Morphological and Biochemical Studies of the Yellow and Purple–red Petal Pigmentation in *Paeonia delavayi*

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Abstract. *Paeonia delavayi* is a species endemic to Southwest China and an important genetic resource for flower color breeding of tree peonies. The mechanisms underlying the flower coloration of this plant have not been fully elucidated. In this article, the petals of yellow-colored individual (PI) and purple–red-colored individual (Pd) of *P. delavayi* were studied. And anatomical observations revealed that a large amount of yellow protoplasts and a small amount of colorless protoplasts were located in the yellow-colored PI petals, whereas a mixture of purple, red, and pink protoplasts were observed in the purple–red-colored Pd petals. The PI cells were subrotund and flat, whereas the Pd cells were irregularly polygon-shaped and bulging. Chemical analyses were performed, and the results indicated that significant differences occurred between the cell sap pH of the PI and Pd flowers and large differences occurred in the contents of Fe and Al between PI and Pd. Cyanidin- and peonidin-based anthocyanins with flavones and flavonols as copigments determined the Pd flower color, whereas chalcone 2'-G with apigenin 7-O-neohesperidoside and chrysoeriol 7-O-glucoside as copigments determined the yellow color of PI. Correspondingly, the genes dihydroflavonol 4-reductase (*DFR*) and anthocyanidin synthase (*ANS*) were significantly highly expressed in Pd, whereas chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavone synthase (*FNS*), flavonol synthase (*FLS*), flavonoid 7-O-glucosyltransferase (*7GT*), and 2'-4'-6'-4-tetrahydrochalcone 2'-glucosyltransferase (*THC*) had high transcript levels in PI relative to Pd. The results indicate that the color variation of *P. delavayi* petals may be related to a delicately controlled balance of the aforementioned factors.

Paeonia delavayi is an unique wild tree peony species with variously colored individuals including yellow, orange, red, dark red, and purple–red occurring both within and between populations, and the yellow colored individuals are considered the most precious resource for cultivar breeding (Hong and Pan, 1999, 2005a, 2005b, 2007; Hong et al., 1998; Li et al., 2011). Moreover, the

antioxidant activity of the yellow-colored flowers of *P. delavayi* is higher than that of other yellow flowers, and this activity indicates its potential for use in drug development or functional food development (Li et al., 2009).

In certain species, flavonoids, particularly anthocyanidins, are the primary contributors to the different coloration of flowers (Tanaka and Ohmiya, 2008; Suzuki et al., 2016). To date, more than 30 different flavonoids, including anthocyanins and multifunctional glycosides of flavones and flavonols, have been identified and quantified from different groups and several wild species of tree peony

(Hosoki et al., 1991; Wang et al., 2001a, 2001b, 2005; Zhang et al., 2007). Wang et al. (2001a) analyzed the composition and content of flower pigments of seven wild tree peonies and found that pelargonidin (Pg)-based anthocyanins were not detected in any accessions in the subsection Delavayanae and that the purple flowers of *P. delavayi* primarily contained peonidin-3, 5-glucosides (Pn3G5G). Zhou et al. (2011) investigated the pigment composition of the yellow petals of *P. delavayi* wild population in Yunnan Province, China, and found that the primary components were chalcone, flavone, and flavonol, including isosalipurposide, kaempferol (Km), quercetin (Qu), isorhamnetin (Is), chrysoeriol (Ch), and apigenin (Ap) glycosides. In addition, reports have indicated that the petal epidermis cell shape can affect flower color. Conical cells lead to darker flower colors and improved color saturation. On the contrast, flat cells lead to lighter flower colors. For example, the magenta flower color of *Antirrhinum majus* changes to pink when conical epidermal cells are flattened (Noda et al., 1994). The length and arrangement of *Iris tectorum* petal epidermal cells also have certain influence on the flower color (Yoshida et al., 1995), and epidermal cells with protruding papillae can generate a velvet sheen on the petals as in *Anagallis monelli* L. (Quintana et al., 2007). In tree peony, the epidermal cells of *Paeonia rockii* and *Paeonia ostii* were all elongated and explanate (Shi et al., 2017). Flower coloration is also linked to metal ions and vacuolar pH differences, such as in *Tulipa gesneriana* (Momonoi et al., 2009; Shoji et al., 2007, 2010), *Hydrangea macrophylla* (Ito et al., 2009; Schreiber et al., 2010, 2011), and *Ipomoea tricolor* (Yoshida et al., 2005, 2009a, 2009b). Thus, we can infer that the variously colored flowers of *P. delavayi* are the result of certain physiological differences. However, the precise mechanisms involved remain uncertain, and comprehensive studies of color formation in *P. delavayi* or other tree peony flowers have not yet been reported.

