Factors Affecting the Color of Herbaceous Peony

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ABSTRACT. Herbaceous peony (*Paeonia lactiflora* Pall.) is a well-known ornamental plant with abundant flower colors. However, our understanding of the underlying mechanisms of flower color formation is limited. In this study, a wild sample of herbaceous peony (collected from Heze, China) and eight cultivars with different colors were selected for experimental investigation. The Royal Horticultural Society Color Chart was used to determine flower color, and the anatomic structure; cell sap pH value; moisture content (MC); condensed tannin content (Ct); soluble sugar and soluble protein content of the petals; and content and composition of anthocyanin, flavonoids, and carotenoids in the petals were examined. 1) In the white, pinkish white, pale purple, purplish pink, and reddish purple cultivars, deeper color was associated with greater total amounts of anthocyanin (TA). Hypochromic effects were observed for kaempferol-7-O-glucoside (Km7G), myricetin-3-rhamnoside (My3R), and luteolin-7-O-glucoside (Lu7G). The accumulation of quercetin-3-O-glucoside (Qu3G) and lutein affected yellow color formation in the petals. 2) There are papillate epidermal cells in the petals of the wild *P. lactiflora* sample, 'Lanyucangjin', and 'Dongjingnvleng'. 3) Cell sap pH and MC of the petals of white, pinkish white, pale purple, and purplish pink cultivars were greater than those of the purplish red and most of the reddish purple cultivars. 4) The Ct was greatest in the purplish red cultivars, whereas no condensed tannins were detected in the white, pinkish white, and pale purple cultivars. 5) There were no significant correlations among soluble sugar content, soluble protein content, and the other physiological indications.

Flower color is an important characteristic of ornamental plants that results from substances in the petal cells, such as flavonoids, carotenoids, and alkaloids; and various intracellular environmental factors such as the pH value and metal ion content (Lapidot et al., 1999; Yoshida et al., 2009). There are three major groups of plant pigments—flavonoids, carotenoids, and alkaloids—and flavonoids are divided further into anthocyanins, flavonoids, and flavonols (Zhao et al., 2005). Studies on yellow camellia (section *Chrysanthema* Chang), medicinal chrysanthemum (*Chrysanthemum morifolium* Ramat), beach rose (*Rosa rugosa* Thunb.), and tree peony (*Paeonia suffruticosa* Andr.) have shown that the content and components of anthocyanin are closely related to flower color (Jiang et al., 2019; Ma et al., 2016; Zhang et al., 2015b, 2018). The intermolecular cocoloration of anthocyanin and accessory pigments can also affect flower color; the chemical bonding energy between these factors can improve the stability of anthocyanin for changing flower color (Zhuang et al., 2018). Sugars are the main energy source and precursors for anthocyanin synthesis, which regulates the anthocyanin content (Zhang et al., 2015a), and proteins are the products of functional gene expression that control changes in the sugar content (He et al., 2010).

Herbaceous peony (*Paeonia lactiflora*) is a perennial herbaceous plant that exhibits varied flower types and colors. In Tai'an, China (lat. 117°06'N, long. 36°11'E), it blooms from

late April to mid May. Studies on herbaceous peony flower color have focused mainly on the classification and identification of anthocyanin, and analyses of the anthocyanin synthesis pathway and anthocyanin stability (Jia et al., 2008; Zhong et al., 2012). The petal structure and cell contents of herbaceous peonies have never been investigated thoroughly.

In this study, we selected wild plants and eight representative cultivars of herbaceous peony with different colors. We documented the anatomic structure of the petals and determined the cell sap pH value, water content, Ct, soluble sugar and soluble protein contents, and pigment content and composition. We also analyzed the indexes influencing flower color formation to identify the key physiological factors underlying different colors and to elucidate the molecular mechanisms of herbaceous peony flower color, which will aid future breeding efforts of new cultivars.

Materials and Methods

PLANT MATERIALS. The experiments were carried out from Apr. 2018 to Sept. 2019 at four locations at Shandong Agricultural University, Tai'an, China: Garden Center Laboratory of the Horticultural Experiment Station (lat. 36°19'N, long. 117°12'E), Gardening Center Laboratory and the Life Science Center (lat. 36°17'N, long. 117°16'E), Herbaceous Peony of the Horticulture Test Station (lat. 36°16'N, long. 117°16'E), and Experimental Center of the Academy of Life Sciences (lat. 36°16'N, long. 117°16'E). The wild sample of herbaceous peony and eight cultivars (Xuefeng, Jinzanciyou, Lanyucangjin, Dongjingnvleng, Honglou, Dafugui, Foguangzhuying, and Zilingjinpao) were grown for 3 years after vegetative propagation. From 19 Apr. to 7 May 2018, we sampled the plants from 0900 to 1100 hr on days when the ground temperature was more than 20 °C and there was no cloud cover. We collected

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only completely opened flowers and used the fresh petals from the middle layer as samples. ‘Jinzancyu’ and ‘Foguangzhuying’ have variable petal shapes within each flower, so we sampled the inner and outer petals from both cultivars. Fresh samples were used to make paraffin sections, and to determine the cell sap pH value and MC. The remainder of the fresh samples was frozen in liquid nitrogen and preserved at -80°C .

