The PsMYB12L/*PsDFR* Module is Involved in Double-Color Formation in *Paeonia Suffruticosa* ‘Shima Nishiki’

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Abstract

Background: *Paeonia suffruticosa* ‘Shima Nishiki’ is a extremely precious double-color cultivar in the world because of its unique and attractive flower color. However, the underlying molecular mechanisms of its double-color formation have not been completely unravelled until now. In the present study, firstly, the full-length cDNA sequence, genomic DNA sequence, promoter region sequence of the *PsDFR* gene in the red and pink petals of the ‘Shima Nishiki’ cultivar were cloned and analyzed, respectively. Meanwhile, the methylation level of CpG island and promoter region of this gene in the red and pink petals was also measured. Moreover, the identification of regulatory effect of PsMYB114L/PsMYB12L and *PsDFR* was performed.

Results: Here, we found that the full-length cDNA sequence, genomic DNA sequence, promoter region sequence of *PsDFR* were identical in the red and pink petals, respectively. There were some differences for the methylation level of this gene in the red and pink petals, but these differences were little and didn't show obvious regularity. In addition, the regulatory effect of PsMYB12L and *PsDFR* was successfully identified.

Conclusions: Based on these above results, we concluded that PsMYB12L regulating the differential expression of *PsDFR* may be a key reason for the double-color formation. These results will advance our understanding of the molecular regulatory mechanisms of double-color formation in *P. suffruticosa* ‘Shima Nishiki’.

Background

Tree peony (*Paeonia suffruticosa* Andrews) is a famous traditional flower in China. At present, it also has been well known and widely cultivated as a beautiful ornamental plant in the world [1, 2]. Flower color is a very important ornamental trait in tree peony, and which can be generally divided into nine categories (red, pink, purple, yellow, white, black, blue, green and double-color). As for double-color flowers, the germplasm resources with this phenotype are very scarce and difficult to be cultivated by traditional breeding methods. Among them, *P. suffruticosa* ‘Shima Nishiki’ is a wellknown double-color cultivar in the world that its flower color phenotype can be stably inherited [3]. It is a chimeric cultivar derived from the bud mutation of the ‘Taiyoh’ cultivar, and usually shows two kinds of color models (red and pink) on the same flower. The showy double-color phenotype has high ornamental value, which is often favored by many consumers and has great commercial value to a certain extent [4]. Therefore, considering the cultivar that has this trait as a valuable experimental material will be of great significance to further explore and reveal the regulatory mechanisms of the double-color formation, and breed more tree peony cultivars with excellent and novel double-color patterns in the future [5].

Flower color is one of the most important ornamental traits in many plants. As for the colorful petals (such as red, pink, etc.), anthocyanins usually play a key role in determining flower color at the physiological level [6–8]. Furthermore, various studies have found that the anthocyanin biosynthetic

pathway is relatively conserved in different species, which involve multiple metabolic steps catalyzed by many enzymes [9, 10]. At the molecular level, anthocyanin biosynthesis and accumulation are usually directly related to the expression of structural genes in the above pathway, including chalcone synthase gene (*CHS*), chalcone isomerase gene (*CHI*), flavanone 3-hydroxylase gene (*F3H*), and flavonoid 3'-hydroxylase gene (*F3'H*), dihydroflavonol 4-reductase gene (*DFR*), anthocyanidin synthase gene (*ANS*), etc [11–14]. Therefore, the differential expression of anthocyanin biosynthetic structural genes is generally the most direct factor determining flower color difference in various ornamental plants.

In the anthocyanin biosynthetic pathway, the expression pattern and intensity of these structural genes are usually regulated by some transcription factors (TFs) related to anthocyanin biosynthesis [11, 15]. At present, many previous studies have found that the families of main TFs involved in the regulation of anthocyanin biosynthesis include MYB, bHLH, WD40, etc [16–18]. Among them, MYB (especially R2R3-MYB) TFs are the most abundant category in various studies involving the regulation of anthocyanin biosynthesis, and their regulatory effect on flower color is generally also the most important [19, 20]. MYB TFs can individually regulate the expression of structural genes related to anthocyanin biosynthesis in plants, and can also comprehensively regulate their expression of the related structural genes through MYB-bHLH complex or MYB-bHLH-WD40 (MBW) protein complex formed by the interaction of the above three kinds of TFs [14, 21–24].

Many studies have found that the most important pigment related to flower color formation in tree peony is also anthocyanin. As for its category, there are six anthocyanins including cyanidin glucosides (Cy3G and Cy3G5G), pelargonidin glucosides (Pg3G and Pg3G5G) and peonidin glucosides (Pn3G and Pn3G5G), respectively. The composition and content of these six anthocyanins are usually the key metabolic reasons for flower color formation and difference in tree peony [2, 3, 25, 26]. In addition, anthocyanin biosynthesis and accumulation in *P. suffruticosa* petals are generally directly related to the expression levels of anthocyanin biosynthetic structural genes, and the expression of these structural genes is also regulated by upstream transcription factors [3, 11, 14].

