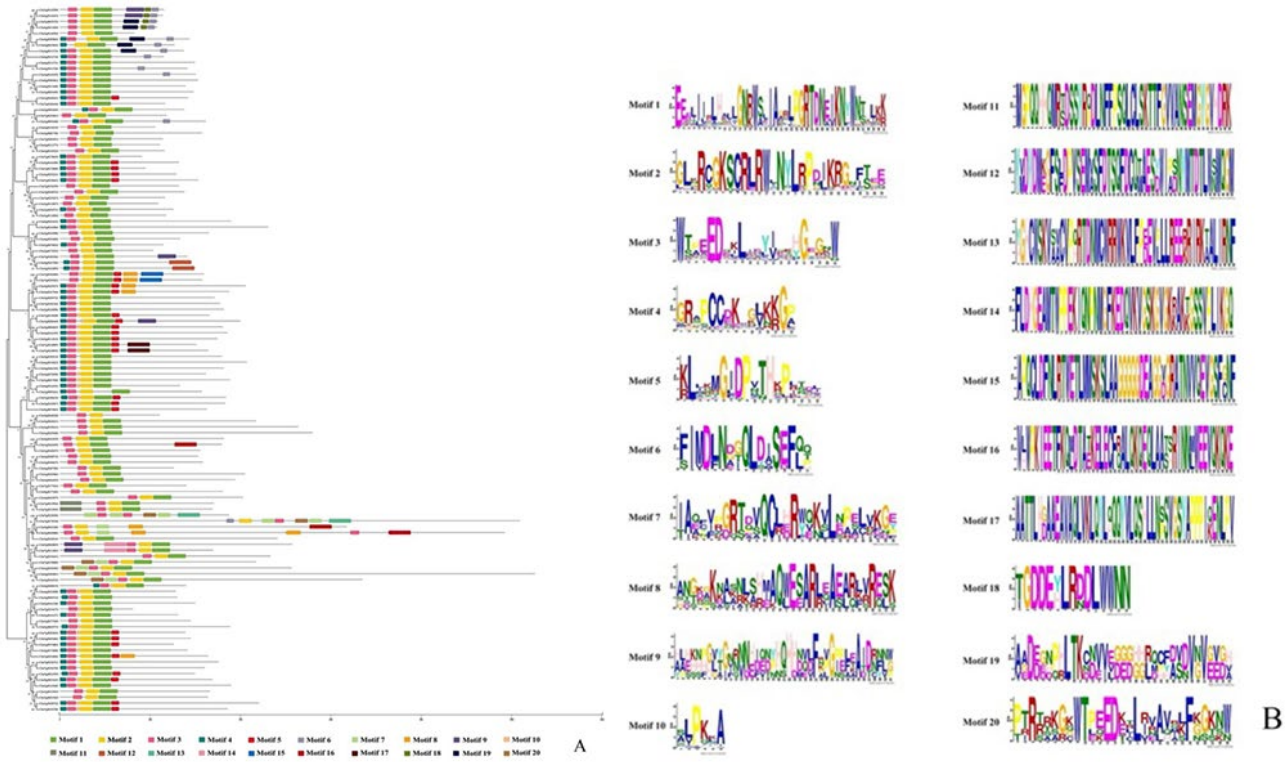

Figure 2 Conserved element prediction of R2R3-MYB family genes in *Rosa chinensis*

Note: A: The conservative element distribution figure; B: The detailed structure of 20 motifs