In the present work, the major factors underlying the formation of various colorations of *P. delavayi* were investigated in individuals with purple–red (Pd) and yellow flowers (PI) from a wild population in Yunnan Province, China, and the anatomy of morphology of petals, protoplast features, and pigment compositions were analyzed. Cell sap pH and metal ion contents of the petals were also investigated. To the best of our knowledge, this work is the first comprehensive exploration of the formation of flower color in *P. delavayi*, and the analysis may provide additional insights into the mechanism of color formation in tree peonies.

Materials and Methods

Plant material. The yellow-colored individuals (PI) and purple–red individual (Pd) of *P. delavayi* were introduced from

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a wild population in Shangri-La County (lat. 27°57'N, long. 99°35'E), Yunnan Province, China, and grown in the germplasm repository of Northwest A&F University, Shaanxi, China, under the same conditions with sufficient light and water (Fig. 1). The flowers opened during five developmental stages (from Stage 1 to Stage 5, i.e., from S1 to S5) as in the previous study of Shi et al. (2015). The petals were picked from five different individuals of PI and Pd during five developmental stages and immediately frozen in liquid nitrogen for the total content measurement of pigments. To measure the pigment contents and conduct gene expression analyses, the petal samples were separately detached at the fully opened stage with exposed anther and immediately frozen in liquid nitrogen and stored at -80 °C before analysis. In addition, petals at the fully opened stage were separately detached and used immediately for the morphological and anatomical observations, protoplast preparation, and pH and metal ion measurements.

Petal color measurement. The petal color at the fully opened stage was determined by comparison with the Royal Horticultural Society Color Chart (RHSCC) and then measured with a chroma meter (CR-400; Konica Minolta Sensing, Inc., Osaka, Japan) using five color parameters, including the indices of lightness (L^*), chromatic components (a^* , b^*), brightness (C^*), and hue angle (h) (Zhang et al., 2007). Six replications were recorded. The mean values and SDS were obtained.

Microscopic observations of the epidermal cells and transverse sections. According to the method of Shi et al. (2017), fresh petals of PI and Pd flowers at the fully opened stage were cross-sectioned with the help of razor blade. The upper and lower epidermal layers were peeled off using a razor blade. The layers were placed onto a glass slide with a drop of water and then immediately observed under a light microscope (BX43; Olympus, Tokyo, Japan) equipped with a DS cooled camera head with FNIS-Elements image processing software.

Scanning electron microscopy observations. The PI and Pd petals of *P. delavayi* were spliced into 5 × 5 mm blocks, fixed in FAA buffer (38% methanol, 5 mL; acetic acid, 5 mL; 70% ethanol, 90 mL) under vacuum pressure for 30 min, and then incubated at 4 °C overnight. Next, the fixed petals were dehydrated in an ascending aqueous ethanol series (30, 50, 70, 80, 90, and 100%), followed by 100% acetone and then treated twice with isopentyl acetate for 30 min. Finally, the samples were dried using a CO₂ supercritical drying technique (K850; Quorum, England), mounted on a specimen stub, and sputter-coated with gold before examination in a scanning electron microscope (SU-3400N; Hitachi Ltd., Japan).

Preparation of free protoplasts. Free protoplast was prepared from PI and Pd fresh petals at the fully opened stage according to Qi et al. (2013) with slight modifications. The middle part of fresh petals was cut into 1 mm filaments using a blade and then placed into 20 mL of enzymolysis solution (0.3 g of 1.5% (w/v) cellulase R-10, 0.06 g of 0.3% (w/v) macerozyme R-10, 2.1869 g of 600 mM mannitol, 0.04264 g of 10 mM MES, 8 µL of 1 mM CaCl₂, 7 µL of 5 mM β-mercaptoethanol, and 0.02 g of 0.1% (w/v) BSA; pH 5.7). After vacuum infiltration for 30 min without shaking, the enzymolysis solution with petal strips was shaken for 1 h at 80 rpm and 25 °C to release the protoplasts. The reaction mixture containing free protoplasts was filtered through a 75-µm nylon mesh and washed with buffer (0.017 g of 4 mM MES-Tris, 2.1869 g of 600 mM mannitol, and 0.0298 g of 20 mM KCl; pH 5.7), then centrifuged in 20-mL round-bottomed tube. As much supernatant liquid as possible was removed, and the protoplast pellet was resuspended in 2 mL of fresh buffer by gentle swirling. The centrifugal separation/resuspension procedure was repeated two times, and the released protoplasts were examined under a microscope (BX43; Olympus, Tokyo, Japan).

