Color characteristics, pigment accumulation and biosynthetic analyses of leaf color variation in herbaceous peony (*Paeonia lactiflora* Pall.)

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Abstract

Herbaceous peony (*Paeonia lactiflora* Pall.) is one of the color-leaved ornamental spring plants, with graceful appearance and splendid color. However, the underlying mechanism of this coloration variation from purple to green has not been studied in *P. lactiflora*. In this study, the leaves in purple, purple–green, and green stages were compared in terms of anatomical, physiological, and molecular. We found that the variation of leaf color from purple to green was mainly determined by the change in pigments distributed in the leaf surface. Physiological experiments showed a significant increase in chlorophyll contents and a notable reduction in anthocyanin contents in leaves from the purple to green stages. We further found that the anthocyanin biosynthesis-related dihydroflavonol 4-reductase (*DFR*) gene and anthocyanin synthase (*ANS*) gene as well as chlorophyll biosynthesis-related glutamyl-tRNA reductase (*HEMA*) gene showed a decreased trend in leaves from purple to green stages, whereas the chlorophyll degradation-related chlorophyll *b* reductase (*NYC*) gene showed a rising trend. Alteration of *DFR* and *ANS* gene expression might reduce anthocyanin accumulation, whereas increased *HEMA* gene expression would enhance chlorophyll biosynthesis and reduced *NYC* gene expression would inhibit chlorophyll degradation. Consequently, reduction in anthocyanins and enhanced deposition of chlorophylls resulted in leaf coloration variation from purple to green in *P. lactiflora*, which could improve our understanding of its mechanism for further studies.

Keywords Herbaceous peony · Anthocyanins · Chlorophylls · Gene expression

Introduction

For ornamental plants, leaf color is a key trait gaining increasing attention for its great ornamental value. Color-leaved plants play an important role in landscaping for their unique leaf color. The leaf color in plants is mainly determined by leaf pigments comprising chlorophylls, carotenoids, and anthocyanins. Chlorophylls, produced in chloroplasts, are the vital photosynthetic pigment of leaves

(Yang et al. 2015). Leaves appearing green, mainly account for high concentrations of chlorophyll, which is relevant to chlorophyll biosynthesis and degradation. Carotenoids are an important class of natural pigments that are ubiquitous in the yellow, orange—red, or red pigments of plants (Su et al. 2014). Anthocyanins are a natural type of water-soluble pigment widely found in plants, ranging in a red to blue color in leaves (Carlson and Holsinger 2010). Furthermore, successful leaf color alteration from purple to green by genetic transformation using several genes that are involved in anthocyanin biosynthesis has already been reported in crab apple (*Malus spectabilis*) (Tian et al. 2015) and apple (*Malus sp.*) (Szankowski et al. 2009).

Recently, studies related to leaf coloration have focused on fall-color trees and leaf color mutants. For example, Field et al. (2001) reported that chlorophyll degradation was accompanied with enormous anthocyanin accumulation, leading to red-colored maple leaves during the late developmental stages. Leaf color mutants occur due to chlorophyll-deficiency, leading to green-deficient leaf color mutations such as yellow—green, light-green, or yellow leaf color in

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crops (Liu et al. 2018a; Zhang et al. 2018), vegetables (Liu et al. 2018b), and ornamental plants (Yu et al. 2016; Li et al. 2015). However, few studies have concentrated on spring color-leaved trees with leaf color alterations from purple to green.

Herbaceous peony (*Paeonia lactiflora* Pall.) is a traditional Chinese ornamental plant and has nowadays been developed as an emerging high-end cut-flower. It enjoys the high reputation of ‘two of the most beautiful flowers’ with peony (*Paeonia suffruticosa* Andr.) due to its graceful appearance and splendid color. To improve its ornamental characteristics and quality, studies have focused on floral coloration (Zhao et al. 2016), floral shape (Wu et al. 2018), tolerance to abiotic and biotic stress (Gong et al. 2015; Hao et al. 2016), and inflorescence stem mechanical strength (Zhao et al. 2019; Tang et al. 2019); however, leaf coloration has not been taken into consideration. *Paeonia lactiflora* is one of the color-leaved ornamental spring plants, which has purple leaves at the early developmental stage and green leaves at the late developmental stage. This trait has brought high value to *P. lactiflora*. However, the mechanism underlying this coloration variation from purple to green has not been studied in *P. lactiflora*. If we could clarify the shift mechanism involved, it would be possible to inhibit the color transformation and to cultivate *P. lactiflora* with purple phenotype, which may contribute to higher commercial value.