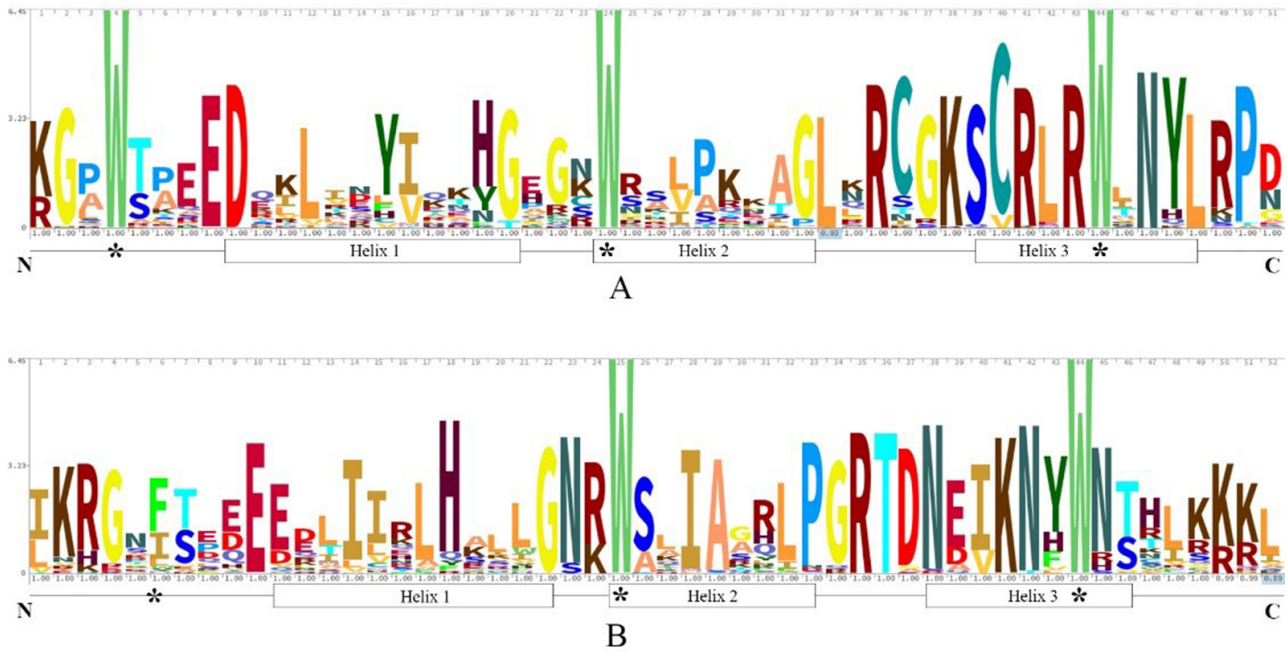

Figure 3 Sequence logos of the R2 (A) and R3 (B) MYB repeats for the full-length alignments of all rose R2R3-MYB domains