Fig. 1. Cellular features of a yellow-flowered individual (PI) and a purple-red-flowered individual (Pd) of *Paeonia delavayi*. Fully opened flowers of PI (A) and Pd (E) of *P. delavayi*. Cross sections of petals of PI (B) and Pd (F) of *P. delavayi*. Epidermal cells of upper petals: epidermal cells of PI (C) and Pd (G) petals. Scanning electron micrograph of epidermal cells from the outer epidermis of petals of PI (D) and Pd (H). Bars = 100 µm.

Table 1. Primers used for quantitative real-time polymerase chain reaction analysis.

Gene name	Unigene ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
CHS	Unigene45464_All	CAATCATGGCAATTGGAACA	GCTCGACCTTGTCTCACTG
CHI	CL7622.Contig1_All	AACAACCTTTCCGAGGCTGTG	CCTAGCTGCCAACTCTGTCT
DFR	CL1376.Contig6_All	GATGCCGAAACCGTGTGT	AAATGCTTCACCTTCTCTGT
F3H	CL2583.Contig1_All	CTTCGCTCTACCGCCTGAT	CAATCTCGCACAGCCTCTC
3GT	CL4890.Contig2_All	GGGAGATAGAGAAAGGAGTGAGG	GAAGAACCACCATCCACCAG
5GT	Unigene16716_All	ATCAAGCCACGAATGCCAAGC	AATCAACCACCTTCCCCACCTC
7GT	CL11011.Contig1_All	CCTATCCGACACAAGGCAAT	TACGCAGAGATGCTGGTTG
THC2'GT	Unigene18441_All	CATCTTGTATCGGCAGTGGA	TCTGTCTGGGTTGAGAAGG
FNS	CL8830.Contig1_All	TTCATCCCAGCACACACACT	ACCCTCACAGCACTCCAAGA
FLS	Unigene8606_All	AGAAGCAGTGGGTGGTGAAG	ATGGGTGAGGGTGGACAAATC
ANS	Unigene13390_All	ACCAAGAGGCTGGGAAGATT	CAGGGAAGACAAGGTGAAG

Table 2. Petal colors and color parameters of *Paeonia delavayi*.

Materials	Petal color	RHSCC	CIEL*a*b* color coordinates				
			L^*	a^*	B^*	c^*	h
PI ²	Yellow	4A	93.256 ± 0.035	43.526 ± 0.014	56.188 ± 0.021	94.8 ± 0.038	90.418 ± 0.032
Pd ³	Deep purple	185A	48.565 ± 0.016	81.295 ± 0.041	15.061 ± 0.008	3.949 ± 0.011	47.208 ± 0.015

²PI, yellow-colored flower of *P. delavayi*.

³Pd, purple-red flower of *P. delavayi*.

Metal ions measurement. The petals of PI and Pd flowers at fully opened stage were rinsed in sterile water and then placed in a drying oven at 105 °C for 30 min, followed by 80 °C for 6 h. Dried petals were finely ground to a powder, and then, 2 mg of dried powder was digested in 5 mL HNO₃ and 1 mL H₂O₂ and then treated in a high performance microwave digestion unit (ICAP Qc; Thermo Fisher Scientific, Waltham, MA) using the procedure of Qi et al. (2013). After complete digestion and acid removal, the samples were diluted with double-distilled water and then analyzed the contents of metal ions using ICP-MS (ICAP Qc; Thermo Fisher Scientific). The mean values and sds were obtained from three biological replications.

Petal vacuolar pH measurement. To measure the vacuolar pH, 2 g of fresh petals of the PI and Pd flowers at the fully opened stage was ground in liquid nitrogen and centrifuged at 18,407 g_n for 5 min. The pH of the supernatant was measured using a flat pH tester (pH5F; Shanghai Sanxin Instrument Corporation, China) at 25 °C (Zhao et al., 2016). The average pH and sds of each flower color was calculated from 10 replications.