As for the cultivars with monochrome-color flowers in tree peony, Zhang et al. [27] found that the lower expression of some structural and regulatory genes (*PsMYB2*, *PsCHS1*, *PsF3H1*, *PsDFR1*, etc.) in in-vase flowers may be responsible for the color fading of petals by analyzing the expression levels of these genes related to anthocyanin biosynthesis of *P. suffruticosa* 'Luoyang Hong' in in-vase flowers and on-tree flowers. Based on comparative analysis of transcriptomes of different color petals in *Paeonia ostii*, Gao et al. [28] found that PoMYB2 and PoSPL1 negatively regulating the expression of downstream structural genes (*PoDFR6* and *PoANS1*) related to anthocyanin biosynthesis may be closely related to flower color intensity variation. Furthermore, Gu et al. [23] performed the analyses of transcriptome sequencing, virus-induced gene silencing (VIGS), yeast one hybrid (Y1H) assay, etc., and they found that PsMYB12 regulating the high expression of *PsCHS* gene at the base of petals should be the key reason for the petal blotch formation in *P. suffruticosa*.

As for tree peony with the double-color flowers, at present, there are two famous and typical cultivars ('Er Qiao' and 'Shima Nishiki') in the world. Zhang et al. [29] found that an indel in *F3'H* and the upregulation of *FLS* in acyanic petals should be two key factors mediating the variegated coloration of the 'Er Qiao' flowers. Furthermore, Zhang et al. [3, 30] preliminarily concluded that *PsDFR* may play a critical role in double-color formation in *P. suffruticosa* 'Shima Nishiki' based on transcriptome sequencing and quantitative Real-Time PCR (qRT-PCR) analyses. Meanwhile, two important TFs (PsMYB12L and PsMYB114L) differentially expressed between the red and pink petals in *P. suffruticosa* 'Shima Nishiki' were also screened, and they found that both of them can positively regulate anthocyanin biosynthesis by overexpression in *Arabidopsis*/apple calli [14]. However, the molecular regulatory mechanisms of double-color formation in *P. suffruticosa* 'Shima Nishiki' have not yet been fully revealed.

In order to further explore and identify the regulatory mechanisms of double-color formation in *P. suffruticosa* 'Shima Nishiki', the full-length cDNA, genomic DNA and promoter region sequences of *PsDFR* in the red and pink petals of its double-color flowers (Fig. 1) were cloned and comparatively analyzed, and the methylation levels of CpG island and promoter regions of *PsDFR* in the petals of these two colors were also determined. Meanwhile, based on Y1H and dual luciferase reporter assays, whether PsMYB12L/PsMYB114L can regulate the differential expression of *PsDFR* was identified. To a certain extent, these results will further advance our understanding of the molecular regulatory mechanisms of double-color formation in *P. suffruticosa* 'Shima Nishiki', and also provide gene reserve and theoretical basis for better regulating flower color traits in tree peony through genetic engineering technology, and breeding more excellent cultivars with novel color patterns and higher commercial value in the future.

Results

Cloning and sequence alignment of the full-length cDNA of *PsDFR* in the red and pink petals

Based on the unigene sequence of *PsDFR* obtained from transcriptome sequencing of *P. suffruticosa* 'Shima Nishiki', the full-length cDNA of its red and pink petals was cloned and sequenced, respectively. The results of electrophoresis showed that the target fragments with single band and the same size were obtained from the petals of these two colors. Furthermore, it was found that there is no difference in the full-length cDNA sequence of this gene between the red and pink petals, and both of their lengths are 1095 bp (Fig. 2).

Cloning and sequence alignment of the genomic DNA of *PsDFR* in the red and pink petals

In view of the above obtained full-length cDNA sequence of *PsDFR*, the genomic DNA of the red and pink petals in *P. suffruticosa* 'Shima Nishiki' was cloned and sequenced. Considering the possible complexity of introns in genomic DNA, it is relatively difficult to clone the full-length genomic DNA at one time. Here, the amplification of this gene was performed by four times. The results of electrophoresis showed that the target fragments with a single band and same size were obtained from the petals of these two colors (Fig. 3a). Sequencing results showed that the genomic DNA sequences of the red and pink petals were identical. According to statistics, the full-length genomic DNA of *PsDFR* was 2896 bp, including 6 exons

and 5 introns. Among them, the third intron sequence was relatively complex, including two TATA repeats (Fig. 3b).