In this study, we compared the leaves of *P. lactiflora* at three different leaf developmental stages including purple, purple–green, and green coloration in terms of color indices, structure, and pigment content. Furthermore, we isolated and sequenced the genes involved in leaf coloration and validated the expression of these genes using quantitative real-time polymerase chain reaction (qRT-PCR). Our results revealed the variation of color characteristics, pigment accumulation, and biosynthesis in leaves from purple, purple–green, and green in *P. lactiflora*.

Materials and methods

Plant materials

Paeonia lactiflora cv. ‘Zilan Xijin’ grown in the field of the germplasm repository of Yangzhou University, Jiangsu Province, China (32° 23' N, 119° 24' E), was used as the experimental material. The leaves at three leaf developmental stages (purple stage (S1), purple–green stage (S2), green stage (S3); Fig. 1a, b) were collected as samples. Anatomical observation as well as color indices and SPAD values determination were performed immediately after each collection, and the samples were then frozen in liquid nitrogen and stored at –80 °C until examination.

Anatomy observation

Microscopic observation of leaf transverse sections was performed using free-hand sections. The leaves were cross-sectioned and placed on a glass slide with a drop of water. They were immediately observed under a light microscope (Olympus CX31RTSF, Tokyo, Japan) and the specific operational method was performed as described by Zhao et al. (2015).

Color indices measurement

Leaf colors including leaf adaxial and abaxial surface colors, were first compared with the Royal Horticultural Society Color Chart (RHSCC), and were then measured using a portable colorimeter, RM200QC spectrophotometer (X-Rite, Switzerland) in the laboratory at room temperature. In total, fifteen different leaves were measured and each measurement was repeated three times. The results were recorded using the color parameters L^* , a^* , and b^* . L^* stands for lightness (0 = black, 100 = white), a^* indicates the red (positive)/green (negative) coordinate, and b^* represents the yellow (positive)/blue (negative) coordinate. Then, SPAD values of these fifteen leaves (also including leaf adaxial and abaxial surface) were detected using a SPAD chlorophyll meter (SPAD-502 PLUS, Konica Minolta, Japan) and each measurement was repeated three times.

Pigment contents measurement

The content of pigments including anthocyanin, flavonoid, chlorophyll, and carotenoid was measured. Anthocyanins were extracted in a methanol and acetic acid mixture (methanol/water/acetic acid = 85:15:0.5, v/v/v), according to the procedure described by Lewis et al. (2003). The absorbance of each supernatant was measured at 530 nm and 657 nm using a UV spectrophotometer (UV BlueStar A, Beijing Lab Tech Co., Ltd., China) to calculate the anthocyanin concentration. The content of flavonoids was measured using a reagent kit (Suzhou Comin Biotechnology Co., Ltd., China). For chlorophylls and carotenoids, approximately 0.1 g of the same samples used for anthocyanin extraction were extracted using an ethanol and acetone mixture (ethanol/acetone = 1:4, v/v). The absorbance of the supernatant obtained by centrifugation was measured using the same spectrophotometer at 470, 645, and 663 nm. The concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophylls, and carotenoids were then calculated according to Lichtenthaler (1987).

Gene isolation and sequencing

According to the cDNA sequence of 11 genes including phenylalanine ammonia-lyase (*PAL*) gene, chalcone synthase

Fig. 1 Phenotype and microscopic observation of leaves in *P. lactiflora* at three leaf developmental stages. **a** Phenotype of the *P. lactiflora* plant. **b** Phenotype of the leaf adaxial and abaxial surface. Bar = 1 cm. **c** Microscopic observation of leaf transverse sections. Bar = 100 μ m

(*CHS*) gene, chalcone isomerase (*CHI*) gene, flavonoid isomerase (*FLS*) gene, dihydroflavonol 4-reductase (*DFR*) gene, anthocyanin synthase (*ANS*) gene involved in flavonoid biosynthesis, and glutamyl-tRNA reductase (*HEMA*) gene, protoporphyrinogen IX oxidase (*PPO*) gene, magnesium chelatase subunit I (*CHLI*) gene, chlorophyll *a* synthase (*CHLG*) gene, and chlorophyll *b* reductase (*NYC*) gene involved in chlorophyll biosynthesis, obtained from the transcriptome data (NCBI accessions: SRP127132), gene-specific primers were designed to isolate the cDNA sequences. The methods that used for gene isolation and sequencing were the same as described by Zhao et al. (2015) with some modifications. The gene-specific primers are shown in

