Identification of Two Novel R2R3-MYB Transcription factors, \textit{PsMYB114L} and \textit{PsMYB12L}, Related to Anthocyanin Biosynthesis in \textit{Paeonia suffruticosa}

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Abstract: Flower color is a charming phenotype with very important ornamental and commercial values. Anthocyanins play a critical role in determining flower color pattern formation, and their biosynthesis is typically regulated by R2R3-MYB transcription factors (TFs). \textit{Paeonia suffruticosa} is a famous ornamental plant with colorful flowers. However, little is known about the R2R3-MYB TFs that regulate anthocyanin accumulation in \textit{P. suffruticosa}. In the present study, two R2R3-MYB TFs, namely, \textit{PsMYB114L} and \textit{PsMYB12L}, were isolated from the petals of \textit{P. suffruticosa} ‘Shima Nishiki’ and functionally characterized. Sequence analysis suggested that \textit{PsMYB114L} contained a bHLH-interaction motif, whereas \textit{PsMYB12L} contained two flavonol-specific motifs (SG7 and SG7-2). Subsequently, the in vivo function of \textit{PsMYB114L} and \textit{PsMYB12L} was investigated by their heterologous expression in \textit{Arabidopsis thaliana} and apple calli. In transgenic \textit{Arabidopsis} plants, overexpression of \textit{PsMYB114L} and of \textit{PsMYB12L} caused a significantly higher accumulation of anthocyanins, resulting in purple-red leaves. Transgenic apple calli overexpressing \textit{PsMYB114L} and \textit{PsMYB12L} also significantly enhanced the anthocyanins content and resulted in a change in the callus color to red. Meanwhile, gene expression analysis in \textit{A. thaliana} and apple calli suggested that the expression levels of the flavonol synthase (\textit{MdFLS}) and anthocyanidin reductase (\textit{MdANR}) genes were significantly downregulated and the dihydroflavonol 4-reductase (\textit{AtDFR}) and anthocyanin synthase (\textit{AtANS}) genes were significantly upregulated in transgenic lines of \textit{PsMYB114L}. Moreover, the expression level of the \textit{FLS} gene (\textit{MdFLS}) was significantly downregulated and the \textit{DFR} (\textit{AtDFR}/\textit{MdDFR}) and \textit{ANS} (\textit{AtANS}/\textit{MdANS}) genes were all significantly upregulated in transgenic lines plants of \textit{PsMYB12L}. These results indicate that \textit{PsMYB114L} and \textit{PsMYB12L} both enhance anthocyanin accumulation by specifically regulating the expression of some anthocyanin biosynthesis-related genes in different plant species. Together, these results provide a valuable resource with which to further study the regulatory mechanism of anthocyanin biosynthesis in \textit{P. suffruticosa} and for the breeding of tree peony cultivars with novel and charming flower colors.

Keywords: \textit{P. suffruticosa}; R2R3-MYB; overexpression; anthocyanin; transcriptional regulation

1. Introduction

\textit{Paeonia suffruticosa} is a very popular ornamental flowering plant that was first cultivated more than 1600 years ago in China and is currently distributed worldwide. This species is in the Paeoniaceae
family and has been named ‘the king of flowers’ for its showy and colorful flowers [1]. Approximately 1500 cultivars of *P. suffruticosa* with a variety of flower colors have been produced by breeders worldwide [2]. Among the many flower colors of this species, most fit into two clusters: monochrome color (red, pink, white, purple, black, blue, green, and yellow) and double color. Cultivars with a double-color phenotype are rarer and more sought after, and thus have great ornamental and commercial value [3]. Among them, *P. suffruticosa* ‘Shima Nishiki’, a well-known chimeric cultivar, was selected from the bud mutation of *P. suffruticosa* ‘Taiyoh’. ‘Shima Nishiki’ usually has red and pink petals on the same flower, and this aesthetically pleasing double-color phenotype can be stably inherited [4]. Therefore, the ‘Shima Nishiki’ cultivar is regarded as an important experimental material with which to study the molecular regulatory mechanism of flower color and in the breeding of new cultivars [5].

Anthocyanins are important soluble flavonoid compounds that are widely distributed in the leaves, flowers, fruits, seeds and other tissues of many plants [6]. Anthocyanin composition and concentration are usually closely related to flower color intensity [7,8]. The anthocyanin biosynthetic pathway is well known to be highly conserved in many ornamental plants [9–14]. Anthocyanin biosynthesis and accumulation are usually regulated by a series of structural genes and regulatory genes [15,16]. The structural genes encode enzymes associated with anthocyanin biosynthesis, including chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylation (F3′H), dihydroflavonol 4-reductase (DFR) anthocyanin synthase (ANS), flavonol synthase (FLS), and anthocyanidin reductase (ANR) [17–19] (Figure 1). Among them, FLS is a dedicated enzyme involved in flavonol biosynthesis, and ANR is a key enzyme for proanthocyanidin biosynthesis. The regulatory genes can be divided into three families R2R3-MYB, bHLH, and WD40 [20–22] and they usually form a regulatory complex to activate the expression of anthocyanin biosynthetic genes [23–25].