Flavonoid and anthocyanidins analysis. The petals of Pd and PI flowers at five different development stages were lyophilized using the lyophilizer (LGJ-25C; Beijing Four-ring Science Instrument Plant Corporation, China). Then, a total of 10 mg of lyophilized petal powder from 10 individual PI and Pd flowers were extracted in 1 mL of 0.1% acetic acid/methanol solution at 4 °C overnight and then centrifuged for 10 min at 10,000 rpm. The supernatant was collected and dried with a vacuum centrifuge concentrator (CV100-DNA; Aijimu, Beijing, China). The identification and quantification of flavonoid and anthocyanin compounds were performed with an ultrahigh performance liquid chromatography–mass spectrometry system (UPLC-MS/MS; Waters, Milford, MA) coupled to a triple quadrupole mass spectrometry system (XEVO®-TQ; Waters) with electrospray ionization (ESI) based on previous methods (Veberic et al., 2015) with modifications. The anthocyanin and flavonoid contents

were measured semiquantitatively according to the method reported by Shi et al. (2017). The mean values and sds were obtained from three biological replications.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted from the fully opened petals of the PI and Pd flowers using Trizol (Invitrogen, Carlsbad, CA). After verifying the RNA purity and integrity, 1 mg of total RNA was reverse-transcribed to first-strand cDNA with a PrimeScript RT reagent kit (Takara, Otsu, Japan). The qRT-PCR assay was conducted using a fluorescent intercalating dye on a Light Cycler 480 with SYBR Green I Master Mix (Roche, Mannheim, Germany), following the procedure of Shi et al. (2017). Specific primers were designed using Primer Premier software (Table 1). The relative expression levels of genes in the petals of the PI and Pd flowers were normalized to the *TUB* gene expression level in the same sample. All reactions were conducted with three biological replications and three technical replications.

Results

Color indices. According to the RHSCC, the colors of Pd and PI flowers were purple–red (185A) and yellow (4A), respectively (Fig. 1A and E; Table 2). The higher L* value in PI indicated that PI petals had a lighter color than those of Pd. The lower a* value in PI than in Pd indicated that the green level was higher in PI, whereas the redness level was higher in Pd. The higher b* value in PI than in Pd indicated that the yellow level was higher in PI, whereas the blue level was higher in Pd. C* represents the saturation of the color, and the higher C* value for PI than for Pd indicated that PI had a much more vivid color, whereas Pd had a grayer color. The hue angle (*h*) of ≈90° in the PI petals represented yellow and the *h* of ≈47° in the Pd petals mean red.

Anatomical analysis. In PI, the yellow cells were located in upper epidermis (Fig. 1). In addition, sporadically colored

cells were in the palisade mesophyll and spongy mesophyll (Fig. 1B). In Pd, the colored cells were primarily located in the upper and lower epidermis and not in the palisade mesophyll and spongy mesophyll (Fig. 1F). Notably, the degree of pigmentation varied slightly between the upper and lower epidermis. To further clarify the mechanism for the various flower colorations of *P. delavayi*, the colored protoplasts were analyzed. A large amount of yellow protoplasts and a small amount of colorless protoplasts were located in the yellow-colored PI petals. However, the purple–red-colored flower was composed of a mixture of purple, red, and pink protoplasts (Fig. 2). These results were in line with the observations from transverse sections.

The shape of the epidermal cells can influence the visual effect of the color. In this study, the PI cells were subrotund and flat, whereas the Pd cells were irregularly polygon-shaped and bulging (Fig. 1C, D, G, and H). These findings suggested that the various flower colors of *P. delavayi* were partly associated with different epidermal cell shapes.

Metal ions and pH analysis. To determine whether metal ions had an effect on flower color formation of *P. delavayi*, eight metal elements (Ca, Mg, Fe, Zn, Cu, Al, Cd, and Mn) related to color formation were measured using inductively coupled plasma mass spectrometry in PI and Pd petals (Yoshida et al., 2009a, 2009b; Qi et al., 2013). As shown in Table 3, the two most abundant metal ion elements in the PI and Pd petals were Ca and Mg, which were followed by Fe and Al. Except for the contents of Fe and Al, which were much higher in Pd than in PI, the other metal elements did not show differences between the PI and Pd petals.

Besides, the pH of the PI and Pd petals was 5.76 ± 0.049 and 4.61 ± 0.015, respectively (Table 4), and the difference was significant. Thus, the sap pH might contribute to the formation of different flower colors.

Qualitative and quantitative analysis of pigments. For tree peony, flavonoids, particularly anthocyanins, are responsible for various flower colors such as white, yellow, red, pink, purple, and so on (Hosoki et al., 1991; Wang et al., 2001a, 2001b, 2005; Zhang et al., 2007; Zhou et al., 2011). The pigment composition and content were measured in the fully opened flowers of PI and Pd using UPLC-MS. According to the ultraviolet-visible absorption characteristics, anthocyanins, flavonol, and chalcone were detected under the wavelength of 520 and 350 nm, respectively (Fig. 3). At 520 nm, no anthocyanins were found in PI, whereas five

Fig. 2. Protoplasts from yellow-flowered individual (PI) and purple–red-flowered individual (Pd) of *Paeonia delavayi*. Bars = 30 μm.