Supplementary Table S1, and the PCR products were sent to TsingKe Biological Technology Co., Ltd. (Beijing, China) for sequencing. Sequence comparison was performed using NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Gene expression analysis

Gene transcript levels were analyzed using qRT-PCR with a BIO-RAD CFX Connect™ Optics Module (Bio-Rad, USA). cDNA was synthesized from RNA using a PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan). *PlActin* was used as an internal control in *P. lactiflora* (Zhao et al. 2012a). All gene-specific primers in this study are shown in

Table 1, and were synthesized by TsingKe Biological Technology Co., Ltd. (Beijing, China). qRT-PCR was performed as reported by Tang et al. (2019). Relative expression levels of target genes were calculated by the $2^{-\Delta\Delta C_t}$ comparative threshold cycle (C_t) method (Schmittgen and Livak 2008). The C_t values of triplicate reactions were gathered using the Bio-Rad CFX Manager V1.6.541.1028 software.

Statistical analysis

All experiments described here were repeated three times in a completely randomized design. Primers were designed using the Primer 5.0 program. All data were means of three replicates with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA).

Results

Anatomical observation and color indices

As shown in Fig. 1a, leaf color was transformed from purple to purple–green to green during the leaf developmental stages. The color of the leaf abaxial surface was more vivid than that of the leaf adaxial surface (Fig. 1b). Anatomical observation demonstrated that red-colored pigments were all distributed in the epidermis at the purple and purple–green stage (Fig. 1c). Specifically, increased red-colored pigments were accumulated in the leaf abaxial surface than in the leaf adaxial surface. Besides, accumulation of red-colored pigments was reduced along with the leaf development, and completely disappeared at the green stage.

Furthermore, the color indices of the leaf adaxial and abaxial surfaces were checked using the RHSCC and the chromameter. With leaf development, the color of the leaf adaxial surface gradually ranged from 200B to 200A

to 137C, whereas that of the leaf abaxial surface changed from 59A to 200C to 138B. The adaxial surface of leaves showed higher L^* and b^* values and lower a^* values than the abaxial surface of leaves. In three different leaf developmental stages, the L^* and b^* values of the leaf surface were gradually increased, whereas the a^* value of the leaf surface was decreased (Fig. 2a). These color indices showed that the colors of the leaf surface were purple and purple–red at the purple stage, respectively, and then gradually faded to green along with leaf development. Collectively, this result was relevant to the visual result.

Pigment contents

Leaf color variation was accompanied with pigment changes including flavonoids (containing anthocyanin), chlorophylls, and carotenoids. For anthocyanin and flavonoid contents, quantitative comparison showed that the leaves accumulated the most anthocyanins at the purple stage, the least anthocyanins at the green stage, and the middle level at the purple–green stage. The anthocyanin level in the leaves at the purple stage was 1.34 times and 2.14 times higher than that at the purple–green and green stage, respectively. Although accumulation of flavonoids demonstrated a decreasing tendency, it did not reach a significant level (Table 2).

During leaf developmental stages, the contents of chlorophyll *a*, chlorophyll *b*, and total chlorophyll sharply rose from the purple stage to green stage. Specifically, the content of chlorophyll *a* was increased by 19.21% and 31.55% at the purple–green and green stage, respectively, compared to that of chlorophyll *a* at the purple stage, whereas the contents of chlorophyll *b* and total chlorophyll were increased by 36.22%, 23.61%, and 82.51%, 44.74% at the purple–green and green stage, respectively, compared to that at the purple stage (Table 2). SPAD value was further calculated and the result showed that the value significantly increased from the purple stage to green stage, with no difference between the