![Figure 1. A general schematic diagram of the metabolic pathway related to anthocyanin biosynthesis.](image-url)

**Figure 1.** A general schematic diagram of the metabolic pathway related to anthocyanin biosynthesis. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; FLS, flavonol synthase; ANR, anthocyanidin reductase.

Many structural genes have been characterized and cloned in *P. suffruticosa* [12,26–28]. In the MYB-bHLH-WDR (MBW) complex, R2R3-MYB transcription factors (TFs) usually play critical roles in anthocyanin biosynthesis and accumulation [29,30]. Many R2R3-MYB TFs involved in anthocyanin biosynthesis have been isolated and characterized from various plants, including
Arabidopsis thaliana [24], Zea mays [31], Vitis vinifera [32], Malus crabapple [33], Petunia hybrida [34], Antirrhinum majus [29], Dendranthema morifolium [35] and Phalaenopsis aphrodite [14]. In P. suffruticosa, most previous studies were focused primarily on the preliminary investigation of R2R3-MYB TFs based on transcriptome sequencing and qRT-PCR analyses [12,36–38], whereas whether and how R2R3-MYB TFs control anthocyanin biosynthesis and accumulation in P. suffruticosa are almost unknown.

In the present study, two novel R2R3-MYB TFs, namely, PsMYB114L and PsMYB12L, were cloned in P. suffruticosa. Subsequently, the expression patterns of PsMYB114L and PsMYB12L were determined at five developmental stages in P. suffruticosa ‘Shima Nishiki’. Furthermore, the function of these two TFs was further verified by heterologous expression in Arabidopsis and apple calli. These results will provide valuable insights into understanding the putative roles of PsMYB114L and PsMYB12L in regulating anthocyanin biosynthesis in P. suffruticosa.

2. Results

2.1. Cloning and Analysis of the PsMYB114L and PsMYB12L Genes

Based on the functional annotation and gene expression analysis of transcriptome sequencing data in P. suffruticosa ‘Shima Nishiki’ [39], we filtered two MYB unigenes exhibiting relatively high expression differences between the red and pink petals as the targeted genes of this study.

The full-length cDNA sequences of the two novel MYB genes were obtained with PCR amplification. By conducting GenBank BLAST searches of the amino acid sequences of these two genes, we found that these genes have the highest homology with transcription factor MYB114-like [Quercus suber] and transcription factor MYB12-like [Juglans regia], respectively. Therefore, we named these genes PsMYB114L and PsMYB12L. Sequencing results revealed that PsMYB114L (Figure S1A,B) and PsMYB12L (Figure S1C,D) contained an open reading frame (ORF) of 600 and 1140 bp encoding 199 and 379 amino acids and that their predicted proteins had a molecular mass of 22.81 and 42.61 kDa and a theoretical isoelectric point (pI) of 8.53 and 4.86, respectively.

Multiple sequence alignment of amino acids revealed that PsMYB114L and PsMYB12L, belonging to the SANT superfamily (which typically consists of tandem repeats of three alpha-helices arranged in a helix-turn-helix motif, with each alpha helix containing a bulky aromatic residue), and other known R2R3-MYB TFs related to anthocyanin biosynthesis contained a highly conserved R2R3 DNA-binding domain. The presence of this conserved domain means that PsMYB114L and PsMYB12L are also R2R3-MYB TFs and may perform similar functions in regulating anthocyanin biosynthesis. Furthermore, PsMYB114L had a bHLH-interaction motif ([D/E]Lx2[R/K]x3Lx6Lx3R) in the R3 domain at the N terminus and did not have any conserved motifs at the C terminus (Figure 2A). Moreover, PsMYB12L did not have any bHLH-interaction motifs at the N terminus, whereas it contained two flavonol-specific motifs [40], namely, SG7 ([K/R][R/x][R/K]xGRT[S/x][R/G]xx[M/x]K) and SG7-2 ([W/x][L/x]LS), at the C terminus (Figure 2B).
Figure 2. Amino acid sequence alignment analysis of the \( \text{PsMYB114L} \) (A) and \( \text{PsMYB12L} \) (B) genes with other known R2R3-MYB TFs. The green and pink long lines indicate the R2 and R3 domain, respectively. The red boxes show the conserved bHLH-interaction, SG7 and SG7-2 motifs. The NCBI GenBank accession numbers of these sequences are as follows: ZmC1, Zea mays, AF320613.3; ZmPL, Zea mays, NM_001112415.1; FtMYB2, Fagopyrum tataricum, JF313346.1; FtMYB1, Fagopyrum tataricum, JF313344.1; AtTT2, Arabidopsis thaliana, NM_122946.3; VvMYBPA2, Vitis vinifera, NM_001281024.1; VvMYBF1, Vitis vinifera, FJ948477.2; AtMYB11, Arabidopsis thaliana, NM_116126.3; AtMYB12, Arabidopsis thaliana, NM_130314.4; AtMYB111, Arabidopsis thaliana, NM_124310.3; EsMYBF1, Epimedium sagittatum, KU365320.1.

