

Identification and Characterization of Anthocyanins by High-performance Liquid Chromatography–Electrospray Ionization–Mass Spectrometry in Herbaceous Peony Species

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ABSTRACT. Petal anthocyanins were systematically identified and characterized by high-performance liquid chromatography (HPLC)–electrospray ionization–mass spectrometry (MS) coupled with diode array detection among nine wild herbaceous peony (*Paeonia* L.) species (15 accessions). Individual anthocyanins were identified according to the HPLC retention time, elution order, MS fragmentation patterns, and by comparison with authentic standards and published data. Six main anthocyanins, including peonidin-3,5-di-*O*-glucoside, peonidin-3-*O*-glucoside-5-*O*-arabinoside (Pn3G5Ara), peonidin-3-*O*-glucoside, pelargonidin-3,5-di-*O*-glucoside, cyanidin-3,5-di-*O*-glucoside, and cyanidin-3-*O*-glucoside (Cy3G), were detected. In addition to the well-known major anthocyanins, some minor anthocyanins were identified in herbaceous peony species for the first time. Detection of the unique anthocyanins cyanidin-3-*O*-glucoside-5-*O*-galactoside and pelargonidin-3-*O*-glucoside-5-*O*-galactoside in both *Paeonia anomala* L. and *P. anomala* ssp. *veitchii* (Lynch) D.Y. Hong & K.Y. Pan indicated these two species should belong to the same taxon. Pn3G5Ara was found only in European wild species and subspecies suggesting different metabolic pathways between European and Chinese accessions. Anthocyanins conjugated with galactose and arabinose were observed in the genus *Paeonia* for the first time. The North American species, *Paeonia tenuifolia* L., had high Cy3G content in flower petals. This anthocyanin composition is distinct from the anthocyanin composition in Asian and European species and possibly is responsible for the vivid red coloration in flowers.

The genus *Paeonia* consists of three sections (*Moutan* DC., *Onaepia* Lindley, and *Paeonia* DC.) and ≈35 species to form an independent family, Paeoniaceae. Section *Paeonia* was further divided into two subsections [*Foliolatae* F.C. Stern and subsection *Dissectifoliae* F.C. Stern (Stern, 1946)] that were disjunctly distributed in eastern Asia, central Asia, the western Himalayas, and the Mediterranean regions. Section *Paeonia* is the largest one, consisting of 22 species, some of which are complex and polyploid. Section *Paeonia* is the evolutionary biome in this genus (Ferguson and Sang, 2001; Pan, 1995; Sang et al., 1997).

China is considered to be the distribution and evolution center of the genus *Paeonia*. The middle region of China is the most concentrated zone of *Paeonia* species and botanical

varieties in the world (Hong et al., 1988; Peng and Jiang, 2000; Yu and Xiao, 1987). Approximately eight species in section *Paeonia* have been identified as originating from China (Hong et al., 2001). Many primitive, distinct characteristics in this section are unique among angiosperms (Peng and Jiang, 2000).

Chinese herbaceous peony is one of the earliest cultivated plants and the most influential germplasm in the peony population accounting for 41% of the world's wild germplasm (Gao and Peng, 2004). In the 19th century, *Paeonia lactiflora* Pallas, the unique wild parent of Chinese cultivars (Yuan, 1999), was introduced into Europe and contributed to many new cultivars by interspecific hybridization with *Paeonia officinalis* L., a native species of Europe (Wister and Wolfe, 1962). Today, *P. lactiflora* has become a world-famous flower with many offspring all over the world. In general, the wild species have white and reddish purple flowers, whereas colors of herbaceous peony cultivars are rich in diversity. Cultivars have been divided into nine groups according to petal color: white, yellow, pink, blue, red, purple, black, green, and double color (Li, 1999; Wang, 2003; Wang and Zhang, 2005; Zhang et al., 2006). However, herbaceous peony lacks vivid red color as compared with that of other flowering plants such as tree peony (*Paeonia suffruticosa* Andrews) and rose (*Rosa centifolia* L.).