Table 1 Gene-specific primers used in qRT-PCR analysis

Gene	Forward primer sequence (5' → 3')	Reverse primer sequence (5' → 3')
<i>PAL</i>	TGAGTCAAGTTGCCAAGAG	GCATTAAAGGGTAAGTAGCG
<i>CHS</i>	ATGACTGGAACCTCGATACTCT	CTCACTCAAAACATGCCTAG
<i>CHI</i>	TTGTAACGGGTCCATTG	TTCGGCATCAGTGTAAGT
<i>FLS</i>	GCTATCTCATCCGCACAA	CTTCAAACCTTCTCTACCAG
<i>DFR</i>	GCACTTTCTCCAATCACAG	CAAATGTAGCGACCCTCT
<i>ANS</i>	AGATTAGAACAAGAAGTCGGTG	GGAGGATGAAAGTGAGGG
<i>HEMA</i>	GAAGTCGTGGCTGCTAAC	GCTTTCCTAGTCTTCTTCGTTA
<i>PPO</i>	TCCACTCTATGGGCGTAA	TTGCAACCCGAAGCTATT
<i>CHLI</i>	AACGTCTTCTACGGCAATC	GAAATCCCAATCCCTCCA
<i>CHLG</i>	GCTGGATTGTTAGATGTG	AAAGGTGGAGCAGAGTAT
<i>NYC</i>	ACAGTTGCGAGAACCCTA	TCAGCCCAGTTTCGTATT
<i>Actin</i>	GTTGCCCTTGATTACGAG	CAGCTTCCATTCCGATTA

Fig. 2 Color parameters and SPAD of leaves in *P. lactiflora* at three leaf developmental stages. **a** Color parameters of the leaves. **b** SPAD of the leaves. S1, purple stage, S2, purple–green stage, S3, green stage. The values represent the means $\pm$ SDs, and different letters indicate significant differences ($P < 0.05$)

leaf adaxial and abaxial surfaces (Fig. 2b). For carotenoids, the accumulation of carotenoids demonstrated a gradually rising trend, but did not reach a significant level (Table 2).

Pigment biosynthesis-related gene isolation and sequence analyses

To investigate the transformation mechanisms of color shift in *P. lactiflora* leaves at the molecular level, six structural genes in the flavonoid biosynthetic pathway including *PAL*, *CHS*, *CHI*, *FLS*, *DFR* and *ANS*, along with five structural genes in the chlorophyll biosynthetic pathway including *HEMA*, *PPO*, *CHLI*, *CHLG*, and *NYC* were isolated (Supplemental Fig. S1–S11). The partial-length cDNA sequence of *PIPAL* (MN869918) was 2206 bp, and contained a partial ORF of 2121 bp encoding a 706 amino acid protein, an untranslated region (UTR) of 28 bp in 5' end, a 3'-UTR of 57 bp. Similarly, the partial-length sequence cDNAs of *PICHS* (MN869919), *PICHI* (MN869920), *PIFLS* (MN869921), *PIDFR* (MN869922), *PIANS* (MN869923), *PIHEMA* (MN869924), *PIPPO* (MN869925), *PICHLI* (MN869926), *PICHLG* (MN869927), and *PINYC* (MN869928) were 1253 bp, 803 bp, 1045 bp, 1186 bp, 1108 bp, 1534 bp, 1357 bp, 1420 bp, 1197 bp, and 1438 bp, respectively. They contained a partial ORF of 1182 bp, 654 bp, 999 bp, 1134 bp, 1065 bp, 1524 bp, 1357 bp, 1272 bp, 1119 bp, and 1837 bp encoding 393, 217, 332, 377, 354, 507, 453, 423, 372, and 307 amino acid proteins, respectively. The high identity and similarity of these proteins was shown with related proteins from other plants (Supplemental Table S2).

Pigment biosynthesis-related gene expression

To further characterise the transformation mechanisms of color shift of *P. lactiflora* leaves, the expression of these 11 structural genes was examined by qRT-PCR. They were all expressed at three different leaf developmental stages, but their expression patterns were different from each other. In the flavonoid biosynthetic pathway, *PICHS* was expressed at the highest level, followed by *PIPAL*, *PIDFR*, and *PIANS*, whereas *PICHI* and *PIFLS* were expressed the lowest levels. During the three leaf developmental stages, the expression patterns of *PIPAL*, *PIFLS*, *PIDFR*, and *PIANS* demonstrated a decreasing trend, whereas that of *PICHS* first increased and then decreased and that of *PICHI* expressed a trend opposite to *PICHS*. Besides, *DFR* catalysed dihydroflavonols into leucoanthocyanidin, and then leucoanthocyanidin was catalysed by *ANS* to colored anthocyanidin. In this study, the expression level of *PIDFR* decreased sharply at the purple–green and green stage compared to that at purple stage which was reduced by 98.27%, 98.41%, and 97.62%, 98.20%, respectively (Fig. 3a).