To better evaluate the phylogenetic relationships of \( \text{PsMYB114L} \), \( \text{PsMYB12L} \) and 16 other known MYB TFs related to the regulation of anthocyanin biosynthesis, a phylogenetic tree was constructed based on the amino acid sequences of these 18 MYB TFs from different species using the neighbor-joining method. The phylogenetic analysis indicated that these 18 MYB TFs were classified into four groups (Flavonol, Anthocyanin, Anthocyanin/Proanthocyanidin and Proanthocyanidin) based on their specific roles in the flavonoid biosynthesis pathway. Among them, \( \text{PsMYB114L} \) had the closest phylogenetic relationship with ZmC1 and ZmPL, which are involved in regulating anthocyanin biosynthesis, whereas \( \text{PsMYB12L} \) belongs to a subgroup of MYB proteins that includes VvMYB1, EsMYBF1, AtMYB11, AtMYB12 and AtMYB111, which regulate flavonol synthesis and had the closest phylogenetic relationship with VvMYBF1 (Figure 3).
2.2. Subcellular Localization of PsMYB114L and PsMYB12L

To examine the subcellular localization of PsMYB114L and PsMYB12L, the recombinant vector (PsMYB114L-GFP/PsMYB12L-GFP) and the control vector (pCAMBIA1301-GFP) were introduced into the tobacco leaves. Our results were basically consistent with those of previous studies [41,42]. The green fluorescent protein (GFP) fluorescence of the control vector was clearly distributed throughout the entire cell (Figure 4A), and the PsMYB114L-GFP/PsMYB12L-GFP vector displayed a strong fluorescence signal in the nucleus and cytoplasm of tobacco cells (Figure 4B,C). Therefore, we speculated that the two R2R3-MYB TFs (PsMYB114L/PsMYB12L) were simultaneously localized and functioned in the nucleus and cytoplasm.
2.3. Expression Patterns of PsMYB114L and PsMYB12L in *P. suffruticosa* ‘Shima Nishiki’

qRT-PCR analysis was conducted to survey the expression patterns of *PsMYB114L* and *PsMYB12L* in *P. suffruticosa* ‘Shima Nishiki’ (Figure 5). Petal samples of this cultivar were collected at five developmental stages (Figure S2). The expression levels of the *PsMYB114L* gene peaked at S3 and then decreased from S3 to S5, whereas the *PsMYB12L* gene exhibited the highest expression at S4. Furthermore, the expression levels of the eight anthocyanin biosynthesis-related genes (*PsCHS*, *PsCHI*, *PsF3H*, *PsF3’H*, *PsDFR*, *PsANS*, *PsFLS*, and *PsANR*) were analyzed. Among these genes, *PsF3’H*, *PsDFR*, and *PsANS* showed a trend similar to that of *PsMYB12L*, whereas *PsFLS* and *PsANR* showed a trend similar to that of *PsMYB114L*. 

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**Figure 4.** Subcellular localization analysis of the *PsMYB114L* and *PsMYB12L* genes. (A) Control vector (pCAMBIA1301-GFP) expressed in epidermal cells of tobacco leaves. (B) Recombinant vector (*PsMYB114L*-GFP) expressed in epidermal cells of tobacco leaves. (C) Recombinant vector (*PsMYB12L*-GFP) expressed in epidermal cells of tobacco leaves. White lines at the bottom right of the picture represent 20 μm in the respective pixel. GFP, GFP fluorescence; Chloroplast, Chloroplast fluorescence; Bright, Bright field; Merged, Superposition of bright field and fluorescence.
AtANS, AtF3, AtF3H, upregulated in transgenic Arabidopsis plants; among them, both of the AtDFR/AtANR genes showed a relatively high difference between the Col-0 and transgenic plants, whereas AtFLS and AtANR were downregulated in transgenic PsMYB114L plants (Figure 6E).