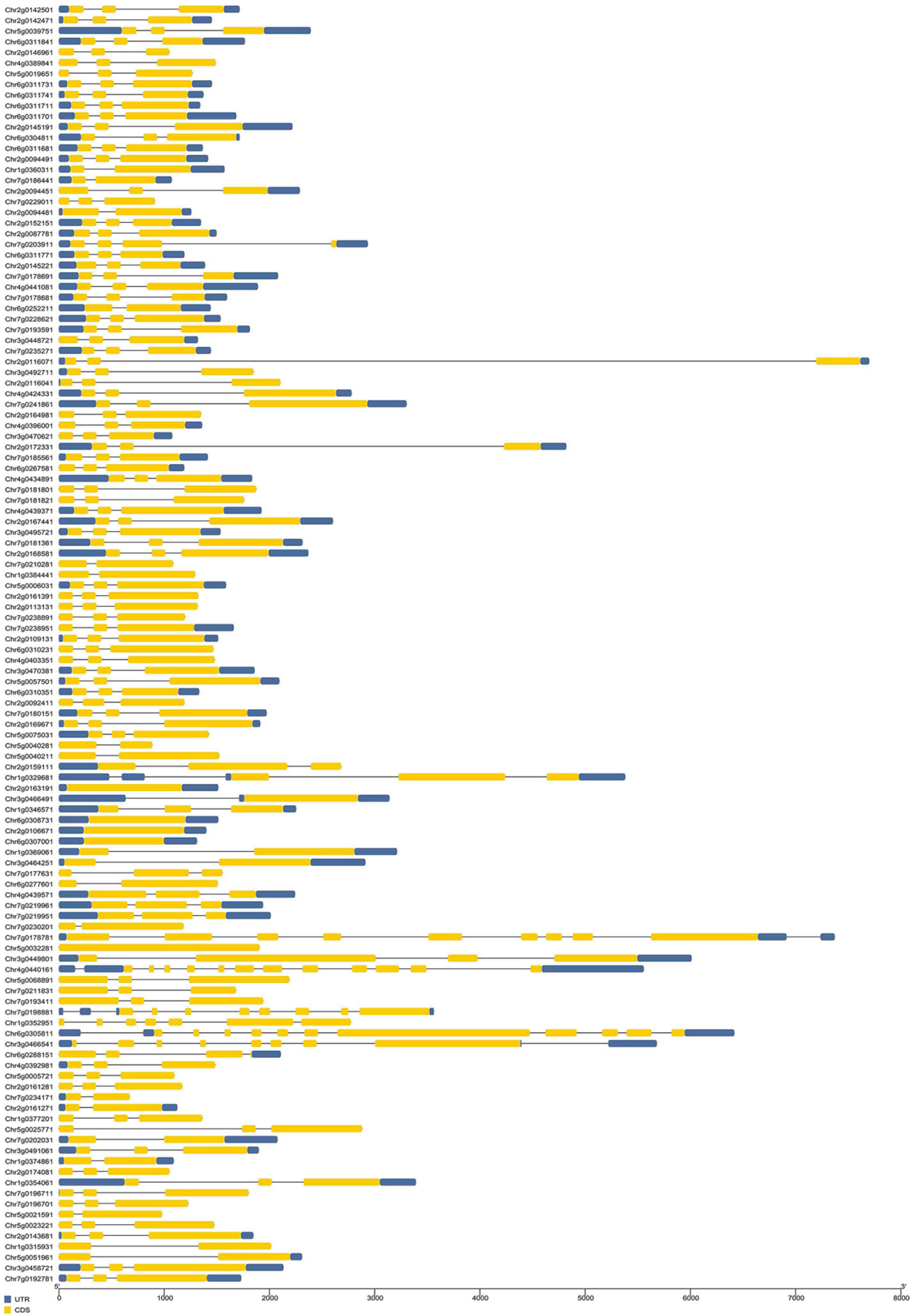

Figure 4 Gene structure of R2R3-MYB genes in *Rosa chinensis*

Note: Yellow lines indicate the CDS of genes; Blue lines indicate the upstream/downstream; Gray lines indicate intron