FLOWER COLOR, PETAL CELL SAP pH, AND MC. The Royal Horticultural Society Color Chart (Royal Horticultural Society, 2015) was used to determine flower color (Li et al., 2018). Five grams of fresh petals were cut into small segments, ground into a homogenate with a mortar and quartz sand, and the cell sap pH was measured with a pH meter (PB-10; Sartorius, Goettingen, Germany) (Xue et al., 2015). Four grams of fresh petals were dried in an electric constant-temperature air blast drying box (DHG-9145a; Shanghai Yiheng Science and Technology, Shanghai, China) at 80°C for 12 h. Finally, the constant weight and MC were calculated as $\text{MC} = (\text{FW} - \text{DW})/\text{FW} \times 100\%$, where FW is the fresh weight and DW is the dry weight.

PETAL MICROSTRUCTURE. Paraffin sections were prepared according to the methods described by Cheng (2008). We selected fresh petals and cut off the middle portion and fixed them with FAA fixative [formalin:glacial acetic acid:50% ethanol (5:5:90)] for 48 h after removing the debris. Sections were made using a pathological microtome (RM2016; Shanghai Leica, Shanghai, China). After dewaxing and washing, the sections were stained with safranin for 2 h, decolorized with alcohol, and stained with fast green for 6 to 20 s. The sections were then immersed in a dimethylbenzene solution for 5 min, and sealed with neutral resin. The sections were observed using an optical microscope (Eclipse E100; Nikon, Tokyo, Japan), and imaged and analyzed with an imaging system (DS-U3, Nikon). An eyepiece micrometer and image-processing software (Image-Pro Plus 6.0; Media Cybernetics, Rockville, MD) were used to measure the thickness of the petal cross-sections, cytoderm, and parenchymatous tissue; and the length and width of the epidermal cells.

BIOCHEMICAL CONSTITUENTS OF THE PETALS. The soluble sugar content was determined via the anthrone colorimetric method (Cang and Zhao, 2013). A fresh sample (0.3 g) was added to 10 mL distilled water and boiled twice for 30 min. After filtration, the volume was adjusted to 50 mL with distilled water. One milliliter of the extract was combined with 1 mL distilled water (2 mL distilled water was added to the control group), 0.5 mL ethyl acetate anthrone solution, and 5 mL sulfuric acid. The mixture was heated in boiling water for 1 min then cooled to room temperature, and the absorbance spectra were measured at 630 nm using an ultraviolet and visible spectrophotometry (UV-Vis) spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan).

The soluble protein content was determined via the Coomassie brilliant blue method (Yu, 2014). A fresh sample (0.5 g) was added to 5 mL phosphate buffer ($0.05 \text{ mol} \cdot \text{L}^{-1}$, pH 7.8) and centrifuged at 4°C for 20 min ($11,354 g_n$). Twenty microliters of supernatant was combined with 3 mL Coomassie brilliant blue solution (20 μL distilled water was added to the control group), and after 2 min, the absorbance spectra were measured at 595 nm using a UV-Vis spectrophotometer (UV-2450).

The Ct was determined using the vanillin–hydrochloric acid method (Yao and Ti, 2010). A fresh sample (0.2 g) was added to 5 mL 70% methanol and, after 24 h, the sample was centrifuged at 4°C for 20 min ($2575 g_n$). The supernatant (0.5 mL) was

combined with 3 mL 4% vanillin methanol solution and 1.5 mL concentrated hydrochloric acid. The mixture was extracted in a 20°C water bath in darkness for 20 min, and the absorbance spectra were measured at 510 nm using a UV-Vis spectrophotometer (UV-2450). Catechin was used as a standard.

The TA was determined by pH-differential spectrophotometry (Wang, 2015). A fresh sample (0.25 g) was added to 5 mL 1% methanol hydrochloride solution. After 24 h of extraction in darkness at 4°C , the sample was centrifuged at 4°C for 10 min ($6591 g_n$). One milliliter of supernatant was combined with 4 mL KCl buffer (pH 1.0) and 4 mL sodium acetate buffer (pH 4.5). After extraction at 4°C in darkness for 15 min, absorbance was measured at 510 and 700 nm using a UV-Vis spectrophotometer (UV-2450). The control group was treated with the same blank extract without extract as the experimental group.

The total amount of flavonoids (TF) was determined by spectrophotometry (Wang, 2015). A fresh sample (0.1 g) was added to 10 mL 95% ethanol. After ultrasonic extraction at 20°C for 60 min, the sample was centrifuged at 4°C for 10 min ($6591 g_n$) and was then left to rest for 4 h. Two milliliters of supernatant was combined with 4 mL distilled water and 1 mL 5% sodium nitrite. After 6 min, 1 mL 10% aluminum nitrate was added and, after an additional 6 min, 10 mL 5% sodium hydroxide was added. The volume was adjusted to 25 mL with distilled water, and the absorbance spectra were measured at 500 nm after 15 min using an UV-Vis spectrophotometer (UV-2450). The control group was treated with the same blank extract without extract as the experimental group.

The total amount of carotenoids (TC) was also determined by spectrophotometry (Li et al., 2000). A fresh sample (0.2 g) was added to 10 mL extract [anhydrous ethanol:acetone (1:1)] and, after ≈ 30 h of extraction in darkness at 4°C , the plant tissue turned white. Absorbance was measured at 665, 649, and 470 nm using an UV-Vis spectrophotometer (UV-2450). The control group was treated with the same blank extract without extract as the experimental group.