For PsMYB12L overexpression in Arabidopsis, the expression levels of all eight genes were upregulated in transgenic PsMYB12L plants. Among them, the four genes (AtCHS, AtCHI, AtDFR, and AtANS) all showed a relatively high difference between the Col-0 and transgenic plants (Figure 6F).

**2.4. Overexpression of PsMYB114L and PsMYB12L in Arabidopsis**

To characterize the functions of PsMYB114L and PsMYB12L, these two genes under the expression of the 35S promoter were genetically transformed into Arabidopsis. Phenotypic investigations of the transgenic lines of PsMYB114L and PsMYB12L revealed that their leaves were much deeper in color than those of Col-0 and showed a purple-red color (Figure 6A). Meanwhile, these transgenic lines of the two genes were confirmed by PCR analysis (Figure 6B). Furthermore, the total anthocyanin content results indicated that the transgenic lines of PsMYB114L and PsMYB12L produced much more anthocyanin than Col-0 (Figure 6C, D).

Additionally, the expression levels of anthocyanin biosynthesis-related genes (AtCHS, AtCHI, AtF3H, AtF3′H, AtDFR, AtANR, AtFLS, and AtANK) in the Col-0 and the transgenic Arabidopsis plants of PsMYB114L and PsMYB12L were analyzed with qRT-PCR experiments. Compared with the Col-0, overexpression of PsMYB114L upregulated the expression of most of the genes (AtCHS, AtCHI, AtF3H, AtF3′H, AtDFR, and AtANK) in transgenic PsMYB114L plants; among them, both of the AtDFR/AtANR genes showed a relatively high difference between the Col-0 and transgenic plants, whereas AtFLS and AtANK were downregulated in transgenic PsMYB114L plants (Figure 6E).

The expression patterns of the PsMYB114L gene, PsMYB12L gene and anthocyanin biosynthesis-related structural genes in P. suffruticosa ‘Shima Nishiki’. S1, flower bud emerging stage; S2 small bell-like flower-bud stage; S3, large bell-like flower-bud stage; S4, bell-like flower-bud extending stage; S5, color exposing stage. Different lowercase letters indicate significant differences at \( p < 0.05 \).
PsMYB114L downregulated the expression of most of the genes, specifically, PsMYB114L and PsMYB12L were all accumulated markedly higher amounts of anthocyanins than did the WT (Figures 7C,D and 8C,D).

2.5. Overexpression of PsMYB114L and PsMYB12L in Apple Calli

For further functional validation, the two genes (PsMYB114L and PsMYB12L) were ectopically expressed in the calli of ‘Orin’ apple. Interestingly, after light and low-temperature treatments, the WT had almost no phenotypic changes, but an especially obvious color change was observed in the transgenic lines of PsMYB114L and PsMYB12L (Figures 7A and 8A). PCR amplification confirmed that these transgenic apple calli carry PsMYB114L and PsMYB12L (Figures 7B and 8B). With regard to the total anthocyanin content, the transgenic lines of PsMYB114L and PsMYB12L all accumulated markedly higher amounts of anthocyanins than did the WT (Figures 7C,D and 8C,D).

Additionally, the expression levels of anthocyanin biosynthesis-related genes (MdCHS, MdCHI, MdF3H, MdF3′H, MdDFR, MdANS, MdFLS, and MdANR) in the WT and the transgenic lines of PsMYB114L and PsMYB12L were analyzed by qRT-PCR. Compared with the WT, overexpression of PsMYB114L downregulated the expression of most of the genes, specifically, MdCHS, MdCHI, MdF3H, MdF3′H, MdFLS, and MdANR, and upregulated the expression of MdDFR and MdANS in transgenic PsMYB114L calli (Figure 7E).

For PsMYB12L overexpression, the expression levels of most genes, including MdCHS, MdF3H, MdF3′H, MdDFR, MdANS, and MdANR, were upregulated, but those of MdCHI and MdFLS were downregulated in transgenic PsMYB12L calli (Figure 8E).
Therefore, determining how certain R2R3-MYB TFs regulate anthocyanin production in R2R3-MYB TFs in regulating flower color in structural genes involved in the anthocyanin biosynthetic pathway [46,47]. However, the role of factor influencing flower color [43–45]. R2R3-MYB TFs comprise one of the largest gene families in their ornamental and commercial value. Many prior studies have shown that anthocyanins are a key content in transgenic apple calli and the WT. (C) Anthocyanin extraction solutions for transgenic apple calli and the WT. (D) Total anthocyanin content in transgenic apple calli and the WT. (E) Expression analysis of anthocyanin biosynthesis-related genes in transgenic apple calli and the WT. WT, Wild-type ‘Orin’ apple calli; PsMYB114L-1 and PsMYB114L-2, two transgenic lines of the PsMYB114L gene. Different lowercase letters indicate significant differences at p < 0.05.

