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Cloning and Expression of One Anthocyanin-Related R2R3-MYB Gene in *Rosa rugosa*

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Abstract

Based on the transcriptome of *Rosa rugosa*, one anthocyanin-promoting R2R3-MYB gene, *RrMYB*10.1 (Accession Nos:MH717244), was cloned from the petals of *Rosa rugosa* 'Zizhi'. Sequence analysis results showed that *RrMYB*10.1 had a full length opening reading frame of 747bp, encoding 249 amino acids. Sequence analysis revealed that RrMYB10.1 contained the conserved R2R3-MYB domain, two atypical anthocyanin-promoting motifs and a conserved amino acid signature for the interaction with bHLH protein. The results of phylogenic tree revealed that RrMYB10.1 showed high homology with other anthocyanin-promoting proteins in *Rosacea*, and sharing the highest identity (98.39%) with RhMYB10. RT-PCR results showed that *RrMYB*10.1 was mainly expressed in petals among various tissues and expressed significantly higher in petals in bud stage than in opening period. To sum up, these results showed that *RrMYN*10.1 may play a key role in regulating anthocyanin concentration, thus providing a certain foundation on regulating flower color formation in *Rosa rugosa*.

Keywords

Rosa rugosa, Anthocyanin, R_2R_3-MYB, Gene Expression

1. Introduction

Rosa rugosa, as a deciduous shrub in *Rosacea*, has many prominent traits such as intense perfume, strong resistance to winter hardiness, drought, disease, pests and salinization, which makes it highly valued in landscaping application and own a great market potential. But monotonous flower color (most flower is rose

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red, rare is pink and white, lack yellow, orange and other complex color) has seriously restricted its application in gardens for a long time. Studies have shown that the color of plant tissues is mainly determined by anthocyanins, which is water-soluble pigment that belongs to the flavonoid family of compounds and giving red, blue and purple colors in a range of flowers, fruits, foliage, seeds and roots [1]. Anthocyanin biosynthesis is regulated by structural genes and regulatory genes. Structural genes synthesize anthocyanins by encoding a series of biosynthetic enzymes in the anthocyanin pathway, while regulatory gene is a transcription factor, which can activate or inhibit the temporal and spatial expression of structural genes through the specific DNA-protein and protein-protein interactions, thus regulating the synthesis of anthocyanins. There are three main types of transcription factors involved in the regulation of anthocyanin biosynthesis: MYB, b HLH and WD-40 protein. As the most important transcription factors, MYB TFs can not only regulate anthocyanin biosynthesis but participate in many other life activities such as secondary metabolism, stress resistance, cell differentiation and regulation of cell differentiation and cycle, cell wall formation, lignin precipitation, biosynthesis of glucosinolates, plant germination and development of vegetative reproductive organs, and regulation of transcription factors [2]-[8]. Anthocyanin-regulating MYBs have been isolated from many species to date, including *Arabidopsis thaliana AtMYB*75 or *PAP*1, *AtMYB*90 or *PAP*2, *AtMYB*113 and *AtMYB*114 [9], *Solanum lycopersicum ANT*1 [10], *Petunia hybrida AN*2 [11], *Capsicum annuum A* [12], *Vitis vinifera VvMYB*1*a* [13], *Zea mays P* [14], *Oryza saliva C*1 [15], *Ipomoea batatas IbMYB*1 [16], *Antirrhinum majus ROSEA*1, *ROSEA*2 and *VENOSA* [17], *Gerbera hybrid GhMYB*10 [18], *Picea mariana MBF*1 [19], *Garcinia mangostana GmMYB*10 [20], *Malus* × *domestica MdMYB*10, *MdMYB*1*/MdMYBA* [21] [22] [23], and *Gentian GtMYB*3 [24]. Although structural genes operating in this biosynthetic pathway in *Rosa rugosa* have been well characterized [25] [26], to date there is little research on the transcriptional level in regulating anthocyanin biosynthesis.

Basing on the transcriptome of *R. rugosa*, we cloned one R2R3-MYB gene associated with anthocyanin biosynthesis, named *RrMYB*10.1, then analyzed its bioinformatics and constructed phylogenetic tree to predict its functions. The expression level of *RrMYB*10.1 in different tissues and different flower developments was identified by using qPCR technology. By figuring out how *RrMYB*10.1 works in anthocyanin biosynthesis, we may provide a certain foundation on moderating the accumulation of anthocyanin and further design the petal color of *R. rugosa*.