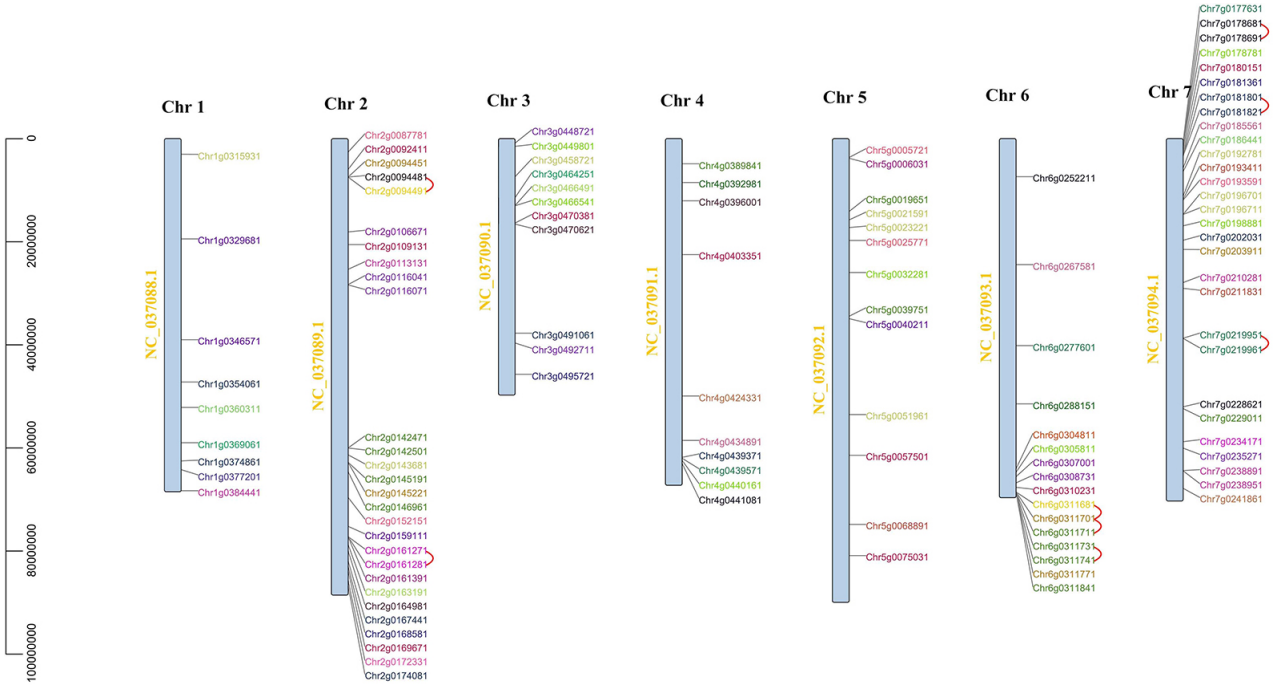

Figure 5 Chromosome location of R2R3-MYB genes in *Rosa chinensis*

Note: Gene name colors indicate different subgroups, and red lines indicate tandem repeats

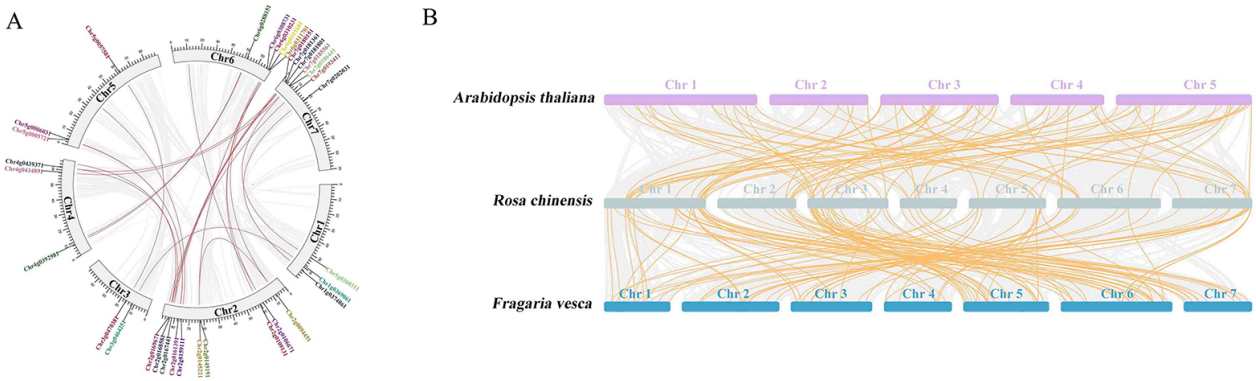

Figure 6 Synteny analysis of R2R3-MYB genes

Note: A: Synteny analysis of R2R3-MYB genes in Rose, gray lines mark the position of genes on chromosomes, curved lines indicate collinearity relationship among genes, whereas the red lines suggest duplicated MYB gene pairs, gene name colors indicate different subgroups; B: Synteny analysis of R2R3-MYB genes between rose as well as Arabidopsis and strawberry, the yellow lines suggest duplicated MYB gene pairs

1.5 Expression analysis of R2R3-MYB gene in *Rosa chinensis*

Based on the RNA-Seq database of *Rosa chinensis* 'Old Blush' at different parts and periods, the expression patterns of R2R3-MYB family genes were analyzed. A total of 120 R2R3-MYB family genes were divided into six groups according to the expression of genes at 13 different stages and positions (BFL, OFT, SEN, NDB, RAC, DBO, FTN, IMO, IFL, CYN, FTS, DET, FTB) (Figure 7). Among them, group I contained 24 genes, which were expressed in different tissues and periods. It was speculated that these genes were involved in the whole growth and development of plants. Among them, the S9 subfamily gene *Chr2g174081* and the S6 subfamily gene *Chr3g0448721* were significantly expressed in BFL and OFL flower color transition periods. As the homologous gene of *RhMYB10*, *Chr3g0448721* was speculated to have the function of promoting anthocyanin synthesis. There were 26 genes in group II, mainly expressed in NDB, DBO, IMO, DET and flower development period. Compared with group I, the expression of these genes was specific, mainly concentrated in axillary buds, flower organs and other parts. There were 18 genes in group Ⅲ, among which the expression of IMO and FTS increased