Figure 8. Overexpression analysis of the PsMYB12L gene in apple calli. (A) Colors observed in transgenic apple calli and the WT. (B) Results of positive PCR detection in transgenic apple calli. (C) Anthocyanin extraction solutions for transgenic apple calli and the WT. (D) Total anthocyanin content in transgenic apple calli and the WT. (E) Expression analysis of anthocyanin biosynthesis-related genes in transgenic apple calli and the WT. PsMYB12L-1 and PsMYB12L-2, two transgenic lines of the PsMYB12L gene. Different lowercase letters indicate significant differences at p < 0.05.
3. Discussion

Flower color is a very important trait in many ornamental plants and has a close association with their ornamental and commercial value. Many prior studies have shown that anthocyanins are a key factor influencing flower color [43–45]. R2R3-MYB TFs comprise one of the largest gene families in plants and play key roles in regulating anthocyanin accumulation by activating the expression of structural genes involved in the anthocyanin biosynthetic pathway [46,47]. However, the role of R2R3-MYB TFs in regulating flower color in *P. suffruticosa* has seldom been functionally verified. Therefore, determining how certain R2R3-MYB TFs regulate anthocyanin production in *P. suffruticosa* would aid in breeding improved cultivars with desirable flower colors.

In the present study, two novel R2R3-MYB TFs (PsMYB114L and PsMYB12L) possibly involved in anthocyanin biosynthesis were successfully cloned and characterized from the petals of *P. suffruticosa* ‘Shima Nishiki’ and found to contain full-length cDNA of 600 and 1140 bp encoding 199 and 379 amino acids, respectively. The amino acid sequence alignment between PsMYB114L/PsMYB12L and other known R2R3-MYB TFs involved in anthocyanin regulation indicated that the R2R3 domain distributions of these R2R3-MYB TFs were highly similar, but a bHLH-interaction motif ([D/E]Lx2[R/K]x3Lx6Lx3R) existed in the R3 domain of PsMYB114L, whereas PsMYB12L did not contain this motif for interaction with bHLH proteins. In *Arabidopsis*, based on a similar function, 125 TFs of R2R3-MYB gene-family members were classified into more than 25 subgroups [48]. Furthermore, many previous studies demonstrated that subgroup 7 [49,50], characterized by both the SG7 ([K/R][R/x][R/K]xGRT[S/x][R/G]xx[M/x[K]) and SG7-2 ([W/x][L/x]LS) motifs, specifically regulated flavonol biosynthesis. *PsMYB12L* contained these two motifs (SG7 and SG7-2) at the C terminus of the protein, but *PsMYB114L* lacked these two motifs.

Phylogenetic analysis indicated that *PsMYB12L* and 5 flavonol-regulating R2R3-MYB TFs (*VvMYBF1*, *EsMYBF1*, and *AtMYB11/12/111*) belonging to subgroup 7 [30,51] were clustered together, and *PsMYB114L* and certain R2R3-MYB TFs belonging to subgroup 5 (*AtTT2*, *ZmC1*, *VvMYBPA2*, etc.) [52–55] had relatively higher homology. Based on the motif analysis of amino acid sequences and phylogenetic analysis, *PsMYB114L* might regulate anthocyanin production by combinatorially interacting with a basic helix-loop-helix (bHLH) factor [25,56,57]. *PsMYB12L* might independently regulate the expression of anthocyanin biosynthesis-related genes without the MBW complex [58].

In addition, we conducted further ectopic transgenic studies by overexpressing *PsMYB114L*/PsMYB12L in *Arabidopsis* and apple calli. In contrast to the green-colored leaves of the Col-0 A. *thaliana* ecotype and the white-colored WT apple calli, the leaves of these transgenic *Arabidopsis* plants turned purple-red and the transgenic calli of *P. suffruticosa* *PsMYB12L* were red, which was in agreement with their remarkably higher anthocyanin content. The color and total anthocyanin content analyses of *Arabidopsis* and apple calli indicated that these two R2R3-MYB TFs contribute to anthocyanin accumulation in transgenic lines.