2. Materials and Methods

The experiment was conducted at the flower germplasm resource nursery of Shandong Agricultural University and the Flower Institute of Forestry College.

2.1. Plant Material

We chose healthy individuals with stable floral inheritance of *Rosa rugosa* 'Zizhi' as experiment material, which grown in the field of rose germplasm resource nursery. The petals in different stages of bud development (identified as S1-S5 in Table 1 and Figure 1) and flowering periods (identified according to the method of Chen Chen, the flower opening period is divided into five stages: bud stage (S5), early opening stage (S6), half opening stage (S7), full blooming stage (S8), and late blooming stage (S9)) were harvested in May for qPCR to analyze its expression in petals of different developments. Stages, Sepals, stems, leaves, stamens, pistils and petals in early opening stage were collected for gene spatial expression. All samples were collected and frozen in liquid nitrogen immediately, then stored at −80˚C until used.

2.2. Methods

2.2.1. RNA Extraction, cDNA Synthesis and Gene Cloning

The extraction of RNA from petals in early opening period was operated according to the instructions of EASYspin plant RNA rapid extraction kit, the integrity of RNA was detected by 1% agarose gel electrophoresis and its concentration and purity were tested by ultraviolet spectrophotometer. The cDNA was synthesized by RNA reverse transcription kit according to the description of abm reverse transcription kit. Based on the transcriptome, one R2R3-MYB gene was selected, the sequence was identified as complete CDS by BLAST. The specific primers for complete ORF amplification (Table 2) were designed by DNAMAN software. The synthesized cDNA was used as template, and the reaction system was as follows: 2 × Gflex PCR Buffer 12.5 μL, the target gene upstream and downstream primers each 1 μL, template cDNA 1 μL, TKS Gflex DNA Polymerase 0.5 μL, sterilization ddH2O supplement to 25 μL; The PCR reaction conditions are as follows: 94˚C for 1 min; 98˚C for 10 s, 55˚C for 15 s, and 68˚C for 30 s for a total of 30 cycles. The PCR products was detected by 1% agarose gel electrophoresis, and the target product was reclaimed according to the description of Hipure Gel Pure DNA Mini Kit(Magen), then ligated with the carrier pMD18-T to transform Escherichia coli DH5*α*. Positive clones were selected and sequenced at Ruibiotech.

Table 1. Identification and characteristics of different stages in bud development.

Name	Bud diameter (mm)	The proportion of exposed petal to the total surface of petal	Petal color
S1	5	0	green
S2	6	Less than 1/5	Faint yellow
S3	8	1/4 ~ 1/3	Faint pink
S4	10	1/3 ~ 1/2	Rose purple
S5	12	1/2 ~ 2/3	Deep rose purple

Table 2. Primers used to clone and expression analysis of *RrMYB*10.1 in *R. rugose*.

Primer name	Nucleotide sequence(5'-3')	Purpose
RrMYB10.1-F	ATGGGTGGTATTCCATGGAC	Gene cloning
RrMYB10.1-R	TTATGAAGAATCAGTAACCCA	
RrMYB10.1-S-F	ACTAGTATGGGTGGTATTCCATGGAC	Vector construction
RrMYB10.1-B-R	GGTGACCTTATGAAGAATCAGTAACCCA	
RrMYB10.1-Q-F	GCTGAGGAAATGCATAGAGAAG	RT-PCR
RrMYB10.1-Q-R	AAAGCTCCCTCTCTTGATGTTC	
Actin-F	TGAGGCCATTTACGACAT	
Actin-R	AGATCACAGGAGCATAGGAG	

(a)

(b)

Figure 1. Characteristics of petals in different development in bud stage. (a) Diameter of bud in different developments; (b) petal color in n different developments in bud stage.

2.2.2. Bioinformatics Analysis

The ORF finder (NCBI) was used to search for an open reading frame, and the Conserved Domains database (NCBI) was used to analyze the conserved domains. ExPaSy-SOPMA was used to predict protein secondary structure. We used BLASTX (NCBI) to study the homology of the nucleotide sequence and conducted multiple sequence alignment by DNAMAN, and then constructed phylogenetic tree with other anthocyanin-promoting proteins by MEGA5.0 software. The phylogenetic tree was constructed according to the neighbor-joining method, and tested by bootstrap, which was repeated 1000 times.