According to the results from previous studies (Liu et al., 2019; Yang et al., 2018; Zhao et al., 2016; Zhong et al., 2012) and those from preliminary tests, we selected particular biochemical substances and measured their contents. These included eight anthocyanins [cyanidin-3-O-glucoside (Cy3G), cyanidin-3,5-di-O-glucoside (Cy3G5G), peonidin-3-O-glucoside (Pn3G), peonidin-3,5-di-O-glucoside (Pn3G5G), pelargonidin-3-O-glucoside (Pg3G), pelargonidin-3,5-di-O-glucoside (Pg3G5G), delphinidin-3-O-glucoside (Dp3G), and delphinidin-3,5-di-O-glucoside (Dp3G5G)], seven flavonol glycosides [kaempferol-3-O-glucoside (Km3G), Km7G, isorhamnetin-3-O-glucoside (Is3G), isorhamnetin-3-O-galactoside (Is3Gal), Qu3G, quercetin-7-O-glucoside (Qu7G), and My3R], one flavonoid glycoside (Lu7G), and two carotenoids (β -carotene and lutein).

We used the high-performance liquid chromatography (HPLC)–UV method to identify the anthocyanins, flavonoids, and carotenoids.

For the anthocyanin measurement, 0.2 g fresh sample was added to 6 mL extract [methanol:water:hydrogen chloride (70:29.9:0.1)]. After 24 h of extraction at 4°C in darkness, the sample was extracted by ultrasound for 30 min, centrifuged at 4°C for 10–15 min ($148 g_n$), and filtered using 0.22- μm filter paper. HPLC was performed using a liquid chromatograph (LC 20-a) with a 5- μm , 4.6×100 -mm column (Venusl XBP C18; Agela Technologies, Tianjin, China). The mobile phase was 8%

formic acid–water [(0.08% trifluoroacetic acid):acetonitrile (0.08% trifluoroacetic acid) (95:5)], the flow rate was 0.6 mL·min⁻¹, and the detection wavelength was 520 nm. The standard concentration was 1 µg·mL⁻¹, the solvent was 8% formic acid–water (0.08% trifluoroacetic acid), and the injection volume was 20 µL. The standards used were cyanidin-3-O-glucoside chloride, cyanidin-3,5-diglucoside, peonidin-3-O-glucoside chloride, peonidin-3,5-O-β-diglucopyranoside, pelargonidin-3-O-β-D-glucoside, pelargonidin-3,5-diglucoside, delphinidin-3-β-D-glucoside, and delphinidin-3,5-diglucoside.

For the flavonoid measurement, 0.3 g fresh sample was added to 5 mL 0.2% butylated hydroxytoluene acetone solution for extraction. After 24 h of extraction at 4 °C in darkness, the sample was extracted by ultrasound for 10 to 20 min, centrifuged at 4 °C for 10 min (2575 g_n), and filtered using 0.22-µm filter paper. HPLC was performed using a liquid chromatograph (LC 20-a) with a 5-µm, 4.6 × 150-mm column (Inertsil ODS-3, Shimadzu). The mobile phase was 1% formic acid:methanol (1:1), the flow rate was 0.8 mL·min⁻¹, the detection wavelength was 355 nm, and the column temperature was 35 °C. The standard concentration was 1 µg·mL⁻¹ and the injection volume was 10 µL. The standards were Km3G, kaempferol-7-O-β-D-glucoside, Is3G, Is3Gal, Qu3G, quercetin-7-O-β-D-glucoside, myricitrin, and luteolin-7-O-β-D-glucoside.

For the carotenoid measurement, 0.5 g fresh sample was added to 25 mL extract [80% methanol:dimethyl sulfoxide (1:1)]. After 24 h of extraction at 4 °C in darkness, the sample was extracted by ultrasound for 30 min, centrifuged at 4 °C for 10 min (2085 g_n), and filtered using 0.22-µm filter paper. HPLC was performed using a liquid chromatograph (LC 20-a) with a 5-µm, 4.6 × 100-mm column (Venusil XBP C18, Agela Technologies). The mobile phase was an acetonitrile:methanol:Tris-HCl buffer

0.05 mol·L⁻¹ (72:8:3, pH 7.5), methanol:n-hexane (5:1), the flow rate was 0.8 mL·min⁻¹, the detection wavelength was 440 nm, and the column temperature was 35 °C. The standard concentration was 1 µg·mL⁻¹ and the injection volume was 10 µL. The standards were β-carotene and lutein.

STATISTICAL ANALYSIS. All measurements were repeated three times. IBM Statistics (version 19.0; IBM, Armonk, NY) was used for one-way analysis of variance and correlation analyses, and the least significant difference test and Duncan's new multiple range test were used to compare the differences between samples. The plots were generated in Microsoft Excel (Office 2016; Microsoft, Redmond, WA) and Origin 2017 (OriginLab, Northhampton, MA).

Results

COLOR DETERMINATION. Flower color was determined from the Royal Horticultural Society Color Chart (Royal Horticultural Society, 2015). The result is shown in Fig. 1. 'Xuefeng' is white; the outer petals of 'Jinzanciyu' is pinkish white and the inner petals are yellow; 'Lanyucangjin' is pale purple; 'Dongjingnvleng' is purplish pink; the wild sample, 'Honglou', 'Dafugui', and 'Foguangzhuying' are reddish purple; and 'Zilingjinpao' is purplish red.