Subsequently, qRT-PCR analysis of seven anthocyanin biosynthesis-related genes (MdCHS, MdCHI, MdF3H, MdF3’H, MdDFR, MdANS, MdFLS, and MdANR) was further performed in *Arabidopsis* and apple calli. In terms of *PsMYB114L*, the qRT-PCR results in *Arabidopsis* showed that the expression levels of *AtDFR* and *AtANS* were significantly upregulated, whereas *AtFLS* and *AtANR* were downregulated to a certain extent compared with the levels in the Col-0. Furthermore, the qRT-PCR results in apple calli showed that the expression levels of *MdDFR* and *MdANS* were upregulated to a certain extent, whereas *MdFLS* and *MdANR* (especially *MdFLS*) were significantly downregulated compared with the levels in the WT. Meanwhile, based on the results of expression patterns of *PsMYB114L* in *P. suffruticosa* ‘Shima Nishiki’, we have known that *PsMYB114L* have a positive correlation with *PsFLS* and *PsANR*. By comparing these three qRT-PCR results in *Arabidopsis*, apple calli, and *P. suffruticosa*, we found differences in the expression patterns of some anthocyanin biosynthesis-related genes. Previous studies have showed that many R2R3-MYB TFs usually regulate flavonoid biosynthesis by interacting with the promoter of the targeted structural genes [55,58]. For promoter region, in general, the sequence of the same structural gene in different plant species...
We considered that with the expression of anthocyanin biosynthesis-related genes documented in the abovementioned PpMYB15 study on the overexpression of AtANS/MdANS were consistent with the significantly higher anthocyanin accumulation in transgenic plants. Moreover, the expression levels of MdDFR, PsMYB12L, and apple calli, and considered that the expression difference of the PsMYB12L gene in Arabidopsis and apple calli, and P. suffruticosa should be a specific transcriptional regulator on DFR and ANS genes in these three species. Furthermore, we also found differences in the expression patterns of the FLS gene in Arabidopsis and apple calli, and considered that the expression difference of the FLS gene is likely caused by the promoter sequence specificity of this gene in these two species [61]. Based on the motif analysis of PsMYB12L, we speculated that the TF may be a flavonol-specific MYB regulator. Many flavonol-specific MYB TFs have been isolated and functionally verified in various plants, such as A. thaliana, Vitis vinifera, and Epimedium sagittatum [50,62,63]. Furthermore, many flavonol-specific MYB TFs negatively regulate anthocyanin accumulation by inducing higher expression of the FLS gene. By overexpressing AtMYB12 in tobacco, the expression of NICHS, NICH1, and NIFFLS was specifically activated; moreover, the flowers of the transgenic plants were paler in color than their wild-type counterparts [64]. Ectopic expression analysis of EsMYBF1 in transgenic tobacco indicated that NICHS, NICH1, NIF3H, and NIFFLS were upregulated but NIDFR and NITANS were significantly downregulated, and the accumulation of anthocyanins in transgenic tobacco flowers was also remarkably decreased [63]. A study on the overexpression of PpMYB15 in tobacco showed that it can significantly activate the expression of NICHS, NICH1, NIF3H, and NIFFLS, while it had no effects on the expression of NIDFR and NITANS, resulting in pale-pink or pure white flowers in transgenic tobacco plants [40]. Compared with the expression of anthocyanin biosynthesis-related genes documented in the abovementioned studies, in this study AtICHS/MdICHS, AtICH1, AtF3H/MdF3H, and AtFLS had a somewhat similar expression pattern and MdFLS, AtDFR/MdDFR and AtANS/MdANS exhibited the opposite pattern. However, the lower expression of the MdFLS gene and the higher expression of AtDFR/MdDFR and AtANS/MdANS were consistent with the significantly higher anthocyanin accumulation in transgenic lines of PsMYB12L. Because PsMYB12L has the flavonol-specific motif and lacks the bHLH-interaction motif, it alone enhances anthocyanin production by regulating the expression of these key genes (AtDFR/MdDFR, AtANS/MdANS, and MdFLS) independently of bHLH cofactors in Arabidopsis and apple calli.

4. Materials and Methods

4.1. Plant Materials

The tree peony cultivar P. suffruticosa ‘Shima Nishiki’ was grown in the experimental nursery of Forestry College, Shandong Agricultural University, Tai’an, Shandong, China. Flower samples were...
collected at five early flower-bud developmental stages (flower bud emerging stage (S1), small bell-like flower-bud stage (S2), large bell-like flower-bud stage (S3), bell-like flower-bud extending stage (S4), and color exposing stage (S5)) (Figure S2) [65]. All these samples were immediately frozen in liquid nitrogen and then stored at –80 °C for further experiments.

The A. thaliana ecotype Columbia (Col-0) was used for genetic transformation and phenotypic analysis in the present study. The plants were grown under a 16 h light / 8 h dark photoperiod at 23 °C/21 °C. Furthermore, calli of the wild type (WT) of ‘Orin’ apple were subcultured on Murashige and Skoog (MS) medium with 1.5 mg L⁻¹ 6-benzyl adenine (6-BA) and 0.5 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D) at room temperature (24 °C) in a continuous dark environment at 15-day intervals [66]. Subsequently, the calli were used for genetic transformation and phenotypic analysis.