Table 2 Pigment contents of *P. lactiflora* at three different leaf development stage

Category	Content (mg g ⁻¹ DW)		
	Purple stage (S1)	Purple–green stage (S2)	Green stage (S3)
Anthocyanins	0.937 ± 0.177 ^a	0.701 ± 0.129 ^b	0.439 ± 0.122 ^c
Flavonoids	6.719 ± 0.482 ^a	6.657 ± 0.849 ^a	6.225 ± 0.546 ^a
Chlorophyll <i>a</i>	3.495 ± 0.302 ^c	4.166 ± 0.285 ^b	4.597 ± 0.200 ^a
Chlorophyll <i>b</i>	1.220 ± 0.167 ^c	1.662 ± 0.294 ^b	2.227 ± 0.250 ^a
Chlorophylls	4.715 ± 0.451 ^c	5.828 ± 0.574 ^b	6.824 ± 0.397 ^a
Carotenoids	1.462 ± 0.058 ^a	1.679 ± 0.232 ^a	1.698 ± 0.109 ^a

The values represented mean ± SE, and different letters marked significant differences ($P < 0.05$)

Among the six genes in the chlorophyll biosynthetic pathway, the expression level of *HEMA* was the highest, whereas that of the other genes was lower. During the three leaf developmental stages, the expression levels of *PIHEMA* presented a sharp-upward trend; *PIPPO* and *PINYC* exhibited a decreasing tendency, whereas *PICHLI* and *PICHLG* attained the minimum level at the purple–green stage and then increased. The expression patterns of *PIHEMA* and *PINYC* were the most obviously changed during the leaf developmental stages. Specifically, the expression levels of *PIHEMA* increased by 4.36 and 11.66 times, from the purple–green stage to the green stage, and that of *PINYC* at the purple–green and green stage was reduced by 84.61% and 90.86% compared to the levels at the purple stage (Fig. 3b).

Discussion

Leaf color is a key trait of many ornamental plants, and has gained increasing attention for its commercial value. Plants that are always colorful or present red/yellow colors at spring or autumn bring great value to landscape viewing. *Paeonia lactiflora* is a traditional kind of flower in China, and in this research, we found that the new leaves of *P. lactiflora* presented purple color at spring, similar to *M. spectabilis* (Yang et al. 2018). With leaf development, the purple coloration gradually faded and finally the mature leaves presented green color. Leaf color is judged by the pigment concentration or distribution in plant leaves (Ewa 2009), and the new leaves presenting purple color are due to the red-colored pigments concentrated in leaf blade surfaces including the adaxial and abaxial surfaces compared to the mature green leaves. This was consistent with the colored pigment distribution in cabbage (*Brassica oleracea*) (Leahu et al. 2018), but was inconsistent with that in *Anthurium andraeanum* Lind. for the red-colored pigment randomly distributed in mesophyll cells resulting in pink colored leaves (Yang et al. 2015). Besides, increased red-colored pigments accumulated in the abaxial surface of leaf blade, whereas a decreased amount was deposited in the adaxial surface of the leaf blade. As a result, the new purple leaves

were due to the accumulation of red-colored pigments in the leaf surfaces, and the loss of the red-colored pigment and the increase in green colored pigment contributed to the green mature leaves.

There are three types of pigments in plant leaves including chlorophylls, carotenoids, and anthocyanins (Field et al. 2001). In general, a high concentration of chlorophylls contributes to the green coloration of leaves, whereas carotenoids and anthocyanins accumulation is responsible for leaves representing brighter colors (Carlson and Holsinger 2010). Specifically, anthocyanins, the components of flavonoids, determined whether the leaves appear red, blue, and purple (Winkel-Shirley 2001). Leaf coloration depends mainly on flavonoids/anthocyanins when their ratio to chlorophylls is high enough to dissimulate the green color (Shen et al. 2018). In this research, the concentration of flavonoids was higher in purple and purple–green leaves than that in green leaves, but that of anthocyanins was very low. The anthocyanins content showed a significant reduction along with the leaf color transformation from purple to green, which is consistent with the results in *M. spectabilis* (Yang et al. 2018). This was similar with the study on red and green colored leaves in *A. andraeanum* (Yang et al. 2015), *Pelargonium crispum* (Kanemaki et al. 2018), and *Camellia sinensis* L. (Wei et al. 2016; Shen et al. 2018) that the anthocyanin content was lower in green colored leaves than that in red-colored leaves. Combined with the anatomical observation, this suggested that the concentrated distribution of higher levels of anthocyanin in the leaf surface masks the green color of chlorophylls, resulting in the presentation of purple in new leaf coloration.