PIGMENTS IN THE TEST SAMPLES. As shown in Fig. 2, a deeper color in the white, pinkish white, pale purple, purplish pink, and reddish purple cultivars was associated with greater amounts of anthocyanin. The TA was the greatest in the outer petals of reddish purple 'Foguangzhuying' and was the least in white 'Xuefeng'. The composition of anthocyanin is presented in Table 1. Pg3G5G and Pn3G5G were the main anthocyanins in white 'Xuefeng', pinkish white 'Jinzanciyu' (outer petals), and yellow 'Jinzanciyu' (inner petals). Pn3G5G and Cy3G were the

Fig. 1. Flower and Royal Horticultural Society Color Chart (Royal Horticultural Society, London, UK) value of herbaceous peony.

Fig. 2. Total amount of pigments in herbaceous peony petals. FW = fresh weight.

Table 1. Composition and content of anthocyanins in herbaceous peony petals.

Color group	Sample	Content (mg/100 g FW)							
		Pn3G5G	Cy3G5G	Pg3G5G	Dp3G5G	Pn3G	Cy3G	Pg3G	Dp3G
White	'Xuefeng'	0.155	0.000	0.179	0.000	0.000	0.000	0.000	0.000
Pinkish white	Outer petal of 'Jinzanciye'	0.196	0.033	0.052	0.000	0.000	0.000	0.000	0.000
Yellow	Inner petal of 'Jinzanciye'	0.067	0.009	0.051	0.000	0.002	0.000	0.000	0.000
Pale purple	'Lanyucangjin'	2.683	0.000	0.057	0.000	0.000	0.098	0.000	0.000
Purplish pink	'Dongjingnvleng'	11.715	2.378	2.556	0.000	0.000	0.000	0.124	0.000
Reddish purple	Wild sample	61.824	3.861	4.165	0.000	0.326	0.000	0.394	0.000
Reddish purple	'Honglou'	0.000	8.529	2.771	0.000	0.098	91.922	0.840	0.000
Reddish purple	'Dafugui'	56.175	7.596	7.561	0.000	0.014	0.000	0.060	0.000
Reddish purple	Outer petal of 'Foguangzhuying'	137.367	12.815	8.077	0.000	0.231	0.000	1.156	0.000
Reddish purple	Inner petal of 'Foguangzhuying'	48.988	2.280	4.136	0.000	0.011	0.000	1.064	0.000
Purplish red	'Zilingjinpao'	4.801	0.151	1.039	0.000	0.000	0.000	0.000	0.057

FW = fresh weight; Pn3G5G = peonidin-3,5-di-O-glucoside; Cy3G5G = cyanidin-3,5-di-O-glucoside; Pg3G5G = pelargonidin-3,5-di-O-glucoside; Dp3G5G = delphinidin-3,5-di-O-glucoside; Pn3G = peonidin-3-O-glucoside; Cy3G = cyanidin-3-O-glucoside; Pg3G = pelargonidin-3-O-glucoside; Dp3G = delphinidin-3-O-glucoside.

main anthocyanins in pale-purple ‘Lanyucangjin’, and Pn3G5G and Pg3G5G were the main anthocyanins in purplish pink ‘Dongjingnvláng’. Among the reddish purple samples, Pn3G5G and Pg3G5G were the main anthocyanins in the wild sample and the inner petals of ‘Foguangzhuying’, Cy3G and Cy3G5G were the main anthocyanins in ‘Honglou’, and Pn3G5G and Cy3G5G were the main anthocyanins in ‘Dafugui’ and the outer petals of ‘Foguangzhuying’. Pn3G5G and Pg3G5G were the main anthocyanins in purplish red ‘Zilingjinpao’. Cy3G was only detected in pale-purple ‘Lanyucangjin’ and reddish purple ‘Honglou’, and Dp3G was only detected in purplish red ‘Zilingjinpao’.

The TF was the greatest in the wild sample and the least in the pinkish white petals of ‘Jinzanciyu’ (Fig. 2). The components and contents of the flavonoids are shown in Table 2. Km7G and Qu3G were the main flavonoids in white ‘Xuefeng’, and Is3G and Km7G were the main flavonoids in pinkish white ‘Jinzanciyu’ (outer petals). The main flavonoids of the yellow inner petals of ‘Jinzanciyu’ were Qu3G and Is3G. Km7G and Qu3G were the main flavonoids in pale-purple ‘Lanyucangjin’ and purplish pink ‘Dongjingnvláng’. Among the reddish purple samples, the main flavonoids in the wild sample and the outer petals of ‘Foguangzhuying’ were Qu3G and Is3G, Km7G and Qu3G in ‘Honglou’ and ‘Dafugui’, and Is3G and Km7G in the inner petals of ‘Foguangzhuying’. The main flavonoids in purplish red ‘Zilingjinpao’ were Km7G and Qu3G. The contents of Qu7G and Lu7G were the greatest in the reddish purple wild sample, and My3R was the greatest in ‘Honglou’. Km3G and Is3Gal were not detected in any of the samples, and My3R was not detected in the reddish purple wild sample or purplish pink ‘Dongjingnvláng’.

Trace amounts of carotenoids were detected in all samples (Fig. 2). The carotenoid content was the greatest in white ‘Xuefeng’, the second-greatest in yellow ‘Jinzanciyu’ (inner petals), and the least in purplish red ‘Zilingjinpao’. As shown in Table 3, lutein was the main carotenoid component of all the cultivars. The contents of lutein and β -carotene were the greatest in reddish purple ‘Honglou’ and second-greatest in yellow ‘Jinzanciyu’ inner petals.