2.2.3. Expression Analysis of *RrMYB*10.1 in Different Tissues and Different Flower Developments

The cDNA synthesized from RNA in different tissues and different flower developments was used as template, and it was carried out following the instruction of SYBR®Premix ExTaqTM kit by CFX96TM Real-Time System RT-qPCR instrument. The suitable primers of *RrMYB*10.1 for RT-PCR were selected by standard curve method. The specific primers and internal reference primers (Actin) are shown in Table 2. The reaction system is: Mix10ul, cDNA 1 μL, 0.4 μL each of upstream and downstream primer, adding sterilizing ddH_2O up to 20 μL. The reaction procedure was: predenaturation at 95˚C for 30 s; 95˚C for 30 s, 60˚C for 30 s for a total of 39 cycles. The relative expression level was calculated by the method of $2^{-\Delta\Delta CT}$. And the data analysis was graphed through Sigmaplot10.0.

2.2.4. Construction of Expression Vector

Choosing *Spe* I and *Bst* EII as restriction enzymes, designing primers with restriction enzyme sites and then cloning the full length. Then the *RrMYB*10.1 and expression vector pCAMBIA1304 were digested by restriction endonucleases *Spe* I and *Bst* EII, and the target fragments were recycled and connected with solution I. Positive clones were selected and sequenced at Ruibiotech. The diagram of pCAMBIA1304 were shown in Figure 2.

3. Results and Analysis

3.1. Cloning and Sequence Analysis of *RrMYB*10.1

One R2R3-MYB TF, *RrMYB*10.1 was cloned from the petals in early opening stage of *Rosa rugosa* 'Zizhi'. We acquired a clear single target band about 750bp by PCR (Figure 3). Multiple sequence alignment analysis showed that *RrMYB*10.1 had a full length of 750bp, encoding 249 amino acids. The alignment showed that the conserved ANDV motif (Box A in Figure 4) in R3 domain and conserved KPRPR[S/T]F motif (Box B in Figure 4, which were identified to contribute to anthocyanin biosynthesis, were present in RrMYB10.1 and were modified to GNDV and RPQASKY respectively [27] [28]. A conserved amino acid signature (EL × 2R × 3L × 6L × 3R) (the locations indicated by the arrows in Figure 4) has been shown to be functionally important for the interaction between MYB and R/B-like bHLH proteins [29]. The phylogenic tree constructed with anthocyanin-promoting MYBs revealed that RrMYB10.1 showed high similarity with other proteins in *Rosacea* and sharing the highest identity (98.39%) with RhMYB10. And the MYBs from dicot species cluster into one group, while monocot species (Rice and Maize) grouped into another clade (Figure 5).

3.2. Bioinformatics Analysis of *RrMYB*10.1 Gene

There are 249 amino acids encoded by *RrMYB*10.1. The protein's formula is $C_{1248}H_{1970}N_{374}O_{395}S_{14}$ with a molecular weight of 28982.48 and a theoretical pI:

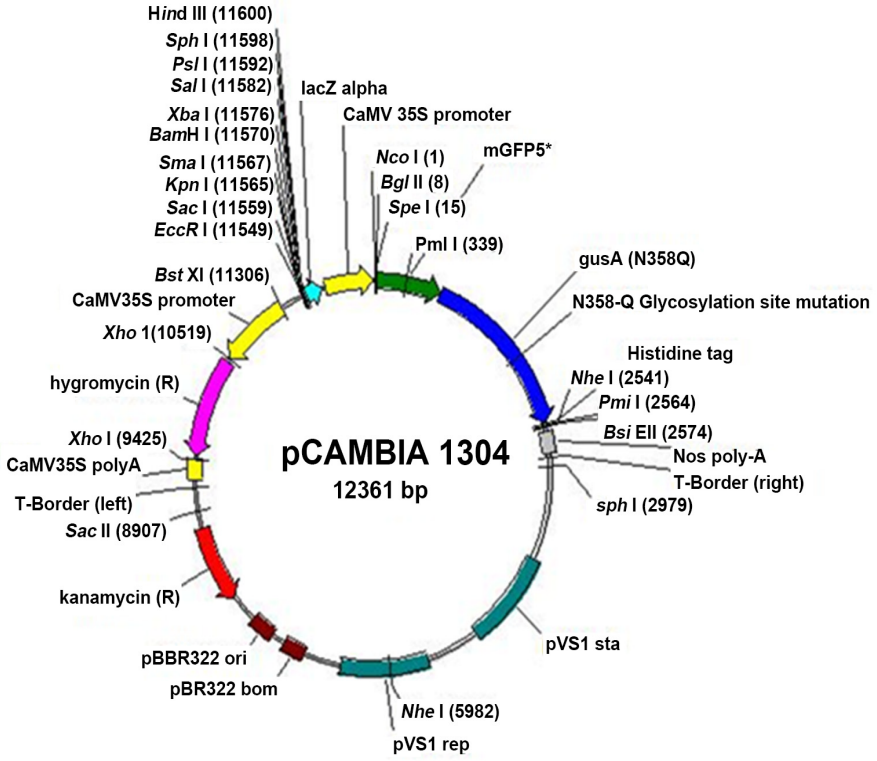


Figure 2. Diagram of pCAMBIA1304.

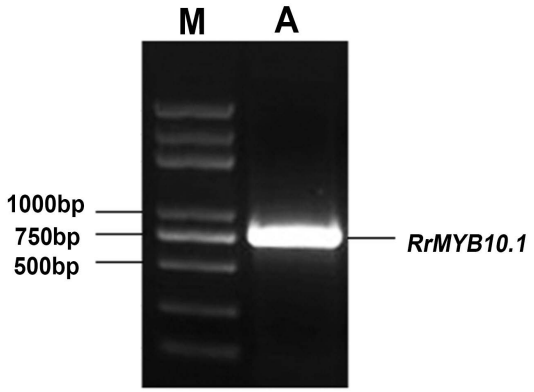


Figure 3. *RrMYB*10.1 gene cloning. M: Marker A: *RrMYB*10.1.

5.86. There are 39 negatively charged residues (Asp + Glu), 34 positively charged residues (Arg + Lys), and 176 neutral amino acids. RrMYB10.1 belongs to unstable protein with an instability index of 55.13 and it's a hydrophilic protein with the total average hydrophobic index at −0.847. The secondary structure of RrMYB10.1 demonstrates that there are 73α-helix, 150 random coil, 14 extended peptide chain, and 12 β-turn. The phosphorylation site prediction results reveals that there are 18 Ser phosphorylation sites, 6 Thr phosphorylation sites, and 5 Tyr phosphorylation sites, so we can infer that it may participate in phosphorylation control.