significantly, which was inferred to be mainly related to plant meristem. In group IV, there were 28 genes with low expression in most tissues and parts, which may not be involved in plant growth and development. There were 15 genes in group V, most of which were expressed in NDB, FTN, IMO, CYN and DET, indicating that these genes are related to the formation of young tissues in early axillary bud and flower development. In addition, *Chr3g0458721* and *Chr7g0234171* were specifically expressed in RAC, indicating that these genes may also be involved in the development of young roots in plant tissues. There were six genes in group VI, and their tissue-specific expression level was not high, and they were ubiquitously expressed in 13 periods. Among them, the S14 subfamily gene *Chr6g0310351* was highly expressed in young roots, suggesting that the function was related to root development. Different from group I, the gene expression in group VI was higher in different tissues and periods. It was speculated that group VI had a more extensive function and played a more important role in the normal growth and development of plant tissues (roots, stems, leaves, flowers).

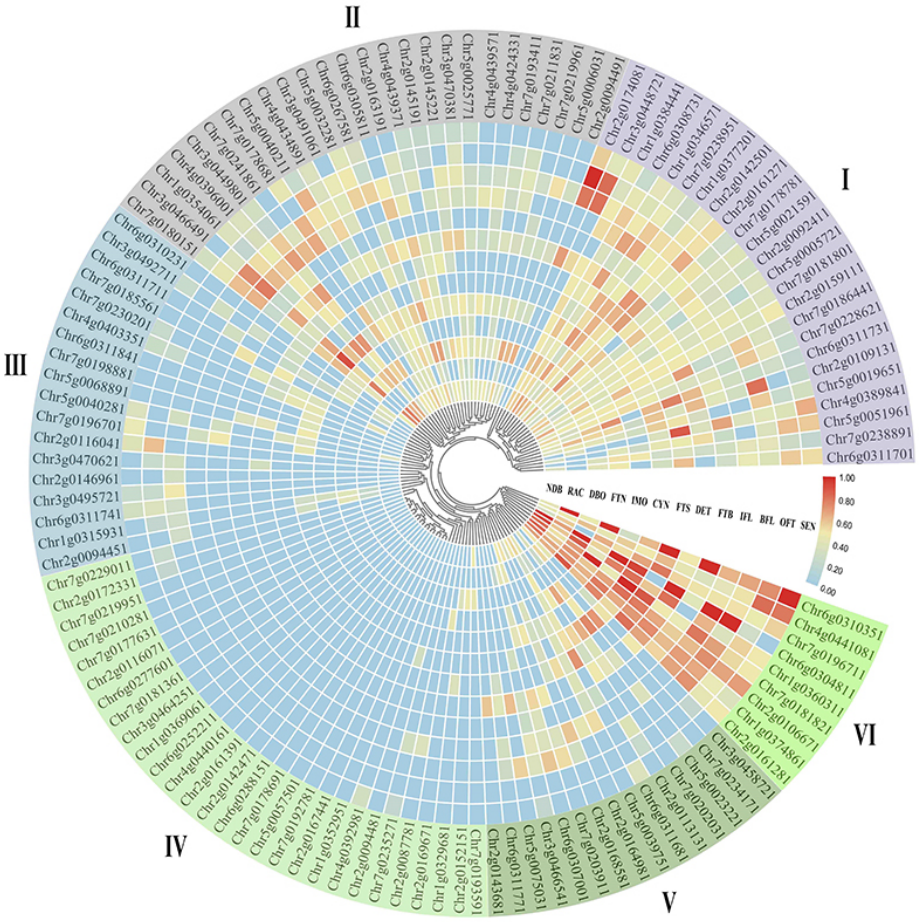

Figure 7 The heatmap of expression of RcR2R3-MYB family genes in *Rosa chinensis* 'Old Blush'