PETAL MICROSTRUCTURE. As shown in Fig. 3A, the petals were composed mainly of two layers of epidermal cells and parenchymal tissue. The upper and lower epidermal cells are similar in morphology and tightly arranged. The parenchymal cells are loosely arranged, and their intercellular spaces are visible. Petal thickness (PT) ranged from 0.11 to 0.44 mm (Table 4). The largest PT was observed in pale-purple ‘Lanyucangjin’, reddish purple ‘Foguangzhuying’ (outer petals), and purplish red ‘Zilingjinpao’. The outer petals of ‘Foguangzhuying’ and ‘Lanyucangjin’ had thicker parenchymal tissues, and the outer petals of ‘Foguangzhuying’ and ‘Zilingjinpao’ had thicker cytodermis. The epidermal cells had four distinct shapes: flat, dome, elongated dome, and papillate. Two types of epidermal cells were observed in pale-purple ‘Lanyucangjin’, purplish pink ‘Dongjingnvláng’, and the reddish purple wild sample, but there was only one cell type in the other samples (Fig. 3B; Table 4). In ‘Jinzanciyu’, the shape and color of the inner and outer petals were different, and the shape of their epidermal cells also varied. The shape of the inner and outer petals was also different in ‘Foguangzhuying’, but the color of the petals and the shape of the epidermal cells were consistent. In ‘Jinzanciyu’, ‘Lanyucangjin’, the wild sample, ‘Dafugui’, and the inner petals of ‘Foguangzhuying’, white stripes were noted on the outer surface of the epidermal cell wall (Fig. 3C).

MOISTURE CONTENT AND pH. The MC of the tested samples varied (Fig. 4). The MC was the greatest in reddish purple ‘Honglou’ (83.04%) and the least in reddish purple ‘Foguangzhuying’ inner petals (70.25%). The MC of the white, pinkish white, pale purple, and purplish pink samples was greater than that of the yellow, purplish red, and most reddish purple samples (except ‘Honglou’).

The results in Fig. 4 show that the cell sap of the samples was slightly acidic, with a pH between 5.84 and 5.05. Yellow ‘Jinzanciyu’ (inner petals) had the highest pH (5.84), whereas reddish purple ‘Honglou’ had the lowest pH (5.05). The cell sap pH of the yellow, white, pinkish white, pale-purple, and purplish pink samples was higher than that of the purplish red and most reddish purple samples (except for the inner petals of ‘Foguangzhuying’).

SOLUBLE SUGAR AND SOLUBLE PROTEIN CONTENTS. The soluble sugar content of the reddish purple wild sample was the greatest ($6.96 \text{ mg}\cdot\text{g}^{-1}$), whereas that of the yellow ‘Jinzanciyu’ inner petals was the least ($3.78 \text{ mg}\cdot\text{g}^{-1}$; see Fig. 5). There was no difference in the soluble sugar content of pale purplish ‘Lanyucangjin’ and reddish purple ‘Foguangzhuying’ (outer petals), but there were significant differences between that of the other samples. The soluble protein content was the greatest in the reddish purple ‘Foguangzhuying’ inner petals ($25.17 \text{ mg}\cdot\text{g}^{-1}$) and the least in the outer petals of ‘Foguangzhuying’ ($11.09 \text{ mg}\cdot\text{g}^{-1}$). The soluble sugar and soluble protein contents of the ‘Foguangzhuying’ inner petal were significantly greater than those of the outer petals, but the pattern was reversed between the petal types of ‘Jinzanciyu’.

CONDENSED TANNIN CONTENT. There were significant differences in Ct (Fig. 2). The Ct in purplish red ‘Zilingjinpao’ was the greatest ($14.00 \text{ mg}\cdot\text{g}^{-1}$), whereas that in the reddish purple ‘Foguangzhuying’ inner petals was the least ($0.19 \text{ mg}\cdot\text{g}^{-1}$). In the other samples, the Ct was $\leq 2.41 \text{ mg}\cdot\text{g}^{-1}$.

CORRELATION ANALYSIS. The results of the correlation analysis are shown in Table 5. The TA correlated positively with the TF, PT, and cytoderm thickness (CT), but correlated negatively with the TC. Moreover, TF correlated positively with PT. The Ct correlated negatively with the MC. There was a significant correlation between PT, parenchyma tissue thickness (PtT), and CT.

Discussion

EFFECT OF PIGMENTS ON FLOWER COLOR. The type and content of anthocyanin are critical factors influencing flower color. The flowers of blooming roses appear white when the anthocyanin content is low, but as the anthocyanin content increases, the flowers gradually turn pink and purple (Zhang et al., 2015b). In tree peony, flower color deepens as the TA increases, and the composition of anthocyanin also affects flower color; paeoniflorin makes the petals red and purple whereas cyanidin makes the petals peach (Liu et al., 2019; Wang and Yuan, 2015). The accumulation of delphinidin can significantly increase the reddish color of petals; a greater delphinidin content in daylily (*Heremacallis* L.) petals results in a reddish brown appearance (Huang, 2013). In our study, deeper color in the white, pinkish white, pale-purple, purplish pink, and reddish purple samples was associated with a greater TA. The TA was the least in white ‘Xuefeng’ and the greatest in reddish purple ‘Foguangzhuying’ (outer petals). The diversity of anthocyanin components in white ‘Xuefeng’, pinkish white ‘Jinzanciyu’ (outer petals), and pale-purple ‘Lanyucangjin’ was

Table 2. Composition and content of flavonoids in herbaceous peony petals.