Table 3. Concentration of metal elements in *Paeonia delavayi* petals.

Materials	Concn of metal ions (mg·kg ⁻¹)							
	Ca	Mg	Fe	Zn	Cu	Al	Cd	Mn
PI ^z	1,614.56 ± 45.712	1,088.47 ± 27.655	51.185 ± 5.075	35.308 ± 3.290	8.766 ± 0.420	37.170 ± 3.648	0.024 ± 0.002	6.140 ± 0.312
Pd ^y	1,636.22 ± 47.236	1,075.22 ± 25.761	64.497 ± 6.434	39.271 ± 3.872	8.166 ± 0.401	55.806 ± 5.512	0.028 ± 0.003	6.633 ± 0.325

^zPI, yellow-colored flower of *P. delavayi*.

^yPd, purple–red flower of *P. delavayi*.

Table 4. pH value and concentrations of flavonoid in *Paenonia delavayi* petals.

Materials	pH	Anthocyanidin (mg·g ⁻¹)					Flavonol (mg·g ⁻¹)					Chalcone (mg·g ⁻¹)				
		S1 ^z	S2 ^z	S3 ^z	S4 ^z	S5 ^z	S1	S2	S3	S4	S5	S1	S2	S3	S4	S5
Pl ^y	5.76 ± 0.049	n.d.	n.d.	n.d.	n.d.	n.d.	0.755 ± 0.084	0.871 ± 0.096	1.073 ± 0.112	1.004 ± 0.106	0.867 ± 0.092	1.001 ± 0.095	1.060 ± 0.102	1.326 ± 0.185	1.306 ± 0.135	1.142 ± 0.122
Pd ^x	4.61 ± 0.015	0.125 ± 0.02	0.330 ± 0.045	0.500 ± 0.052	1.207 ± 0.121	1.252 ± 0.125	0.400 ± 0.051	0.593 ± 0.064	0.748 ± 0.075	0.849 ± 0.0892	0.791 ± 0.0795	0.267 ± 0.026	0.487 ± 0.053	0.463 ± 0.048	0.396 ± 0.045	0.231 ± 0.024

^zS1, S2, S3, S4, S5 indicate the five different opened stages.^yPl, yellow-colored flower of *P. delavayi*.^xPd, purple-red flower of *P. delavayi*.

obvious peaks (a1, a2, a3, a4, and a5) were detected in Pd, among which a3 and a5 were the highest. On the basis of the maximum absorption wavelength (λ_{max}) and the fragment ions (m/z), the five peaks at 520 nm were presumed to be cyanidin 3, 5-di-*O*-glucoside (Cy3G5G), Pn3G5G, cyanidin 3-*O*-glucoside (Cy3G), cyanidin 3-arabinoside (Cy3A), and peonidin 3-*O*-glucoside (Pn3G), respectively (Table 5). Meanwhile, we detected many of the same but also different flavonoids at 350 nm in these two flower types (Fig. 3; Tables 5 and 6). The same flavonoids identified in these two flowers included a Qu derivative, Km 3, 7-di-*O*-hexoside, Is 3-*O*-glucoside, and chalcononaringenin 2'-*O*-glucoside (chalcone 2'-G). Is 3, 7-di-*O*-glucoside, luteolin (Lu) 7-*O*-glucoside, Ap 7-*O*-neohesperidoside, and Ch 7-*O*-glucoside were only detected in Pl, whereas the Pd petals included Km 3-*O*-glucoside, Is 3-*O*-gallcylglucoside, and Km 7-*O*-glucoside (Tables 5 and 6). These results indicated that the Ap-, Ch-, and 7-*O*-glucoside flavonols were the primary copigments responsible for the yellow coloration and the anthocyanins were responsible for the purple-red coloration.