Anthocyanins, originate from a branch of the flavonoid biosynthetic pathway, and are water-soluble pigments belonging to secondary metabolites (Nguyen and Cin 2009). Anthocyanin accumulation is generally controlled by structural gene expression (Jaakola 2013). In *P. lactiflora*, the structural genes involved in the anthocyanin biosynthetic pathway can be distributed into two groups: the upstream biosynthesis genes (*PIPAL*, *PICHS*, *PICHI*, flavanone 3-hydroxylase (*PIF3H*), flavonoid 3'-hydroxylase (*PIF3'H*) and *PIFLS*), and the downstream biosynthesis

Fig. 3 Expression patterns of genes associated with pigment biosynthesis in *P. lactiflora* leaves at three developmental stages. **a** Expression patterns of genes involved in flavonoids biosynthesis. **b** Expression

patterns of genes involved in chlorophyll biosynthesis. S1, purple stage, S2, purple–green stage, S3, green stage

genes (*PIDFR*, *PIANS*, UDP-glucoside (*PIUGT*) and methyl transferase (*PIMT*)) (Zhao et al. 2012b). In *C. sinensis*, the expression levels of these genes including *PAL*, *CHI*, *F3H*, *F3'H*, *FLS*, *DFR*, and *ANS* were all higher in red leaves than in green leaves, with higher accumulation of anthocyanins (Shen et al. 2018). Also, Liu et al. (2016) found that *PAL*, *CHS*, *CHI*, *F3'H*, *DFR*, *ANS*, *UFGT*, and *OMT* were expressed at a higher level in red leaves with more anthocyanins content than in the green leaves of jew (*Tradescantia fluminensis*). However, the expression level

of *CHS* was demonstrated a lower level in *A. andraeanum* (Yang et al. 2015) in pink leaves compared to green leaves, which showed a result opposite to that of Liu et al. (2016). In this research, a similar result was achieved, wherein *PIPAL*, *PICHS*, *PICHI*, *PIFLS*, *PIDFR*, and *PIANS* demonstrated the highest expressions at S1 except for *CHS*, which was increased from the purple to purple–green stage and was decreased from the purple–green to green stage. Combined with the dissimilar expression patterns of *PICHS*, the block of anthocyanidin biosynthesis might be less likely relevant

to *PICHS* expression despite the highest expression level. Among the remaining genes, successful leaf color alteration by genetic transformation using *PIDFR*, *PIFLS*, and *PIANS* has already been reported as follows. In *M. spectabilis*, over-expression of *McDFR*, or silencing of *McFLS*, promoted anthocyanin accumulation and resulted in leaves presenting red color. On the contrary, it resulted in *M. spectabilis* leaves appearing green (Tian et al. 2015). Through silencing *ANS* in a red-leaved *Malus sp.* cultivar, anthocyanin biosynthesis was almost completely blocked and this was accompanied by a reduction in the profile of anthocyanin (Szankowski et al. 2009). Considering the lower expression of *PIFLS*, the enzymatic activities of *PIDFR* and *PIANS* resulted in the *P. lactiflora* leaves demonstrating red coloration, and a block in anthocyanin biosynthesis from purple young leaves to green mature leaves, resulted in reduced anthocyanin accumulation.

The variation of *P. lactiflora* leaf color from purple to green might also be owing to changes in chlorophylls. Chlorophyll is the primary photosynthetic pigment, and leaves appear green mainly due to high concentrations of chlorophyll (Shen et al. 2018). In *C. sinensis*, Shen et al. (2018) found that more chlorophyll *a*, chlorophyll *b*, and total chlorophyll were accumulated in the green colored leaves compared to the red-colored leaves, which was accordance with the report by Liu et al. (2016) in *T. fluminensis* leaves. In our study, we reached a similar result showing that during leaf development, or, with the variation of leaf coloration from purple to green, accumulation of chlorophyll was significantly increased. This result suggested that the accumulation of chlorophyll contributed to a higher concentration of chlorophyll, leading to the leaves appearing green during leaf development.