Color group	Sample	Content (mg/100 g FW)							
		Km7G	Qu3G	Is3G	Km3G	Qu7G	Is3Gal	My3R	Lu7G
White	'Xuefeng'	69.448	40.042	11.311	0.000	4.442	0.000	0.485	1.123
Pinkish white	Outer petal of 'Jinzanciyu'	20.724	0.542	58.895	0.000	2.197	0.000	0.186	0.508
Pale purple	Inner petal of 'Jinzanciyu'	33.118	76.479	33.922	0.000	2.198	0.000	1.030	0.687
Purplish pink	'Lanyucangjin'	122.499	25.179	12.311	0.000	3.408	0.000	1.026	1.156
Reddish purple	'Dongjingnvláng'	57.858	31.264	10.566	0.000	2.194	0.000	0.000	0.579
Reddish purple	Wild sample	74.981	120.423	100.914	0.000	6.590	0.000	0.000	3.144
Reddish purple	'Honglou'	69.120	27.204	9.722	0.000	6.169	0.000	2.252	2.068
Reddish purple	'Dafugui'	42.790	7.999	6.592	0.000	4.298	0.000	0.260	0.596
Reddish purple	Outer petal of 'Foguangzhuying'	22.674	49.754	61.245	0.000	2.463	0.000	1.127	1.236
Reddish purple	Inner petal of 'Foguangzhuying'	33.646	28.025	47.941	0.000	2.293	0.000	1.542	1.771
Purplish red	'Zilingjinpao'	24.838	23.333	12.473	0.000	3.777	0.000	1.077	0.861

FW = fresh weight; Km7G = kaempferol-7-O-glucoside; Qu3G = quercetin-3-O-glucoside; Is3G = isorhamnetin-3-O-glucoside; Km3G = kaempferol-3-O-glucoside; Qu7G = quercetin-7-O-glucoside; Is3Gal = isorhamnetin-3-O-galactoside; My3R = myricetin-3-O-rhamnoside; Lu7G = luteolin-7-O-glucoside.

Table 3. Composition and content of carotenoids in herbaceous peony petals.

Color group	Sample	Content (mg/100 g FW)	
		Lutein	β-Carotene
White	'Xuefeng'	0.000400	0.000200
Pinkish white	Outer petal of 'Jinzanciyu'	0.000510	0.000291
Pale purple	Inner petal of 'Jinzanciyu'	0.002014	0.000387
Purplish pink	'Lanyucangjin'	0.001061	0.000214
Reddish purple	'Dongjingnvláng'	0.000340	0.000240
Reddish purple	Wild sample	0.000204	0.000134
Reddish purple	'Honglou'	0.002420	0.000823
Reddish purple	'Dafugui'	0.000287	0.000186
Reddish purple	Outer petal of 'Foguangzhuying'	0.000195	0.000171
Reddish purple	Inner petal of 'Foguangzhuying'	0.000627	0.000102
Purplish red	'Zilingjinpao'	0.000560	0.000155

FW = fresh weight.

Fig. 3. Petal microstructure of herbaceous peony petals. (A) Petal cross-section at ×40 magnification. (B) Papillate epidermal cell of the petals at ×200 magnification. (C) White stripes on the outside of the petals at ×200 magnification.

less than that in the other color samples. Reddish purple 'Honglou' had a greater Cy3G content than the other reddish purple samples; the color of 'Honglou' is redder than 'Dafugui', but lighter than the wild sample and 'Foguangzhuying'. A small amount of Dp3G was detected in purplish red 'Zilingjinpao', but not in the other cultivars. Flavonoids and flavonols can make the tree peony appear white, pale yellow, or yellow. Kaempferol and luteolin may serve

Table 4. Microstructure parameters of herbaceous peony petals.