Cloning and sequence alignment of the promoter region of *PsDFR* in the red and pink petals

Based on these cDNA and genomic DNA sequences of *PsDFR*, firstly, the promoter region of *PsDFR* in the red petals was cloned by chromosome walking method. In this study, the total fragment length of promoter region of *PsDFR* obtained was 4748 bp, which was amplified by five times. The length of these five fragments was 1698 bp, 743bp, 1434 bp, 951 bp and 421 bp, respectively (Fig. 4a).

By referring to the promoter sequence of *PsDFR* obtained from the red petals in the previous step, the promoter region of *PsDFR* in the pink petals was also cloned and verified by the method of segmented amplification. The length of the fragments amplified was 875 bp, 1493 bp, 1489 bp and 1336 bp, respectively (Fig. 4b). Sequencing results showed that the promoter region sequences of *PsDFR* in the red and pink petals were all the same (Fig. 4c).

Analysis of cis-acting elements in promoter region of *PsDFR*

In view of the identified promoter sequence of *PsDFR*, the cis-acting elements in the promoter sequence with 2000 bp of the start codon (ATG) upstream of *PsDFR* were analyzed by using plantCARE online database. The results showed that many elements were predicted in promoter region of *PsDFR*, including light response element, abscisic acid response element, gibberellin response element, etc (Table 1). Among them, there were three MYB-binding sites at these positions of -405 bp, -503 bp and - 1758 bp of the start codon upstream, respectively (Table 1 and Fig. 5).

Table 1
Cis-acting elements in promoter region of *PsDFR*.

Element name	Position from ATG	Sequence (5'-3')	Function
A-BOX	-553	CCGTCC	cis-acting regulatory element
ABRE	-151, -180, -481, etc	ACGTG	cis-acting element involved in the abscisic acid responsiveness
ARE	-1749/complementary strand	AAACCA	cis-acting regulatory element essential for the anaerobic induction
BOX-4	-254/complementary strand	ATTAAT	part of a conserved DNA module involved in light responsiveness
CAAT-box	-119/complementary strand,-653, etc	CCAAT	common cis-acting element in promoter and enhancer regions
CAT-box	-1969	GCCACT	cis-acting regulatory element related to meristem expression
CGTCA-motif	-562/complementary strand,-978	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
G-Box	-124/complementary strand, etc	CACGTG	cis-acting regulatory element involved in light responsiveness
GT1-motif	-19,-361, etc	GGTTAA	light responsive element
MBS	-1758	CAACTG	MYB binding site
MYB-like	-405,-503, etc	TAACCA	MYB binding site
TATA-box	-80/complementary strand,-255, etc	ATTATA	core promoter element
TATC-box	-1803/complementary strand	TATCCCA	cis-acting element involved in gibberellin-responsiveness
TCCC-motif	-341	TCTCCCT	part of a light responsive element

Determination of methylation level of CpG island and promoter region of *PsDFR* in the red and pink petals

Based on the cloning and sequence alignment of the *PsDFR* gene in the red and pink petals, it was preliminarily concluded that the double-color formation of *P. suffruticosa* 'Shima Nishiki' should not be caused by transposon insertion into *PsDFR* or base deletion of *PsDFR*. In addition, previous studies also have shown that the methylation level of key transcriptional regulatory regions of structural genes related to anthocyanin biosynthesis may affect the formation and difference of flower, leaf and fruit color, especially in CpG island (the GC content of this region is more than 50%, which is usually distributed in the promoter and the first exon region) [31–33]. If the methylation level of some key regions of promoter

or CpG island is very high, the corresponding gene expression can be greatly reduced. Sometimes, it may be completely inhibited.

In this study, we first predicted the location of CpG island in the promoter and first exon region of *PsDFR* by using MethPrimer online software. The results showed that there is a 105 bp CpG island (– 9 bp to 96 bp) near the start codon (ATG) (Fig. 6a).

Firstly, in order to verify whether the color difference of double-color flowers is determined by the differential methylation level of CpG island of *PsDFR*, the methylation level of this position of *PsDFR* in the red and pink petals was detected. The results showed that C-base of all samples sent for sequencing in these petals of two colors changed to T-base after sulfite treatment. Therefore, it is concluded that the methylation of CpG island of *PsDFR* did not occur in the red or pink petals (Fig. 6b).

In addition, based on the prediction analysis of cis-acting elements in the promoter region of *PsDFR*, the methylation level of the key transcription regulatory region and the region containing MYB-binding sites of the start codon (ATG) upstream (–10 bp to –822 bp and – 1732 bp to –2062 bp) was further determined. The results showed that methylation of many sites (CG/CHG/CHH) occurred in these regions of the red and pink petals, and there were a certain degree of differences in some sites. However, on the whole, there was no big difference and obvious regularity for their methylation levels of the red and pink petals (Fig. 6c).