Note: Gene name colors indicate different 6 subgroups with Roman numerals; The colors that is blue to red indicate relative expression data of 13 different periods and tissues

2 Discussion

R2R3-MYB family is a kind of gene family with R2 and R3 domains, and its encoded proteins are generally involved in regulating flower color, fruit color or response to stress (Zhu et al., 2016). To comprehensively understand the R2R3-MYB family genes in *Rosa chinensis*, 120 R2R3-MYB genes were identified based on the published rosa genome data in this study. The phylogenetic tree of R2R3-MYB family in *Rosa chinensis* was constructed by cluster analysis, and 120 genes in *Rosa chinensis* were divided into 34 subgroups. Due to the conservation of R2R3-MYB gene, genes with similar or identical functions will be classified into the same subgroup, which provides a reliable basis for the study of the function of the gene family related genes. In Arabidopsis S6 subgroup, *AtMYB75* (*PAP1*), *AtMYB90* (*PAP2*), *AtMYB113* and *AtMYB114* are the main

transcription factors regulating anthocyanin biosynthesis in *A. thaliana*. *Chr3g0448721*, *Chr7g0235271*, *Chr2g0116071*, *Chr3g0492711* and *Chr2g0116041* are clustered into the same branch with them, and it is speculated that there are similarities in their functions, which may have important functions in the regulation of anthocyanin in *Rosa chinensis*. In Arabidopsis S4 subgroup, *AtMYB4* can inhibit the expression of phenylalanine pathway *C4H* gene (Zhou et al., 2015b), and it is speculated that *Chr7g0178691*, *Chr4g0441081*, *Chr7g0178681*, *Chr6g0252211* and *Chr7g0228621*, which are close to its genetic distance, also have similar function (Dubos et al., 2010). Arabidopsis S14, S16, S19, S21, and S22 subgroups have important functions for plant nutrition and reproductive growth. In addition, in S1 subgroup, *AtMYB30*, *AtMYB60* and *AtMYB96* can activate transcription factors that respond to stress and play an important role in plant stress growth (Dubos et al., 2010). It is speculated that *Chr3g0495721*, *Chr7g0181361* and *Chr2g0168581* can respond to stress conditions. R2R3-MYB genes are widely involved in plant growth and development. Understanding the structure and function of these genes is of great significance for future rosa breeding and life science research.

The N-terminal structure of R2R3-MYB gene in *Rosa chinensis* is highly conserved, and the specificity at the C-terminal of the sequence is strong, which may be the reason for gene functional differentiation (Duan et al., 2020). Through amino acid alignment and MEME analysis of 120 R2R3-MYB genes in *Rosa chinensis*, it was found that there are two highly conserved unequal repeat domains R2 and R3 at the N-terminal of the sequence, which directly interact with the target gene DNA. If the mutation occurs, the gene function will be lost. There was conservative [DE]Lx2[RK]x3Lx6x3R residues in the R3 repeat sequence. These residues were essential for the interaction with bHLH transcription factors, which was also the main way for MYB transcription factors to regulate anthocyanin and PA. In the C-terminus of the sequence, the conserved domain showed a large difference, most of the repressor transcription factor C-terminus will exist C1-motif (GIPD-motif), C2-motif (EAR-motif), TLLLFR motif, C1-motif, C2-motif, which have been confirmed to have a repressor effect, but the role of TLLLFR motif is not clear (Ma and Constabel, 2019). Arabidopsis S9, S11 subfamily has C1-motif, S4 subfamily has EAR-motif, conservative domain is LxLxL, the same found rosa S4, S9, S11 subgroup of 16 genes also have similar conservative structure, speculated that these genes have inhibitory effect on plant growth and development. Other genes do not have such structures, which may be one of the reasons for gene functional differentiation.