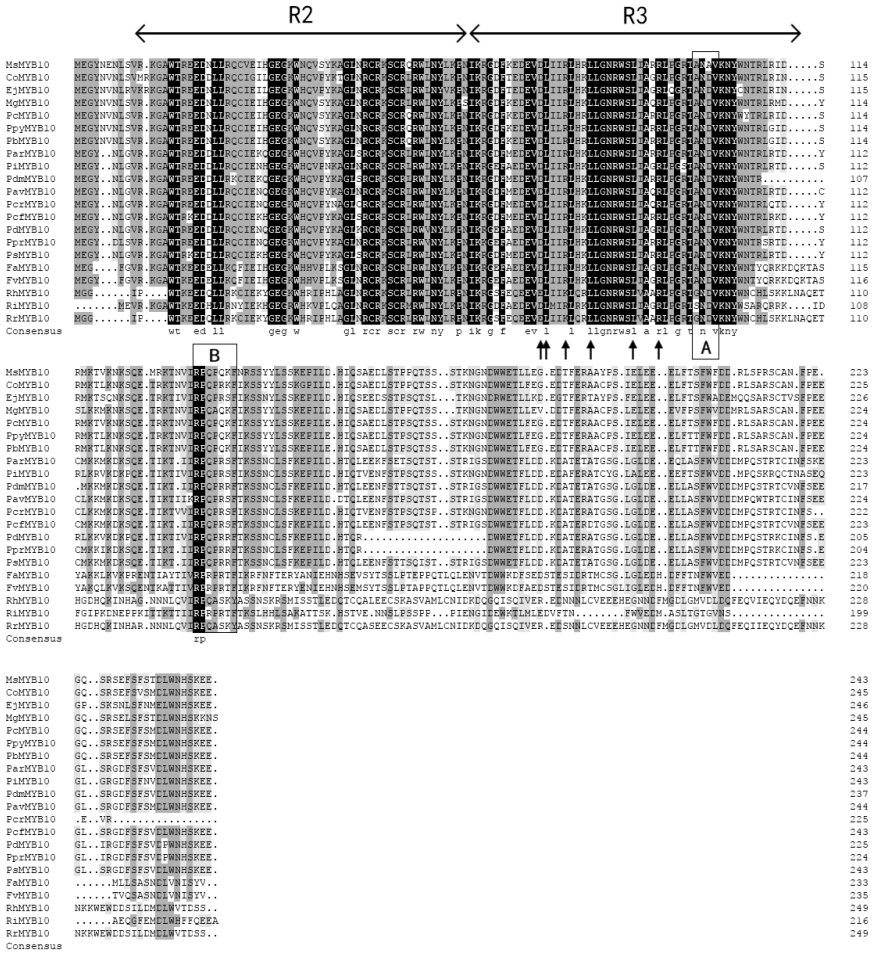


Figure 4. Multiple alignment of theRrMYB10.1 with other MYB TFs. Box (A) a conserved motif [A/S/G]NDV in the R2R3 domain for dicot anthocyanin-promoting MYBs. Box (B) a C-terminal-conserved motif [R/K] Px [P/A/R] xx [F/Y] for anthocyanin-promoting MYBs [27]. Arrows indicate specific residues that contribute to a motif implicated in b HLH co-factor interaction [29].

3.3. Expression Analysis of *RrMYB*10.1 in Different Tissues and Different Flower Developments

The RT-PCR results in different tissues showed that the expression level of *RrMYB*10.1 was significantly high in petals (at the early opening stage) , while it could be detected in other tissues(stems, leaves, stamens , sepals and pistil) with little difference but was relatively low (**Figure 6(a)**). When it comes to the flower development, the expression level increased from stage 1 in bud stage, which was relatively low, to the highest transcript level at stage 5 in bud stage, then declined rapidly with the flower opening (**Figure 6(b)**). Totally, the transcript level of *RrMYB*10.1 in petals in bud stage was significantly higher than in opening period.

3.4. Construction of Plasmid for Transient Gene Expression Assay

The *RrMYB*10.1 and expression vector pCAMBIA1304 were digested by restriction endonucleases *Spe* I and *Bst* EII, and the target fragments were recycled and