Expression analysis of PsMYB114L and PsMYB12L in different tissues

In order to understand the tissue specificity of the *PsMYB114L* and *PsMYB12L* genes, the expression level of these two genes in seven different tissues (root, stem, leaf, flower, sepal, stamen and pistil) in *P. suffruticosa* ‘Shima Nishiki’ was quantitatively analyzed. The qRT-PCR results showed that the expression level of *PsMYB114L* was the highest in leaves, followed by roots, flowers, sepals and pistils, and the lowest in stems and stamens. The expression level of *PsMYB12L* was relatively high in sepals, leaves and flowers, followed by roots and stems, and relatively lower in pistils and stamens (Fig. 7).

Y1H validation of PsMYB114L and PsMYB12L binding to PsDFR promoter

Firstly, the pGADT7 empty vector, the full-length coding sequence (CDS) of *PsMYB114L*/*PsMYB12L* (restriction sites: *Sac*I and *Mlu*I), the pHIS2 empty vector and the promoter fragment of *PsDFR* (restriction sites: *Nde*I and *Xho*I) were successfully amplified using the corresponding primers (Supplemental Table S1) and double-digested (Fig. 8a), and then recombined, respectively. Subsequently, these three recombinant vectors of pGADT7-*PsMYB114L*, pGADT7-*PsMYB12L* and pHIS2-*PsDFR* (Fig. 8b) were verified successfully by PCR and sequencing.

In addition, the results of co-transformation showed all Y187 strains of the treatment group I (pGADT7-*PsMYB114L*, pHIS2-*PsDFR*), treatment group II (pGADT7-*PsMYB12L*, pHIS2-*PsDFR*), control group III (pGADT7, pHIS2-*PsDFR*), negative control group IV (pGADT7, pHIS2) and positive control group V

(pGADT7-Rec2-53, pHIS2-p53) could grow normally on the medium without Leu-Trp (SD/-Leu/-Trp), respectively (Fig. 8c).

Finally, the results on the medium without His-Leu-Trp (SD/-His /-Leu /-Trp) showed that under the concentration of 0 mM and 50 mM 3-AT, all the other groups grew well except for the negative control group IV. Furthermore, under the concentration of 100 mM and 200 mM 3-AT, the results suggested that in addition to the normal growth of the positive control group V, the treatment group II (pGADT7-*PsMYB12L* and pHIS2-*PsDFR*) also showed good growth status, while the treatment group I (pGADT7-*PsMYB114L* and pHIS2-*PsDFR*) and the other control groups did not grow (Fig. 8c). As for *PsMYB114L* and *PsMYB12L*, it was preliminarily concluded that only *PsMYB12L* can be combined with the promoter of *PsDFR*.

Double luciferase report validation of *PsMYB114L* and *PsMYB12L* on *PsDFR* promoter activation

Based on the method of seamless cloning, the full length coding sequence (CDS) of *PsMYB114L* and *PsMYB12L* (*Bam*HI and *Eco*RI) and the promoter fragment of *PsDFR* (*Sal*I and *Hind*III) with restriction sites were obtained with the special primers (Additional file 1: Table S1) using the plasmids of recombinant vector constructed by Y1H assay as templates. At the same time, the pGreenII62-SK empty vector and pGreenII0800-LUC empty vector were double-digested with special enzymes (Fig. 9a), and then recombined with the corresponding candidate genes, respectively. Subsequently, these three recombinant vectors of pGreenII62-SK-*PsMYB114L*, pGreenII62-SK-*PsMYB12L* and pGreenII0800-LUC-*PsDFR* (Fig. 9b) were also verified successfully by PCR and sequencing.

Tobacco leaves were respectively injected with the combination of *Agrobacterium tumefaciens* solution of the recombinant vectors, including 4 control and 2 treatment groups, and then the relative activity of luciferase was detected. As for *PsMYB114L* and *PsDFR*, it was found that their relative activities of luciferase were lower in the control group containing two empty vectors (pGreenII62-SK, pGreenII0800-LUC) and the single empty vector (pGreenII62-SK-*PsMYB114L*, pGreenII0800-LUC), and their relative activities of luciferase were relatively higher but the differences were very small (Fig. 9c) in the control group containing single empty vector (pGreenII62-SK, pGreenII0800-LUC-*PsDFR*) and the treatment group 1 (pGreenII62-SK-*PsMYB114L*, pGreenII0800-LUC-*PsDFR*).

As for *PsMYB12L* and *PsDFR*, their results of luciferase activity showed a similar trend compared with the above results of *PsMYB114L* and *PsDFR*. However, the relative activity of luciferase in the treatment group 2 (pGreenII62-SK-*PsMYB12L*, pGreenII0800-LUC-*PsDFR*) was about 2.3 times higher than that in the control group containing single empty vector (pGreenII62-SK, pGreenII0800-LUC-*PsDFR*) (Fig. 9d). In summary, these results indicated that only *PsMYB12L* could activate the expression of *PsDFR*.