Sample	Microstructure parameters [mean $\pm$ SE (mm)]						Shape of epidermal cells
	Petal thickness	Parenchyma tissue thickness	Cytoderm thickness	Epidermal cell length	Epidermal cell width		
'Xuefeng'	0.2049 $\pm$ 0.0034	0.1203 $\pm$ 0.0017	0.0054 $\pm$ 0.0002	0.0314 $\pm$ 0.0024	0.0493 $\pm$ 0.0018		Elongated dome
Outer petal of 'Jinzanciyu'	0.1125 $\pm$ 0.0025	0.0434 $\pm$ 0.0020	0.0046 $\pm$ 0.0003	0.0237 $\pm$ 0.0017	0.0342 $\pm$ 0.0015		Elongated dome
Inner petal of 'Jinzanciyu'	0.1774 $\pm$ 0.0019	0.1093 $\pm$ 0.0048	0.0045 $\pm$ 0.0002	0.0354 $\pm$ 0.0024	0.0335 $\pm$ 0.0016		Dome
'Lanyucangjin'	0.2854 $\pm$ 0.0041	0.2124 $\pm$ 0.0057	0.0051 $\pm$ 0.0005	0.0316 $\pm$ 0.0022	0.0323 $\pm$ 0.0023		Dome
'Dongjingnvlang'	0.1457 $\pm$ 0.0027	0.0995 $\pm$ 0.0033	0.0057 $\pm$ 0.0003	0.0291 $\pm$ 0.0030	0.0435 $\pm$ 0.0033		Papillate
				0.0545 $\pm$ 0.0021	0.0332 $\pm$ 0.0007		Flat
Wild sample	0.2149 $\pm$ 0.0016	0.1231 $\pm$ 0.0031	0.0049 $\pm$ 0.0004	0.0614 $\pm$ 0.0087	0.0379 $\pm$ 0.0061		Papillate
				0.0314 $\pm$ 0.0015	0.0413 $\pm$ 0.0024		Elongated dome
'Honglou'	0.1963 $\pm$ 0.0038	0.1314 $\pm$ 0.0061	0.0044 $\pm$ 0.0002	0.0364 $\pm$ 0.0025	0.0620 $\pm$ 0.0039		Papillate
'Dafugui'	0.1444 $\pm$ 0.0056	0.0854 $\pm$ 0.0029	0.0068 $\pm$ 0.0002	0.0642 $\pm$ 0.0053	0.0648 $\pm$ 0.0029		Dome
Outer petal of 'Foguangzhuying'	0.442 $\pm$ 0.0081	0.3247 $\pm$ 0.0088	0.0169 $\pm$ 0.0011	0.0261 $\pm$ 0.0010	0.0318 $\pm$ 0.0015		Dome
Inner petal of 'Foguangzhuying'	0.1921 $\pm$ 0.0045	0.1249 $\pm$ 0.0048	0.0057 $\pm$ 0.0002	0.1086 $\pm$ 0.0058	0.0604 $\pm$ 0.0048		Flat
'Zilingjinpao'	0.2724 $\pm$ 0.0008	0.1431 $\pm$ 0.0046	0.0119 $\pm$ 0.0013	0.0607 $\pm$ 0.0017	0.0387 $\pm$ 0.0034		Flat
				0.1432 $\pm$ 0.0131	0.0687 $\pm$ 0.0017		Flat

Fig. 4. Cell sap pH and water content of herbaceous peony petals.

Fig. 5. Soluble sugar and soluble protein content of herbaceous peony petals. FW = fresh weight.

as accessory pigments to reduce the purity of red, changing the color to pink and/or purple (Wang and Yuan, 2015). Studies have shown that quercetin is the primary pigment component of the yellow petals of yellow camellia (Li et al., 2019; Tanikawa et al., 2008). In our study, the main flavonoids were, for the most part, consistent. The Qu3G content in yellow 'Jinzanciyou' (inner petals) was greater than that in the other samples, suggesting that Qu3G plays an essential role in yellow-petal production. The contents of Km7G and My3G in pale-purple 'Lanyucangjin' was greater than those in purplish pink 'Dongjingnvleng', and the contents of Lu7G and My3G were relatively high in reddish purple 'Honglou', which is lighter than the other reddish purple samples. These results indicate that flavonoids (especially Km7G, Lu7G, and My3G) have a hypochromic effect on flower color in the herbaceous peony, making the flowers appear lighter in color. These results are consistent with those of Zhong et al. (2012).

Carotenoids appear yellow or orange in plant petals. Studies of the stock plant and bud mutational branches of *Osmanthus* (*Osmanthus fragrans* Lour.) have shown that carotenoids are the key pigments of petal color change (Wang et al., 2017). However, changes in rose flower color were not the result of carotenoids (Zhang et al., 2015b). Carotenoids were detected in all samples tested in our study, and the proportion of lutein was relatively high in the yellow petals of 'Jinzanciyou'. We speculate that the accumulation of lutein was related to the formation of yellow petals in 'Jinzanciyou'.

EFFECT OF PETAL STRUCTURE ON FLOWER COLOR. The shape of plant epidermal cells affects the appearance of color. Mudalige et al. (2003) observed four types of epidermal cells in dendrobium (*Dendrobium* Sw.) petals: flat, dome, elongated dome, and papillate. When the epidermal cells of butterfly orchid (*Phalaenopsis* Bl.) petals have a tapered structure, the ratio of incident light entering the cells increases, which aids the appearance of pigments within the cells (Xiao et al., 2017).

In our study, the pale-purple 'Lanyucangjin', purplish pink 'Dongjingnvleng', and reddish purple wild sample had papillate epidermal cells, and their colors were brighter. The epidermal cell morphology of the wild sample is different from the other reddish purple samples; this may be a characteristic of the wild species. The microstructure and flower color of the inner and outer petals of 'Jinzanciyou' and 'Foguangzhuying' showed that the epidermal cell shape might affect the

appearance of color, and PT correlated positively with TA and TF. Moreover, the PT and epidermal cell volume of purplish red 'Zilingjinpao' were greater than those in the other samples. This may affect the coloration of the petals. The white stripes on the outer surface of some tree peony petals may be attributed to a raised cuticle (Zhang et al., 1998), which could explain the variable texture of the petals.

EFFECT OF PETAL pH AND MC ON FLOWER COLOR. The cell sap pH of the petal plays an important role in the presence of anthocyanins in the plant organs (Zhuang et al., 2018). In yunnan camellia (*Camellia reticulata* Lindl.) petals, the pH of deep-red petals was less than that of pink petals (Xue et al., 2015); and in yellow camellia, when the cell sap pH approaches neutrality, the flowers turn yellow (Jiang et al., 2019). In our study, yellow 'Jinzanciyou' inner petals had the highest pH, and the pH of pinkish white 'Jinzanciyou' (outer petals), white 'Xuefeng', purplish pink 'Dongjingnvleng', and pale-purple

Table 5. Correlation analysis of physiological indexes of herbaceous peony petals.