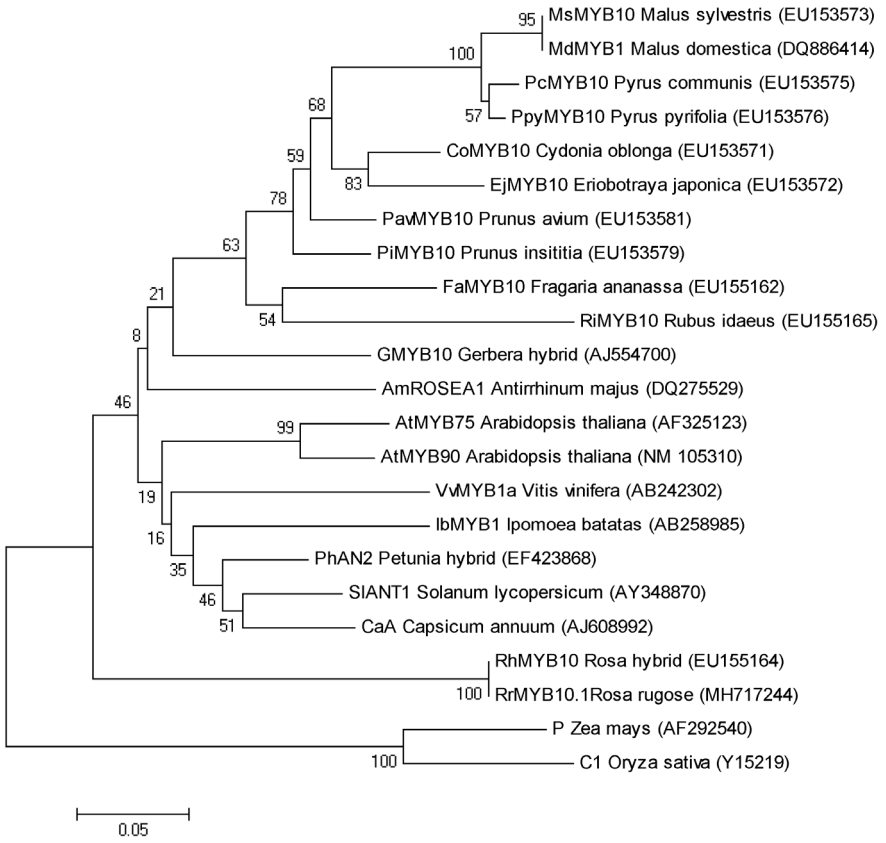
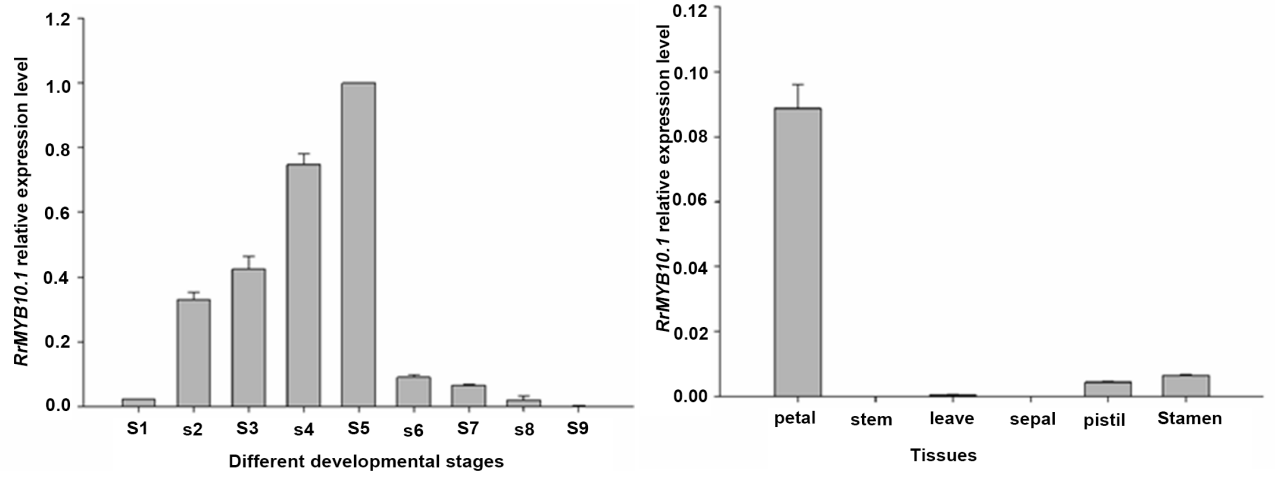


Figure 5. Phylogenetic relationships between RrMYB10.1 and other anthocyanin-related MYBs of rosaceous and other species.



(a) (b)

Figure 6. Relative expression level of *RrMYB*10.1. (a) *RrMYB*10.1 relative expression level in different development stages; (b) *RrMYB10.1* relative expression level in different tissues.

connected with solution I. Enzyme digestion analysis and DNA sequencing showed that the co-expressed protein expressing vectors were constructed successfully. Next, we are going to transfer the recombinant plasmid *RrMYB*10.1-pCAMBIA1304 to Arabidopsis thaliana to verify its function.

3.5. Construction of Plasmid for Transient Gene Expression Assay

The *RrMYB*10.1 and expression vector pCAMBIA1304 were digested by restriction endonucleases *Spe* I and *Bst* EII, and the target fragments (as shown in Figure 7) were recycled and connected with solution I. Enzyme digestion analysis and DNA sequencing showed that the co-expressed protein expressing vectors were constructed successfully. Next, we are going to transfer the recombinant plasmid *RrMYB*10.1-pCAMBIA1304 to Arabidopsis thaliana to verify its function.