Discussion

Based on our previous studies [3, 14, 30], *PsDFR* was preliminarily identified as a key structural gene for double-color formation in *P. suffruticosa* 'Shima Nishiki'. In ornamental plants, there are many reasons for

the formation of double-color flowers at the molecular level. At present, various studies have shown that the insertion of transposon into structural genes related to anthocyanin biosynthesis is usually a key reason contributing to double-color formation in many plants. As for these transposons, there are generally three kinds of insertion sites, including exon, intron and promoter region[34–36].

In this study, the *PsDFR* gene was cloned and sequenced from three levels including exon, intron and promoter. Firstly, the full-length cDNA of *PsDFR* from the red and pink petals was cloned. The results showed that their sequences of *PsDFR* were identical, with the length of 1095 bp, which indicated that there was no transposon insertion in the exon region of the petals of these two colors. Moreover, the full-length genomic DNA of *PsDFR* in the red and pink petals was cloned. The results showed that their genomic DNA contained six exons and five introns, with the third intron containing two TATA repeats, and the full-length (2896 bp) sequences of their genomic DNA were all the same. These results indicated that there was no transposon insertion, deletion and mutation of some bases in the intron region of *PsDFR* in the red and pink petals.

In view of the fact that there is no high-quality genome sequence in tree peony until now, the promoter region of *PsDFR* gene was cloned by chromosome walking method. The ‘Shima Nishiki’ cultivar was selected from the bud mutation of *P. suffruticosa* ‘Taiyoh’ [14]. The flower color phenotype of the ‘Taiyoh’ cultivar is red, and that of ‘Shima Nishiki’ is double-color (red and pink) [1]. Therefore, we speculated if transposon insertion exists in the promoter region, it should occur in the pink petals. In general, the length of the inserted transposons is usually a few bp to a few tens of kb [34–39]. In order to avoid the difficulty of chromosome walking amplification, the promoter region of the red petal was cloned first, and a total of 4748 bp sequence was obtained. According to the promoter sequence of *PsDFR* in the red petals, the promoter region of this gene in the pink petals was also successfully amplified. Interestingly, by comparing the promoter sequences of *PsDFR* in the petals of these two colors, it was found that their sequences were identical. These results indicated that there was no transposon insertion in the promoter region (4748 bp) of *PsDFR* in the red and pink petals of *P. suffruticosa* ‘Shima Nishiki’.

Based on the fact that there was no sequence difference of *PsDFR* in exon, intron and promoter regions between the red and pink petals, it was preliminarily concluded that the double-color formation of *P. suffruticosa* ‘Shima Nishiki’ was not caused by the insertion of transposon into *PsDFR*. Furthermore, the results of cis-acting element analysis in the promoter region of *PsDFR* showed that there were several MYB-binding sites in this region. In addition, on basis of flower color difference possibly caused by methylation of promoter [31, 32, 40], the methylation level of *PsDFR* promoter region in the red and pink petals was further determined. The results showed that the position of both CPG island and one MYB-binding site (TACCA, -503 bp) of the petals of these two colors were not methylated. Meanwhile, many CG/CHG/CHH sites in other regions were methylated to some extent,, including the other two MYB-binding sites (TACCA, -405 bp and CAACTG, -1758), but regrettably there was little difference between the red and pink petals. Therefore, the double-color formation of *P. suffruticosa* ‘Shima Nishiki’ should also not be caused by the methylation level difference of promoter region of *PsDFR* between the red and pink petals. Based on the above results, it is speculated that the color difference of double-color flowers in *P.*

suffruticosa 'Shima Nishiki' is not caused by *PsDFR* itself, but by the differential expression of its upstream MYB transcription factor, which further regulates the differential expression of *PsDFR*.

As for the main transcription factors (MYB, bHLH, WD40, etc) related to flower color, they often indirectly affect anthocyanin biosynthesis and accumulation, and then mediate flower color formation and difference by regulating the expression of structural genes related to anthocyanin biosynthesis [41]. Among these transcription factors, R2R3-MYB has a relatively greater influence on the formation of flower color phenotype [42–44]. It can generally regulate structural genes individually or by forming complexes with bHLH and WD40, and then regulate anthocyanin biosynthesis [45, 46]. In *Arabidopsis thaliana*, AtPAP1 can individually promote anthocyanin accumulation in leaves by regulating the related structural genes of anthocyanin biosynthetic pathway, and then make it present dark-purple phenotype [21]. In *P. suffruticosa*, PsMYB12 can regulate the expression of *PsCHS* by forming a MYB–bHLH–WD40 (MBW) regulatory complex with bHLH and WD40, and then activate anthocyanins biosynthesis and mediate petal blotch formation [23].