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Anthocyanins are a group of widespread natural pigments in plants that are mainly distributed among flowers, fruit, and vegetables and are responsible for bright colors such as red, purple, and blue. Anthocyanins are composed of anthocyanidin (aglycone) and sugar(s) and one optional component, the acylated group(s). Chemical structures of petal anthocyanins and chemotaxonomy of anthocyanins in tree peony have been studied extensively. In comparison, no comprehensive study of petal anthocyanins of herbaceous peony has been conducted despite its long breeding history and extensive distribution. Only a Japanese research team has made some preliminary studies on color measurement and identification of the flower anthocyanins in four wild *Paeonia* species [*P. lactiflora*, *P. tenuifolia*, *P. obovata* Maximowicz, and *P. japonica* (Makino) Miyabe & Takeda] and some Japanese and western herbaceous peony cultivars by thin-layer chromatography (TLC) (Hosoki and Seo, 1991). Six anthocyanins, peonidin-3,5-di-*O*-glucoside (Pn3G5G), pelargonidin-3,5-di-*O*-glucoside (Pg3G5G), cyanidin-3,5-di-*O*-glucoside (Cy3G5G), peonidin-3-*O*-glucoside (Pn3G), cyanidin-3-*O*-glucoside, and pelargonidin-3-*O*-glucoside (Pg3G), detected were found to be identical with those of tree peony. However, these results were not comprehensive as judged by TLC (mobility). Use of more highly analytical equipment and more diverse plant material may uncover additional anthocyanins in peony flower petals.

The most effective technique of identifying anthocyanins is by mass spectrometry (Chirinos et al., 2006; Montoro et al., 2006; Zhang and Cheng, 2006). To our knowledge, there have been no published data of anthocyanins in wild herbaceous peony species available. Therefore, the objective of our study was to identify and characterize the anthocyanins in herbaceous peony species from different regions of the world using reverse-phase high-performance liquid chromatography (HPLC) with diode array detection in tandem with electrospray ionization-mass spectrometry (ESI-MSⁿ) and to clarify the anthocyanin composition. The final results may provide valuable information on the mechanism of their flower coloration and guidelines on breeding new cultivars with novel flower colors in future.

Materials and Methods

Standards and solvents

Malvidin-3,5-di-*O*-glucoside (Mv3G5G) was obtained from Extrasynthese (Genay, France). Cy3G5G, Pg3G5G, Pn3G5G, Cy3G, Pg3G, and Pn3G were obtained and identified previously from the Chinese tree peony cultivar Qing Long Wo Mo Chi and a Japanese tree peony cultivar Hohki (Wang et al., 2001a; Zhang et al., 2007). Rutin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile and methanol were HPLC grade. Methanol, acetic acid, trifluoroacetic acid, phosphoric acid, and formic acid were analytical reagent grade. HPLC-grade water (deionized water) was obtained from a Milli-Q System (Millipore, Billerica, MA).

Plant materials

Plants used in this study included 14 wild herbaceous *Paeonia* accessions [*P. lactiflora* with two different colors (pink and white); *P. obovata* with two different colors (red and white); *P. intermedia* C.A. Meyer; *P. anomala* ssp. *veitchii*; *P. anomala*; *P. mascula* (L.) Miller; *P. daurica* ssp. *wittmanniana* (Hartwiss & Lindley) D.Y. Hong; *P. mlokosewitschii* Lomakin; *P. officinalis* ssp. *humilis* (Retz.) Cullen & Heywood; *P. mascula* ssp. *russi* (Biv.) Cullen & Heywood; *P. daurica* Andrews; and *P. tenuifolia*], which originated from multiple locations (Fig. 1; Table 1) and a hybrid of wild herbaceous peony species with the code number H02222 (*P. daurica* × *P. mascula*). All plants were grown at the Chinese Academy of Sciences (lat. 39°48'N, long. 116°28'E, 76 m altitude).

All petals at full bloom stage were collected from mid-April to the beginning of May in 2006 and 2007.

Petal color measurement

The colors of fresh petals were first identified according to the Royal Horticultural Society Color Chart [RHSCC (Royal Horticultural Society, 2001; Sakata et al., 1995)]. Petal color parameters in the middle portion of the upper epidermis were measured with a spectrophotometer NF333 (Nippon Denshoku



Fig. 1. The map illustrates the locations of nine herbaceous peony species (15 accessions) in the world. (1) Dahaituo National Nature Reserves, Chicheng County, Hebei Province, China; (2) Kuandian Manzu Minority Autonomous Region, Dandong City, Liaoning Province, China; (3) Tacheng Wild Almond Nature Reserve, Yumin County, Xinjiang Uygur Autonomous Region, China; (4) Lanzhou Peace Peony Garden, Yuzhong County, Gansu Province, China; (5) Kanas National Nature Reserve, Xinjiang Uygur Autonomous Region, China; (6) northern Turkey; (7) southern Caucasus region; (8) mideastern Caucasus region; (9) southern France; (10) La Corse, Mediterranean Sea; (11) Hatay, Turkey; (12) Amasya, Turkey; and (13) Kartli, Igoeti, GA.