4. Discussion

One MYB TF, *RrMYB*10.1, was cloned from *Rosa rugosa* 'Zizhi'. And the multiple sequence analysis showed that RrMYB10.1 had conservedR2R3 domain, two anthocyanin-promoting motifs and a conserved amino acid signature for the interaction with b HLH proteins. These results showed that RrMYB10.1 belonged to R2R3-MYB subfamily and may interact with b HLH proteins to regulate anthocyanin biosynthesis. Studies have shown that R2R3-MYB plays a key role in regulating anthocyanin biosynthesis in many plants such as *Arabidopsis thaliana* [30], *Prunus avium* [31], *Daucus carota* [32] and *Muscari spp* [33]. Some R2R3-MYB TFs can regulate specific structural genes individually, however, many reports show that the R2R3-MYB TFs interact with common bHLH and WD-40 factors form a MYB–bHLH–WD40 (MBW) transcriptional activator complex to regulate the anthocyanin biosynthesis. For example, In *Arabidopsis thaliana*, *AtPAP*1 (MYB), *AtTT*8 (b HLH) and *AtTTG*1(WD-40) form a ternary complex to activate the structural genes *AtANS, AtDFR, AtUFGT* to regulate the anthocyanin concentration in seed coat [34]. In *Malus domestica*, *MdMYBA* bind to the promoter regions of *MDANS* [35], while *MDMYB*1 and *MDBHLH*3 directly bound to the promoter regions of *MDUFGT* and *MDDFR* to regulate anthocyanin accumulation [36]. In *Grape hyacinth*, the expression of MaMybA alone cannot activate the expression of early genes, but it can activate the expression of late structural genes *MaDFR* and *MaANS*. While *MaAN*2 can't activate structural genes alone, but can activate *MaDFR* and *MaANS* significantly when binding with *MabHLH*1 or *AtTT*8 [33]. What's more, R2R3-MYB TFs also participant in many biological processes, such as cell morphogenesis and response to abiotic and biological stress. The phylogenic tree showed that MYB10.1 clustered according to their taxonomic relationship, for RrMYB10.1 is closely related to the members in *Rosacea* with the highest homology to RhMYB10 of 98% identify. And the MYBs from dicot species cluster into one group, while monocot species (Rice and Maize) grouped into another clade.

The RT-PCR results showed that *RrMYB*10.1 exhibited a tissue-specific expression, for its expression level in petals was significantly higher than in other tissues. In bud stage, the expression level of *RrMYB*10.1 increased with the petal color deepening, and reached the highest level at late bud stage, then it decreased rapidly in initial opening period and continued to decline slightly with flower

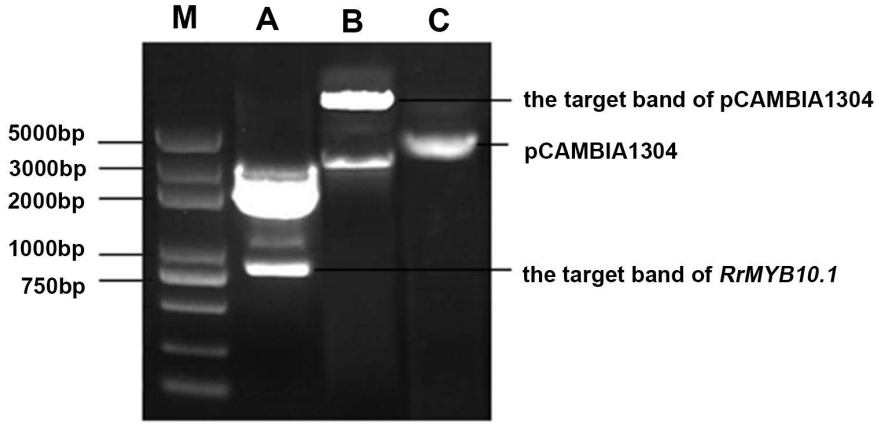


Figure 7. Identification of recombinant expression vector by double enzyme. M: Marker; A: *RrMYB10.1* by *Spe* I and *Bst* EII double enzyme; B: pCAMBIA1304 by *Spe* I and *Bst* EII double enzyme; C: pCAMBIA1304 with no enzyme

opening, finally reached the minimum expression in late blooming period. It indicated that *RrMYB*10.1 was mainly expressed in the petals in bud stage and expressed at a low level in flower opening stage, which was consistent with the anthocyanin accumulation trend in *Rosa rugosa*. Study has shown that anthocyanin has synthesized completely in late bud stage, and some anthocyanin is degraded with petals opening when exposed to sunlight [37]. So we may draw a conclusion that *RrMYB*10.1 plays a key role in the pathway of anthocyanin biosynthesis in *Rosa rugosa*.

5. Conclusion

In summary, one R2R3-MYB TF, *RrMYB*10.1, was cloned and found to play a key role in regulating anthocyanin concentration in *Rosa rugosa*, thus providing a certain foundation on regulating flower color formation. Finally, it needs further study on the function of *RrMYB*10.1 and how it interacts with b HLH proteins to co-regulate specific structural genes to control anthocyanin biosynthesis in *Rosa rugosa*.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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