The expression patterns of R2R3-MYB gene family members were different in different periods and tissues. Group I genes are expressed in all tissues and developmental stages and play an important role in plant growth and development. Group VI genes are more strongly expressed, which may be the main genes involved in the regulation of plant growth and development. Group II genes show the same expression pattern in different stages of IMO, BFL, OFT and SEN flower color development, and the expression level of OFL is the highest at the flowering stage, while *MdMYB10* and *MdMYB110a* are highly expressed in apples with the deepening of pericarp color (Chagne et al., 2013). It is speculated that genes in group II are related to flowering process and anthocyanin synthesis. The gene expression of group III and group IV in different tissues and periods is low, even not expressed, may not participate in plant growth and development. There are great differences in gene expression patterns among different groups, suggesting that different groups of R2R3-MYB play different roles in plant development. In addition, the R2R3-MYB genes were widely distributed in clusters on chromosomes, mainly concentrated in the upstream/downstream of chromosomes 2, 6 and 7, and gene duplication was found. Through the analysis of repetitive genes and gene expression data, it was found that repetitive genes had the same expression pattern to *Chr1g0369061-Chr3g0464251*, *Chr4g0392981-Chr6g0288151*, *Chr1g0360311-Chr7g0186441*, *Chr2g0106671-Chr6g0308731*, *Chr2g0167441-Chr7g0181801*, and its expression was mainly concentrated in the process of flower development. It was speculated that they participated in the flower development of *Rosa chinensis*. The difference of expression patterns of other repetitive MYB genes reflected the sub-function or new function of repetitive genes.

3 Materials and Methods

3.1 Sequence screening of R2R3-MYB gene in *Rosa chinensis*

The genome and protein sequences of *Rosa chinensis* were downloaded from *Rosa chinensis* 'Old Blush' genome website (https://lipm-browsers.toulouse.inra.fr//pub/RchiOBHm-V2/). A total of 126 protein sequences of the identified Arabidopsis R2R3-MYB gene family were obtained from previous literature and NCBI (https://www.ncbi.nlm.nih.gov/). BLASTP ($E<10^{-7}$) was used for comparative search in the *Rosa chinensis* protein database and the repeated sequences were removed. The MYB domain sequences (PF00249) of all species were downloaded from the Pfam database (http://pfam.xfam.org/), and then the HMMER 3.0 software (http://hmmer.org/) was used for further comparative search. The parameters were set as the default parameters. The sequences obtained from the two searches were crossed, and further domain analysis was performed using Pfam and SMART (http://smart.embl.de/), and finally the R2R3-MYB family gene in *Rosa chinensis* was determined.

3.2 R2R3-MYB family classification and protein domain analysis of *Rosa chinensis*

Multiple sequence alignment of the identified R2R3-MYB family protein sequences in *Rosa chinensis* and 126 R2R3-MYB genes in *Arabidopsis thaliana* was performed with the help of MUSCLE program of MEGA 6. Neighbor-joining (NJ) method was used to construct the phylogenetic tree. Bootstrap method was set to 1 000, and other parameters were set to default parameters. Introns, exons and genomic location information of MYB family genes are from the rosa genome database. The gene structure map is drawn by online software GSDS (http://gsds.cbi.pku.edu.cn/index.php), and the MYB conserved domain of R2R3-MYB protein in *Rosa chinensis* is analyzed by sequence analysis software DNAMAN 5.0 and online software Skylign (http://skylign.org/). The online software MEME (http://meme-suite.org/tools/meme) was used to predict the motif of R2R3-MYB protein. The number of predicted parameters was set to 20, and the other parameters were set as the default parameters.

3.3 Chromosome location and repeat gene analysis

Based on the genome database of rosa, MapChart software was used to map the position of MYB gene family on chromosomes. MCscanX software was used to analyze the replication events of MYB gene family of *Rosa chinensis* in the genome.

3.4 Expression analysis of R2R3-MYB in *Rosa chinensis*

The related expression data of R2R3-MYB family genes in *Rosa chinensis* were extracted from the published transcriptome data of *Rosa chinensis* 'Old Blush' (https://lipm-browsers.toulouse.inra.fr/plants/R.chinensis). TBtools tool (Chen et al., 2020) was used to draw the expression heatmap.

Authors' Contributions

LML was the executor of the experimental design and research in this study. LML, ZXN and LSN completed the data analysis, and the first draft of the paper. WQS participated in the experimental design and the analysis of experimental results. BMZ and FXP was the project designer and director, guiding experimental design, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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