Meanwhile, the spatial expression patterns of the *PsMYB114L* and *PsMYB12L* genes in *P. suffruticosa* 'Shima Nishiki' were quantitatively analyzed. The expression level of *PsMYB114L* in leaves and roots was relatively higher, followed by petals, sepals, etc. The results indicated that PsMYB114L may mainly regulate leaf color (or regulate flower color to a certain extent), and specifically regulate the biosynthesis of secondary metabolites in roots (Cortex Moutan). Furthermore, the expression level of *PsMYB12L* was the highest in sepals, leaves and petals, suggesting that PsMYB12L may mainly regulate the formation of sepal/leaf/flower color.

In order to further explore whether PsMYB114L and PsMYB12L can regulate the expression of the *PsDFR* gene, and lead to double-color formation of *P. suffruticosa* 'Shima Nishiki', we performed the validation analyses of Y1H and double luciferase report assays at the same time. These two results also showed that PsMYB12L could bind to the promoter region of *PsDFR* and activate the expression of this gene.

Conclusions

Therefore, we preliminarily concluded that PsMYB12L regulating the differential expression of *PsDFR* may be a key factor mediating double-color formation. These results of this study would provide a valuable resource for further understanding the molecular regulatory mechanisms of double-color formation in *P. suffruticosa*, and benefit molecular breeding of tree peony cultivars with showy and novel flower color in the future.

Methods

Plant materials

The *P. suffruticosa* cultivar 'Shima Nishiki' was grown in the experimental nursery of Forestry College, Shandong Agricultural University, Tai'an City (36°18' N, 117°13' E), Shandong Province, China. The red-

petal and pink-petal samples were separately collected from the same double-color flowers of *P. suffruticosa* 'Shima Nishiki' (Fig. 1), and they were named SN-Red and SN-Pink, respectively. All samples were immediately frozen in liquid nitrogen and then stored at -80°C until further use.

Cloning of genes (full-length cDNA, genomic DNA and promoter region)

Total RNA from the red (SN-Red) petal, pink (SN-Pink) petal, and other tissue samples was respectively extracted using PureLink™ Plant RNA Reagent (Invitrogen, USA) according to the manufacturer's instructions, with slight improvement. Furthermore, cDNA was synthesized with 1 μg of total RNA using 5 $\times$ All-In-One RT MasterMix (with an AccuRT Genomic DNA Removal Kit) (ABM, Canada), and the obtained cDNA was used for the cloning of full-length cDNA. The genomic DNA of the red-petal and pink-petal samples was extracted using Hi-DNAsecure Plant Kit (Tiangen, China), respectively. The obtained DNA was used for the cloning of genomic DNA. In addition, the cloning of promoter region was performed by chromosome walking method.

Analysis of cis-acting elements in promoter region

Cis-acting regulatory elements in promoter region with 2000 bp of the start codon (ATG) upstream of the candidate genes were predicted and analyzed by using PlantCARE online database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/#opennewwindow>) [47].

Determination of methylation level

Sulfite treatment of the obtained genomic DNA was performed using EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH, USA), then cloned and sequenced. There are at least 20 tubes of bacterial solution by sequencing for each treatment. Finally, the methylation levels of different samples were counted by sequence alignment and statistics.

Quantitative Real-Time PCR (qRT-PCR) analysis

qRT-PCR was performed to analyze the expression levels of the candidate genes with a Bio-Rad CFX96™ real-time system (Bio-Rad, USA). The methods of RNA extraction and cDNA synthesis were the same as above. The qRT-PCR experiments were conducted using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, Japan) with three biological replicates. The *Psubiquitin* gene was used as an internal control to normalize the expression data of qRT-PCR [48]. The relative expression levels of genes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method [49]. The gene-specific primers used in this study are shown in Supplemental Table S1.

Y1H assay

According to the method of Tao et al. [50], the regulatory effect of PsMYB12L/PsMYB114L on *PsDFR* was verified by Y1H assay. The full length of PsMYB12L/PsMYB114L (MK518074.1/MK518073.1) and the 2000 bp fragment of *PsDFR* promoter (including the predicted MYB-binding sites) were ligated to pGADT7 and pHIS2 vectors, respectively. Then, plasmids of treatment group I (pGADT7-PsMYB114L,

pHIS2-*PsDFR*), treatment group II (pGADT7-PsMYB12L, pHIS2-*PsDFR*), control group III (pGADT7, pHIS2-*PsDFR*), negative control group IV (pGADT7, pHIS2) and positive control group V (pGADT7-Rec2-53, pHIS2-p53) were cotransformed on the medium without Leu-Trp (SD/-Leu/-Trp), respectively. Finally, the positive individual colonies growing on the medium without Leu-Trp were inoculated on the medium without His-Leu-Trp (SD/-His /-Leu /-Trp) containing 0, 50 mM, 100 mM and 200 mM 3-Amino-1,2,4-triazole (3-AT), respectively, and they were invertedly cultured in 30 °C incubator for about 5 days, and the growth status of fungi colonies was also observed at any time.