4.2. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from all samples according to instructions of the EASY Spin Plant RNA Rapid Extraction Kit (Aidlab Biotech, Beijing, China). The purity and concentration of all RNA samples were assessed using a Nanodrop 2000C spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, DE, USA), and RNA quality was detected using 1 % agarose gels. Furthermore, cDNA was synthesized with 1 µg of total RNA using 5× All-In-One RT MasterMix (with an AccuRT Genomic DNA Removal Kit) (ABM, Vancouver, BC, Canada).

4.3. Cloning of the PsMYB114L and PsMYB12L Genes in P. suffruticosa

In this study, based on the transcriptome sequencing data of P. suffruticosa ‘Shima Nishiki’ in our laboratory, two R2R3-MYB transcription factors were filtered by analyzing the functional annotations of MYB unigenes and performing gene expression analysis.

The cDNA of the ‘Shima Nishiki’ cultivar’s petals was used as the template. The full-length coding sequence (CDS) of the PsMYB114L (MK518073) and PsMYB12L (MK518074) genes was amplified using PCR. The complete 5’ CDS of the PsMYB114L and PsMYB12L genes was identified from the transcriptome sequencing data of P. suffruticosa ‘Shima Nishiki’. The cDNA 3’ end sequence of these candidate genes was obtained using nested PCR technology using PsMYB114L-1-F/PsMYB114L-2-F and PsMYB12L-1-F/PsMYB12L-2-F as forward primers (Table S1), respectively, and B26 was used as the common reverse primer. The full-length cDNA of the PsMYB114L and PsMYB12L genes was amplified with the forward primers PsMYB114L-F1/PsMYB12L-F1 and the reverse primers PsMYB114L-R1/PsMYB12L-R1 (Table S1). The PCR program of gene amplification was as follows: initial denaturation at 95 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 60 °C for 15 s and 68 °C for 60 s. The PCR products were purified and cloned into the pTOPO-Blunt Simple vector for sequencing.

4.4. Subcellular Localization

The full-length cDNA without the termination codon of PsMYB114L/PsMYB12L was amplified with special primers (PsMYB114L-GFPF/PsMYB12L-GFPF and PsMYB114L-GFPR/PsMYB12L-GFPR) (Table S1) with restriction sites (Xba I and Kpn I) and subcloned into the pCAMBIA1301-GFP vector between the Xba I and Kpn I sites to create the PsMYB114L-GFP/PsMYB12L-GFP fusion construct. The recombinant vectors (PsMYB114L-GFP/PsMYB12L-GFP) and control vector (pCAMBIA1301-GFP) were then introduced into tobacco leaves by agroinfiltration. These infiltrated plants were grown for over 72 h in a growth chamber, and the GFP fluorescence of samples was observed under a Nikon C2-ER confocal laser scanning microscope (Nikon, Tokyo, Japan) [67].

4.5. Overexpression Vector Construction

The full-length cDNA of the PsMYB114L and PsMYB12L genes from the petals of P. suffruticosa ‘Shima Nishiki’ was amplified using recombinant primers (PsMYB114L-F2/PsMYB12L-F2 and
PsMYB114L-R2/PsMYB12L-R2) (Table S1) with restriction sites (Spe I and BstE II). Based on the predesigned vector construction procedure, the pCAMBIA1304 empty vector and the pTOPO-Blunt Simple vector containing the target genes (PsMYB114L and PsMYB12L) with restriction sites were double digested separately between the Spe I and BstE II sites and then recombined (Figure S3A–C). Subsequently, the two recombinant vectors pCAMBIA1304-PsMYB114L (Figure S3D) and pCAMBIA1304-PsMYB12L (Figure S3E) were verified successfully by PCR and sequencing with the forward vector validation primer 1304Ve-F and the reverse primers PsMYB114L-R2/PsMYB12L-R2 (Table S1). These two overexpression constructs were also introduced into Agrobacterium tumefaciens strain GV3101 using the freeze-thaw method.

4.6. Stable Transformation of Arabidopsis

The transformation of Arabidopsis was performed using the floral dip transformation method [68]. An A. tumefaciens infection solution (OD600 = 0.8–1.2) containing 5 % sucrose and 0.01 % Silwet L-77 was prepared to infect inflorescences, and the infection time per inflorescence was 15 s. Subsequently, these plants were transferred to a dark treatment for 24 h. These steps were repeated twice more according to the growth state of the plant. Mature T1 seeds were harvested, surface sterilized, and then sown on MS medium with 30 mg L\(^{-1}\) hygromycin B to screen for positive transformants. The resistant seedlings were transplanted into soil and then placed in a light incubator (16 h light/8 h dark at 23 °C/21 °C). When these transgenic Arabidopsis plants had grown to a certain size, they were further verified with gene-specific primers by PCR.