Concerning the pigment content, chalcone 2'-G accumulated most abundantly in Pl and was 5-fold higher than that in Pd. Is 3-*O*-glucoside and Lu 7-*O*-glucoside were also abundantly accumulated in Pl at ≈ 348 and $337 \mu\text{g}\cdot\text{g}^{-1}$, respectively; however, the contents of Ap 7-*O*-neohesperidoside and Ch 7-*O*-glucoside were low at 20 and $53 \mu\text{g}\cdot\text{g}^{-1}$, respectively (Table 6). For Pd, the most abundant anthocyanin was Cy3G ($631 \mu\text{g}\cdot\text{g}^{-1}$), followed by Pn3G ($303 \mu\text{g}\cdot\text{g}^{-1}$), Pn3G5G ($160 \mu\text{g}\cdot\text{g}^{-1}$), and Cy3G5G ($102 \mu\text{g}\cdot\text{g}^{-1}$). In addition, Cy3A was hardly detectable ($8 \mu\text{g}\cdot\text{g}^{-1}$). Interestingly, among the identified flavonols in Pd, chalcone 2'-G was the most abundant ($395 \mu\text{g}\cdot\text{g}^{-1}$), followed by Qu 7-*O*-glucoside ($228 \mu\text{g}\cdot\text{g}^{-1}$) and Is 3-*O*-glucoside ($156 \mu\text{g}\cdot\text{g}^{-1}$) (Table 5). The total content of anthocyanins was rising during development of Pd flowers, whereas the total contents of flavonols and chalcone accumulated without any rules during Pd flower development. However, the total contents of flavonols and chalcone in Pl were always higher than those in Pd at all the developmental stages (Table 4).

Expression analysis of key flavonoid biosynthetic genes. To further investigate the effect of the flavonoid biosynthetic genes on the various flower colorations of *P. delavayi*, qRT-PCR was conducted to test the expression patterns of 12 related structural genes in fully opened petals of Pl and Pd (Fig. 4; Table 1). The 11 genes were *CHS*, *CHI*, *DFR*, *F3H*, *FNS*, *FLS*, anthocyanin 3-*O*-glycosyltransferase (*3GT*), anthocyanin 5-*O*-glycosyltransferase (*5GT*), *7GT*, *THC*, and *ANS*, which are involved in flower pigmentation of *P. delavayi* (Shi et al., 2015). *CHI*, *F3H*, *FNS*, *FLS*, *7GT*, and *THC* were significantly highly expressed in Pl relative to Pd. In particular, *FNS* was barely detected in Pd (Fig. 4). Moreover, the expression patterns of these genes were in agreement with the accumulation levels of the corresponding

flavones and flavonols. However, *CHS*, *DFR*, *3GT*, *5GT*, and *ANS* were expressed strongly in the Pd petals (Fig. 4). Among these genes, the expression of *CHS*, *DFR*, and *ANS*, which are the key enzymes for anthocyanin synthesis (Tanaka and Ohmiya, 2008; Suzuki et al., 2016), was significantly higher in the Pd petals than in the Pl petals. These results demonstrated that the different flower colorations of *P. delavayi* were caused by the different expression patterns of different flavonoid biosynthetic genes, which led to the corresponding differences in the accumulation levels of different pigments.

Discussion

Paenonia delavayi is a special wild tree peony species and has the most previous resource of yellow flowers. The flower coloration involves many factors, such as the petal structure, pigment type and distribution, vacuolar pH, and metal ion complexation (Zhao and Tao, 2015). The shape of epidermal cells affects the pigmentation, with conical cells leading to darker flower color and enhanced color saturation (Noda et al., 1994). In the present study, the petal epidermal cells were different between the yellow-colored Pl and purple-red-colored Pd. As expected, the epidermal cells of Pd were bulging and, therefore, had darker color. Notably, in previous work, we found that the shapes of the petal epidermal cells were different between different tree peony species, with those of the dark purple-colored petals of *P. rockii* elongated and explanate and those of purple-red-colored individuals of *P. delavayi* irregularly polygon-shaped and bulging (Shi et al., 2017). Differences in the shape of the petal epidermal cells also affect the texture of petals and ultimately affect pollinator attraction (Glover, 2000). In wild populations, bees are the most important pollinators for *P. delavayi*, whereas abiotic factors are the most important for *P. rockii* (Li et al., 2013b).

Moreover, anthocyanin can change in relation to the cell sap pH, which affects flower color (Quintana et al., 2007). Because of an increased pH, the reddish-purple petals of *I. tricolor* cv. Heavenly Blue change to blue (Asen et al., 1977). The same results are found in *Petunia hybrid* (Griesbach, 1996). The stability of anthocyanins is greatly influenced by pH and decreases with an increase in pH (Zhao et al., 2005), whereas flavone and flavonol tend to become more yellow as the vacuolar acidity weakens (Fu et al., 2013). Similarly, the pH values in the yellow-colored individuals of *P. delavayi* were much higher than those in the purple-red-colored individuals. Thus, pH might be related to the different flower coloration of *P. delavayi*. Metal ions affect the pigment structure and thus the flower color. The pale yellow flower of *Tagetes patula* changes to golden yellow when treated with alums, to deep orange when treated with Cr, and to brown when treated with Cu, and these changes are considered to be the result of