Dual luciferase reporter assay

According to the method of An et al. [51], the regulatory effect of PsMYB12L/PsMYB114L on *PsDFR* was also further identified by dual luciferase reporter assay. The full length of PsMYB12L/PsMYB114L and the 2000 bp fragment of *PsDFR* promoter (including the predicted MYB-binding sites) were ligated into pGreenII62-SK and pGreenII0800-LUC vectors, respectively. Subsequently, they were transferred into *Agrobacterium tumefaciens*, and then the bacterial fluid of *Agrobacterium tumefaciens* containing different combinations of vectors was slowly compressed into the back of tobacco leaves by syringe, including treatment group 1 pGreenII62-PsMYB114L-SK, pGreenII0800-*PsDFR*-LUC), treatment group 2 (pGreenII62-PsMYB12L-SK, pGreenII0800-*PsDFR*-LUC) and four control groups (pGreenII62-SK, pGreenII0800-LUC; pGreenII62-PsMYB114L-SK, pGreenII0800-LUC; pGreenII62-PsMYB12L-SK, pGreenII0800-LUC; pGreenII62-SK, pGreenII0800-*PsDFR*-LUC). After tobacco plants were cultured for 3 days, the luciferase activity of the sample solution processed by Dual-Luciferase® Reporter Assay System (Promega, USA) was detected by Promega Glomax 20/20.

Abbreviations

CHS: Chalcone synthase gene; CHI: Chalcone isomerase gene; F3H: Flavanone 3-hydroxylase gene; F3'H: Flavonoid 3'-hydroxylase gene; DFR: Dihydroflavonol 4-reductase gene; ANS: Anthocyanidin synthase gene; TFs: Transcription factors; VIGS: Virus-induced gene silencing; Y1H: Yeast one hybrid; qRT-PCR: Quantitative Real-Time PCR; MBW: MYB-bHLH-WD40; SN-Red: Red petal; SN-Pink: Pink petal

Declarations

Acknowledgments

Not applicable.

Authors' contributions

ZDX conceived and designed the research. XPZ and XH participated in the specific design of the study. XPZ and XH performed the experiments and the data analysis, and drafted the manuscript. MYZ, XYX and CL contributed analysis tools and helped analyze the data. All authors contributed to manuscript revision and approved the final version.

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Availability of data and material

The datasets generated and analyzed in the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article and its Supplementary information files. The data on the full-length coding sequence of the *PsMYB114L* (MK518073.1) and *PsMYB12L* (MK518074.1) genes has been deposited in the NCBI database. The data on the full-length cDNA, genomic DNA and promoter region sequence of *PsDFR* can be available in Fig. 2b, Fig. 3b and Fig. 4c, respectively.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The double-color flowers of *P. suffruticosa* 'Shima Nishiki'.

Figure 3

Cloning of the full-length genomic DNA of PsDFR in the red and pink petals. a PCR detection of PsDFR. b Sequence analysis of PsDFR.