In plants, chlorophyll content is affected by the development and division of chloroplasts, and their biosynthesis and degradation (Zhang et al. 2018). The chlorophyll biosynthesis pathway is complex and involves more than 20 genes encoding 16 enzymes (Beale 2005). Leaves appearing green might change due to the expression changes of chlorophyll biosynthetic-related genes. The *HEMA* gene catalyses the initial step of chlorophyll biosynthesis, which plays a vital role in chlorophyll production (Kumar and Söll 2000). Changes in the expression level of *HEMA* might promote the biosynthesis of chlorophylls and add to the accumulation of chlorophylls in green leaves compared to that in the purple leaves in the *C. sinensis* cv. 'Zijuan' (Shen et al. 2018). In this study, a similar result was obtained and the expression level of *PIHEMA* was the highest among chlorophyll biosynthesis-related genes, suggesting that the increased expression of *PIHEMA* promoted the synthesis of chlorophyll and increased the content of chlorophyll in leaves. *PPO* controlled the oxidation of protoporphyrinogen IX and affected the feedback control of chlorophyll

biosynthesis (Koach et al. 2004). The chelation of Mg^{2+} into protoporphyrin IX was catalysed by *CHLD*, *CHLH*, and *CHLI*, which encode Mg-chelatase subunits. *CHLG* controls the final step of chlorophyll biosynthesis. All these genes play an important role in the biosynthesis of chlorophylls, and their deficiency results in yellow—green-leaf mutations (Wu et al. 2007; Deng et al. 2014; Zhang et al. 2015). However, *CHLH* was expressed lower in green leaves compared to that in red leaves (Shen et al. 2018). A similar result was also shown in *CHLG* expression patterns, that a higher level was observed in light-green leaves than in green leaves (Zhu et al. 2015). The opposite result was also reached in this study showing that the expression level of *PIPO*, *PICHLI*, and *PICHLG* demonstrated a decreasing trend from the purple-leaf stage to green-leaf stage, but that their expression was lower than *PIHEMA*. Considering the increased chlorophyll content and that the leaf appeared green, it is reasonable to conclude that the initial stage of chlorophyll biosynthesis was significantly enhanced and was then partially hampered, still accompanied with chlorophyll accumulation. Moreover, the expression level of *PINYC* was notably reduced during leaf development in this research. *NYC* is involved in chlorophyll degradation, catalysing the degradation of chlorophyll *b* (Lai et al. 2015). An increase in the concentration of chlorophyll *b* was observed in *NYC* overexpressing plants (Sato et al. 2009; Jia et al. 2015) and a higher expression level was observed in gold-green leaves compared to that in green leaves (Li et al. 2018). Thus, an increase in chlorophyll accumulation might also be related to the chlorophyll degradation block, resulting in the leaves appearing green.

Apart from anthocyanins and chlorophylls, carotenoids are plant pigments that are responsible for the range from red to yellow color in flowers and fruits (Su et al. 2014). The changes in carotenoid content between red and green leaves were not consistent. For example, a higher level of carotenoid content was reported in leaves with green coloration compared to that in leaves with purple—red coloration in *A. andraeanum* and *T. fluminensis*, whereas an opposite change was observed in *C. sinensis* (Shen et al. 2018). In this study, the result was similar to that in *A. andraeanum* and *T. fluminensis* (Yang et al. 2015; Liu et al. 2016), but did not reach a significant level. As a whole, the result in this study suggests that a high level of anthocyanin content resulted in purple—red-colored new leaves, and that lack of anthocyanin accumulation along with increased chlorophyll accumulation contributed to the variation in leaf color at the physiological level.

In conclusion, we investigated the coloration variation of *P. lactiflora* leaves from purple color to green color with the development of leaves in this study. Higher anthocyanin contents were observed to be concentrated in the surface of the purple leaves at S1. With the development of leaves, the

contents of anthocyanin decreased and the leaves appeared green. Furthermore, qRT-PCR experiments showed that the highest expression of anthocyanin biosynthesis-related genes at the purple stage contributed to high anthocyanin concentration, masking the green coloration of chlorophylls resulting in leaves representing purple coloration at S1. Reduced expression of these genes during leaf development resulted in anthocyanin reduction in the leaves. Increased chlorophyll biosynthesis and weakened chlorophyll degradation contributed to higher chlorophyll contents, resulting in green coloration of leaves. Our findings thus provide insights into the molecular mechanism underlying the coloration variation in plants.

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Authors' contributions JT and DZ planned and designed the experiments. YT, ZF and ML performed the experiments. YT analyzed the data and wrote the manuscript. All authors carefully read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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