4.7. Stable Transformation of Apple Calli

To transform apple calli, 15-day-old WT apple calli were incubated with A. tumefaciens infection solution that carried pCAMBIA1304-PsMYB114L/pCAMBIA1304-PsMYB12L for 20 min, and the apple calli were then cocultured on MS medium supplemented with 0.5 mg L\(^{-1}\) 2,4-D and 1.5 mg L\(^{-1}\) 6-BA for 2 days at 24 °C in the dark. Subsequently, the apple calli were washed three times with sterile water and transferred to a selective medium that contained 15 mg L\(^{-1}\) hygromycin B for transgene selection. The transgenic apple calli were cocultured in the selective medium containing appropriate concentrations of an antibiotic and transferred to a light incubator with constant light (photon flux density of ~100 µmol s\(^{-1}\) m\(^{-2}\)) and low-temperature (15 °C) treatments for phenotypic observation [69,70].

4.8. Measurement of Total Anthocyanin Content

Total anthocyanin were extracted from the rosette leaves of 25-day-old Arabidopsis plants and apple calli cultured for 7 days. Anthocyanin extraction was performed using a methanol–HCl method [71]. Approximately 0.1 g of each sample was incubated in 5 mL of 0.1 % acidic methanol solution (CH\(_3\)OH:HCl:H\(_2\)O = 70:0.1:29.9, v/v/v) overnight in the dark at 4 °C. The absorbance of each extract was measured at 530 and 657 nm with a UV-1600 spectrophotometer (SHIMADZU, Kyoto, Japan). The total anthocyanin content was calculated using the following equation: 

\[
Q_{\text{Total Anthocyanin}} = \frac{(A_{530} - 0.25 \times A_{657}) \times FM}{1}.
\]

There were three biological replicates for each sample.

4.9. Quantitative Real-Time PCR (qRT-PCR) Analysis

qRT-PCR was performed to analyze the expression levels of anthocyanin biosynthesis-related genes in all plant materials in this study. The qRT-PCR experiments were conducted using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Kyoto, Japan) on a Bio-Rad CFX96™ Real-Time system (Bio-Rad, Hercules, CA, USA) with three biological replicates according to the manufacturer’s instructions. The PCR conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 30 s and then a dissociation stage at 95 °C for 10 s, 65 °C for 5 s and 95 °C for 5 s. The Psubiquitin gene, AtActin2 gene and MdActin gene were used as internal controls to normalize the expression levels in P. suffruticosa, A. thaliana and Malus domestica, respectively. All gene-specific primers used in this study
are shown in Table S1 [39,66]. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method [72].

4.10. Sequence and Statistical Analysis

Multiple sequence alignment was performed using DNAMAN 8.0 software (Lynnon Biosoft, San Ramon, CA, USA). Homology search of sequences was carried out using the GenBank BLAST. Phylogenetic tree construction of sequences was performed using MEGA 5.0 software (Arizona State University, Tempe, AZ, USA) with the bootstrap values from 1000 replicates. Primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA, USA). All experiments were repeated three times, and the data are expressed as the mean ± standard error. Variance analyses were performed using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). $p$-values of $<0.05$ were considered statistically significant.

5. Conclusions

In conclusion, two novel R2R3-MYB TFs, namely PsMYB114L and PsMYB12L, were successfully cloned from the petals of $P$. suffruticosa 'Shima Nishiki' and functionally characterized by heterologous expression in Arabidopsis and apple calli. Based on the above results, we preliminarily demonstrated the potential functional roles of PsMYB114L and PsMYB12L in regulating anthocyanin biosynthesis. These results provide a valuable resource for further understanding the molecular regulatory mechanisms of anthocyanin biosynthesis and accumulation in $P$. suffruticosa and breeding improved cultivars of $P$. suffruticosa with desirable flower colors in the future.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/5/1055/s1. Table S1. Primers used in this study. Figure S1. Full-length cDNA amplification of the PsMYB114L and PsMYB12L genes. Figure S2. Flowers of $P$. suffruticosa 'Shima Nishiki' at five developmental stages. Figure S3. Construction of the recombinant expression vectors of pCAMBIA1304-PsMYB114L and pCAMBIA1304-PsMYB12L.

Author Contributions: X.Y. and L.Z. conceived and designed the research. X.Z. and Z.X. participated in the specific design of the study. X.Z. and Z.X. performed the experiments and the data analysis, and drafted the manuscript. M.Z., X.H., and S.Q. contributed analysis tools and helped analyze the data. All authors contributed to manuscript revision and approved the final version.

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