GCAACTTGTGGTAGTCTTATGTATCTGACTGCCACTAGGCCTGATCTTATGTATGTAGTTTGCCTCATCAGTA
 GGTATATGGCTCATCAAAGGAAGCACATTGGGCAGAAGCTAAAAGGATTCTACGTTATCTCAAAGGAACT
 GTTGATTGGGAGTATACTATAGAAGAGAAGTGATTGGTGATTGGTGGGATATTGTGATAGTGATTATGCTG
 GCAATGTTGATGATTCTCGAAGTA**CAACTG**GTTTCGTGTTTCATGTTGAGTGCTGGAGCAATTTCTTGGTCTTC
 ACGAAAACAGCCTATTGTAACCTTTGTCGACTACGGAAGCAGAGTATTTTGCTGCTGCTGCTTGTCTTGTCAG
 TGTGTCTGGATACAGAGAATTCTTGTGAGTCTTGGCTATGAAAAGTGTGTTACTTTATTTTGTGACAA
 TACTTCTGCAATTAAGTTAGCCAAGAACCCTGTGTTTCATGGTAGAAGCAAACACATCAGTGTGAGATTTTAT
 TTCATTGAGATCTTACTAAGGATGGTGTGTGAAACTTGAATTATGTGGGAGCAGTGAGCAGCTCGCAGAC
 ATTTTAACAAAGCCTTTGAAGTTAGAAGCATTGAAAGGTTTCGAAGAATGCTTGGAGTTGTGGCCAAAGA
 AGAAGTAAACTGAACTGTCTTCATCAGCTTCAGTTTAGGGGAAGGATTGTTATTGTTGCTTTGAATTAGTTTG
 TTTTAATTAGTTTGTGTTTGAAGTCAAATAACTCCTGTATTTTAGGAGAAGTTAGAATCAGTCATTAAGTTT
 GTTGTGTTTGTACGAGTCCTTAGATGAGTCGCCACAATCTCAGGGTTGTTGAGATGTGGTTAAGTTTTATT
 TCCCTGTACGCAAATGGCTATTTAAAGTCAGTTTGACATGATCAATAAATATGAAAACAGAGATTTTTCATCAG
 TGTGTCCTTTATAATAACAGGTTTGAGTCCAAAGTTGAGTTTTTGCCTTTTCTTCTCTGGGTGGGCTTTT
 CGTCATCTTCTCTCCTCGGAGTCTGATAAGAAGACTTCTTCTCGCGATCGGCAAGTTGTTTAACAATGTG
 ATCTGGAATCATTCCATCACTGCCAAAAAAATACTGCTCTGTTTCTGGTTCTTGCATAACATCATGGACAAATT
 CATCCGCTCGTGATGGTGGTGGTGTGTTTCTTTGAGTCCATTGTCTGCGACGTTCTTTACCTTCACGAACAATC
 CTTGCTTTATTATCTATCTGATTTTTCTTAGCTCTTCATCTTGTGACTCCCTGCTCGGCTGTGAACTCCTTCG
 GTGCATTGGAGTTATACTCTTCTCGATTCCAATCTCTTTTCGCATCTCTAGGGTTTACGAAGGAGAGGTTTTA
 CTAAACCTGATTTTCTACAGAAAGGGGACTGTAACAGACTTGACGATTCTCCACCGTCCACTTTTTAATTTA
 ACATCAGTTTATGAATAGGTTTACAAGGAAAT**TAACCA**CCTGCAGTTTTTTAAACGTGGGGTATGTATTTCA
 ACATAAATGTTTATTTTATAACAAAGAACTCTGCAAACAGAGGAAAGAAAAAGAT**TAACCA**TGGTGCAGA
 AGACGGCAGGAAAAACAATAGTAAATATAGGTTAATTTTTTAATATTATCTCCCTATTGTTTTTAAATTA
 AAGACCTCAAAATGTGGGATGTATGTTATCAAAATGCAGGGGCATTTTTTGTTCATTAATTATAGGATGAG
 GGTGACAAAATGGTAGATGCACGTAACATCATCTTAATTTTATTCAAACTCTATTCGGAAAACGTGGAGCGG
 GTAGACAATAACAGCCCGTGGCAGCTGGTGAGTTGATCACGTGATTGGGAAGTTAAACGTGTTAGAT
 GGCTCACCTACCGTGTGTATAAAACAAAGCTAGCATGAACCCAAACACAGCCAAAATTCTATTCTCTCCTCG
 TATCGTGGTTAAAAAACAAAGCAA**ATG**

Figure 5

MYB-binding sites of the promoter region of PsDFR.

Figure 6

Methylation levels of CpG island and promoter regions containing MYB-binding sites of PsDFR in the red and pink petals. **a** CpG island prediction of promoter and exon 1 regions of PsDFR. **b** Sequence alignment of CpG island of PsDFR after sulfite treatment. **c** Methylation levels of promoter regions of PsDFR. Red box indicates the methylation levels of 3 MYB-binding sites, respectively.

Figure 7

Expression patterns of the *PsMYB114L* and *PsMYB12L* genes in different tissues of *P. suffruticosa* 'Shima Nishiki'. Different lowercase letters indicate significant differences at $p < 0.05$.

Figure 8

Verification of Y1H assay between PsMYB114L/PsMYB12L and PsDFR. a Double digestion of the empty vector (pGADT7 and pHis2) and candidate genes (PsMYB114L, PsMYB12L and PsDFR). b PCR verification of the recombinant vectors (pGADT7-PsMYB114L, pGADT7-PsMYB12L and pHis2-PsDFR). c Observation on the growth status of fungi colonies of the treatment and control groups on the medium without Leu-Trp (SD/-Leu/-Trp) and His-Leu-Trp (SD/-His/-Leu/-Trp) containing 0, 50 mM, 100 mM and 200 mM 3-Amino-1,2,4-triazole (3-AT), respectively.

Figure 9

Verification of dual luciferase reporter assay between PsMYB114L/PsMYB12L and PsDFR. a Double digestion of the empty vector (pGreenII62-SK and pGreenII0800-LUC). b PCR verification of the recombinant vectors (pGreenII62-SK-PsMYB114L, pGreenII62-SK-PsMYB12L and pGreenII0800-LUC-PsDFR). c Relative luciferase level of PsMYB114L and PsDFR. d Relative luciferase level of PsMYB12L and PsDFR.

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