Fig. 3. Ultrahigh performance liquid chromatography (UPLC) chromatograms of yellow-colored individual and purple-red-colored individual of *Paeonia delavayi* flavonoids at 350 nm and anthocyanins at 525 nm at the fully opened stage (S5). (A) UPLC chromatograms of yellow-colored individual; (B) UPLC chromatograms of purple-red-colored individual at 350 nm; (C) UPLC chromatograms of purple-red-colored individual at 520 nm; f1–f7 indicated identified flavonoids compositions; a1–a4 indicates identified anthocyanin compositions.

a shift between flavonoids and Qu (Li et al., 2013a). Mg^{2+} can increase the content of anthocyanin, thereby enriching flower colors (15% to 70%), such as in the red flower of *Anigozanthos flavidus*, the blue bracts of *Limonium sinuatum*, the pink flowers of *Gypsophila elegans*, and the blue flowers of *Aconitum carmichaelii* (Nissan-Levi et al., 2007). The blue color of *H. macrophylla* is derived from Al^{3+} -complexed anthocyanin and copigments (Ito et al., 2009; Takeda et al., 2005; Yoshida et al., 2003), whereas the sky-blue color of *Meconopsis grandis* and *Centaurea cyanus* is caused by Mg^{2+} - and Fe^{3+} - complexed anthocyanins (Kondo et al., 1994, 1998; Shiono et al., 2005; Takeda et al., 2005; Yoshida et al., 2006; Yoshida and Negishi, 2013). The blue coloration at the bottom of the inner perianth of *T. gesneriana* is attributed to Fe^{2+} (Momonoi et al., 2009; Shoji et al., 2010). In red and yellow petals of *Paeonia lactiflora*, K, Ca, Mg, Mn, and Zn were abundant and showed significant correlation coefficient with the color indice *h* (Zhao et al., 2017). Although Ca, Mg, Fe, and Al were the most abundant metal ions in the Pd and Pl petals, only Fe and Al showed

Table 5. Contents of identified flavonoids and anthocyanins in purple-red-colored individual petals of *Paeonia delavayi* at the fully opened stage (S5) ($\mu g \cdot g^{-1}$).

No. ^z	Identification	λ_{max} (nm)	ESI-(+)-MS (m/z)	Content ($\mu g \cdot g^{-1}$)
f1	Km 3-O-glucoside	347, 254	287, 449	45.056 ± 0.041
f2	Km 3,7-di-O-hexoside	269	287, 449, 633	80.938 ± 0.071
f3	Qu 3,7-di-O-glucoside	256	301, 463, 641	139.174 ± 0.121
f4	Qu 7-O-glucoside	256	303, 487	228.891 ± 0.150
f5	Is 3-O-galloylglucoside	348	317, 653	68.293 ± 0.058
f6	Km 7-O-glucoside	266	287, 471	130.265 ± 0.116
f7	Is 3-O-glucoside	254	317, 501	156.024 ± 0.139
c1	chalcone 2'-G	365	273, 457	395.855 ± 0.311
a1	Cy3G5G	509, 287	449, 287	102.196 ± 0.083
a2	Pn3G5G	515, 287	301, 463, 625	160.076 ± 0.130
a3	Cy3G	509, 287	287, 449	631.384 ± 0.561
a4	Cy3A	509, 287	287, 419	8.592 ± 0.008
a5	Pn3G	515, 287	301, 463	303.992 ± 0.256

^zf1–f7, c1, and a1–a5 indicate the order of the identified flavonoids in yellow-colored flowers of *P. delavayi*.

large differences between Pl and Pd, indicating that the different flower colors of *P. delavayi* might be related to Fe and Al.

Previous studies showed that the opened purple flowers of *P. delavayi* mainly contained Pn3G5G, whereas the yellow flowers of *P. delavayi* primarily contain chalcone, Km, Qu, Is, Ch, and Ap-glycopyranoside

(Wang et al., 2001b; Zhou et al., 2011). As expected, in the present study, the primary pigments of *P. delavayi* were anthocyanin and chalcone. For Pd, anthocyanin, primarily Cy and Pn based, determined the flower color and flavones and flavonols generally acted as copigments to affect the flower color. Although the purple petals also contained

Table 6. Contents of identified flavonoid in yellow-colored individual petals of *Paeonia delavayi* at the fully opened stage (S5) ($\mu\text{g}\cdot\text{g}^{-1}$).