Index	Correlation coefficient										
	TA	TF	TC	Ct	MC	pH	SS	SP	PT	PtT	CT
TA	1										
TF	0.656*	1									
TC	-0.679*	-0.141	1								
Ct	0.515	0.361	-0.433	1							
MC	-0.499	-0.224	0.412	-0.661*	1						
pH	-0.395	-0.419	0.382	-0.226	-0.107	1					
SS	0.108	0.383	-0.014	-0.067	0.045	-0.533	1				
SP	-0.002	0.171	0.163	0.095	-0.214	-0.538	0.584	1			
PT	0.686*	0.680*	-0.285	0.181	-0.144	-0.121	-0.263	-0.363	1		
PtT	0.591	0.566	-0.251	0.019	-0.045	-0.11	-0.287	-0.389	0.974**	1	
CT	0.885**	0.565	-0.508	-0.017	-0.356	-0.131	-0.131	-0.328	0.823**	0.749**	1

*, **Significant at $P \leq 0.05$ or 0.01 , respectively.

TA = total amount of anthocyanins; TF = total amount of flavonoids; TC = total amount of carotenoids; Ct = condensed tannin content; MC = moisture content; SS = soluble sugar; SP = soluble protein; PT = petal thickness; PtT = parenchymal tissue thickness; CT = cytoderm thickness.

‘Lanyucangjin’ were all higher than that of purplish red ‘Zilingjinpao’ and most reddish purple samples (except ‘Foguangzhuying’ inner petals).

The MC of the petals also affects flower color. In golden camellia, the water content of the petals had a slight negative correlation with flower color, indicating that high moisture in the petals interferes with flower color formation (Jiang et al., 2019). In our study, the MC of the white, pinkish white, pale-purple, and purplish pink samples was greater than that of the yellow, purplish red, and most reddish purple samples (except ‘Honglou’). This observation is consistent with the results from golden camellia plants (Jiang et al., 2019) and may be related to the dilution of biochemical substances in the cell sap.

EFFECTS OF SOLUBLE SUGAR AND SOLUBLE PROTEIN ON FLOWER COLOR. The contents of soluble sugar and soluble protein in cells affects flower color. Currently, the relationships between soluble sugar, soluble protein, and flower color are inconsistent. A study on *Magnolia wufengensis* L.Y. Ma et L.R. Wang showed that an increase in the contents of anthocyanin and soluble sugar, and a decrease in the content of soluble protein were associated with deepened color (He et al., 2010). In medicinal chrysanthemum, an increase in the soluble sugar content was associated with a decrease in the anthocyanin content during flower development (Ma et al., 2016).

The correlation analysis used in our study showed there were no significant correlations between the contents of soluble sugar and soluble protein, and the other physiological indices.

EFFECT OF CONDENSED TANNIN ON FLOWER COLOR. Condensed tannins are derivatives of flavanols (Rasmussen et al., 2005) and are often used as complementary substances to change the color of wine. In the purple sweetpotato [*Ipomoea batatas* (L.) Lam.], tannins improve the stability of anthocyanin and make its anthocyanin solution darker (Zhu et al., 2006).

The results from our study showed that the Ct in the purplish red sample was much greater than in the other samples. This suggests that the accumulation of condensed tannins is beneficial to the formation of purplish red color in herbaceous peony.

Conclusions

Taken together, our results demonstrate that the flower color of herbaceous peony is influenced by numerous factors, and that

the content and composition of the pigments are the most important. The TA of the white and pinkish white cultivars was the lowest. In the pale-purple, purplish pink, and reddish purple cultivars, deeper color was associated with a greater TA. The Pg3G5G content was the greatest in white ‘Xuefeng’, the Cy3G content was the greatest in reddish purple ‘Honglou’, and the Pn3G5G content was the greatest in the other tested samples. The composition of flavonoids was consistent across samples; quercetin, isorhamnetin, and kaempferol were the main components. Km7G, My3R, and Lu7G may show hypochromic effects in herbaceous peony flower color formation. All the

samples contained few carotenoids, and the lutein content was greater than that of β -carotene. The content of Qu3G and lutein in yellow ‘Jinzanciyu’ (inner petals) was relatively high, so we speculate that the accumulation of Qu3G and lutein is essential to the appearance of yellow flower color

Other physiological indicators, such as the tissue structure and intracellular environment of the petals, affect the content, concentration, and stability of the pigments, all of which influence the flower color of herbaceous peony. The TA and TF of the cultivars correlated positively with PT, and the samples with papillate epidermal cells in the petals had a brighter color. A greater MC was associated with lighter coloration. The samples with lower cell sap pH or greater Ct’s often showed darker colors, which may be a result of greater anthocyanin stability in the petals.

We analyzed comprehensively the color formation mechanisms of herbaceous peony petals for the contents and composition of the pigments, petal tissue structure, intracellular environment, and biochemical substances. The results presented here add to our limited knowledge of flower color formation; but, because only a limited number of samples were tested, future studies require the inclusion of more plant types. The key genes of the anthocyanin metabolism pathway should be studied to provide physiological and molecular references for herbaceous peony color breeding.

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