No.	Identification	λ_{max} (nm)	ESI-(+)-MS (m/z)	Content ($\mu\text{g}\cdot\text{g}^{-1}$)
f1	Qu derivative	256	303	20.504 ± 0.020
f2	Km 3,7-di-O-hexoside	266	287, 449, 633	85.282 ± 0.079
f3	Is 3,7-di-O-glucoside	255	317, 479, 663	136.611 ± 0.121
f4	Lu 7-O-glucoside	348	287, 449	337.853 ± 0.195
f5	Ap 7-O-neohesperidoside	267	271, 579	20.800 ± 0.02
f6	Is 3-O-glucoside	254	317, 501	348.685 ± 0.183
f7	Ch 7-O-glucoside	346	301, 463	53.496 ± 0.051
c1	chalcone 2'-G	365	273, 457	$1,505.572 \pm 0.585$

^af1–f7, and c1 indicate the order of the identified flavonoids in yellow-colored flowers of *P. delavayi*.

Fig. 4. Expression analyses of flavonoid biosynthetic genes in yellow-colored flower and purple-red flower of *Paeonia delavayi* by quantitative real-time polymerase chain reaction using total RNA from the petals at the fully opened stage (S5). Pl, yellow-colored flower of *P. delavayi*; Pd, purple-red flower of *P. delavayi*. The *TUB* gene was used as an internal control, and relative transcript levels are presented as the means with SDs of three technical and three biological replications. *CHS*, chalcone synthase gene; *CHI*, chalcone isomerase gene; *F3H*, flavanone 3-hydroxylase; *DFR*, dihydroflavonol 4-reductase gene; *FNS*, flavonol synthase; *FLS*, flavonol synthase; *3GT*, anthocyanin 3-O-glycosyltransferase; *5GT*, anthocyanin 5-O-glycosyltransferase; *7GT*, flavonoid 7-O-glycosyltransferase; *THC*, 2',4',6',4'-tetrahydroxychalcone 2'-glucosyltransferase; *ANS*, anthocyanidin synthase gene.

yellow chalcone, highly abundant anthocyanin covered the small amount of chalcone, resulting in a purple-red color. For Pl, the main pigment was chalcone 2'-G, and Ap 7-O-neohesperidoside and Ch 7-O-glucoside were copigments; thus, it appeared yellow, inconsistent with the report of yellow coloration in *P. lactiflora* (Zhao et al., 2014). The same glycoside had different modifications between Pl and Pd. The hydroxylation modification of Qu and Lu can strengthen the red color and darken the flower color and the methylation modification of Cy and Pn can enhance the blue color. The copigment effect of flavones and flavonols in Pd was much higher than in Pl and increased the purple degree, which is consistent with results in Japanese tree peony (Sakata et al., 1995) and rhododendron (Asen et al., 1971). Correspondingly, the downstream structural genes

DFR and *ANS* were significantly highly expressed in Pd relative to Pl, which agreed with the reports of Shi et al. (2015) and Zhao et al. (2015). *THC*, which glycosylates 4, 2', 4', 6'-tetrahydroxychalcone to chalcone 2'-G, was upregulated in Pl (Togami et al., 2011). *FNS* was clearly upregulated in the yellow petals and responsible for flavone production in Pl (Akashi et al., 1999; Martens and Mithöfer, 2005). *CHI* can isomerize 4, 2', 4', 6'-tetrahydroxychalcone to form flavones and flavonols, and anthocyanins. In *Hordeum vulgare* (Marinova et al., 2007), and *Scutellaria baicalensis* (Park et al., 2011), mutations of *CHI* are required for chalcone 2'-G production. As expected, the expression of *CHI* in Pl was almost 2-fold higher than that in Pd. In addition, *FLS* is the key enzyme for Qu and Km, and *7GT* is responsible for the 7-O-glycosylation pattern of flavone (Tanaka

and Ohmiya, 2008). These enzymes were significantly highly expressed in yellow petals, which was consistent with the flavonol and flavone production levels in Pl. Hence, the diversity of the flavone/flavonol and anthocyanin accumulation likely contributes to the different flower coloration of *P. delavayi*.

In conclusion, anatomical, chemical, and gene expression analyses indicated that the epidermal cell shape, vacuolar pH, Fe and Al metal ions, and different pigments all affected the various flower colorations of *P. delavayi*. Therefore, the color variation of *P. delavayi* petals is likely dependent on the delicately controlled balance of these factors. Our results provide a substantial foundation for a full understanding of the mechanisms of *P. delavayi* flower pigmentation.

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