Table 1. Petal coloration of nine wild herbaceous peony species (15 accessions) in the genus *Paeonia*.

| Species | RHSCC ^z | Color series ^y | CIE Lab coordinates ^x | | | | | Location ^w |
|---|--------------------|---------------------------|----------------------------------|-------|-------|------|-------|-----------------------|
| | | | L* | a* | b* | C* | h | |
| Chinese wild species | | | | | | | | |
| <i>P. lactiflora</i> (pink) | 78D | P | 63.9 | 8.6 | 1.9 | 8.8 | 12.2 | 1 |
| <i>P. lactiflora</i> (white) | W | W | 77.8 | -9.4 | 10.2 | 13.9 | 132.7 | 1 |
| <i>P. obovata</i> (red) | 71D | RP | 41.0 | 42.9 | -9.9 | 44.0 | -13.0 | 2 |
| <i>P. obovata</i> (white) | W | W | 71.1 | -5.2 | 11.1 | 12.3 | 115.1 | 2 |
| <i>P. intermedia</i> | 72A | RP | 30.2 | 39.6 | -9.3 | 40.7 | -13.1 | 3 |
| <i>P. anomala</i> ssp. <i>veitchii</i> | 72C | RP | 47.5 | 32.0 | -14.6 | 35.2 | -24.5 | 4 |
| <i>P. anomala</i> | 72A | RP | 38.1 | 38.2 | -15.1 | 41.0 | -21.8 | 5 |
| European wild species | | | | | | | | |
| <i>P. mascula</i> | 64A | RP | 34.9 | 42.7 | -3.8 | 42.8 | -5.1 | 6 |
| <i>P. daurica</i> ssp. <i>wittmanniana</i> | W | W | 74.3 | -5.4 | 12.9 | 13.9 | 112.6 | 7 |
| <i>P. daurica</i> ssp. <i>wittmanniana</i> (flares) | 70C | P | 44.4 | 26.8 | -3.9 | 27.1 | -8.2 | 7 |
| <i>P. mlokosewitschii</i> | 2D | Y | 92.6 | -15.3 | 23.5 | 28.0 | 123.1 | 8 |
| <i>P. mlokosewitschii</i> (fares) | 63D | P | 67.3 | 6.6 | 7.1 | 9.7 | 47.1 | 8 |
| <i>P. officinalis</i> ssp. <i>humilis</i> | 71B | RP | 38.7 | 39.4 | -13.6 | 41.7 | -19.0 | 9 |
| <i>P. mascula</i> ssp. <i>russi</i> | 63C | P | 52.2 | 24.9 | -2.2 | 25.0 | -5.1 | 10 |
| <i>P. daurica</i> | 71B | RP | 34.6 | 44.3 | -8.7 | 45.1 | -11.1 | 11 |
| <i>P. daurica</i> × <i>P. mascula</i> | 61A | RP | 34.5 | 41.7 | -9.5 | 42.8 | -12.9 | 12 |
| North American wild species | | | | | | | | |
| <i>P. tenuifolia</i> | 60A | R | 31.5 | 41.5 | 9.4 | 42.6 | 12.8 | 13 |

^zRoyal Horticultural Society Colour Chart; W: white color.

^yColor series: P: pink series; W: white series; R: red series; RP: reddish purple series; Y: yellow series.

^xL*: Lightness; a*, b*: chromatic components; C*: chroma; Hue angle (*h*) = arctan (b*/a*) (degree).

^wLocation: the explanation of the locations of nine species (15 accessions) were shown in Fig. 1.

Industries, Tokyo) at Commission Internationale de l'Éclairage (CIE) C/2° illumination/viewer condition. CIE made a method to measure and express the colors through value of L*, a* and b*. Within the CIELAB color coordinates, lightness (L*) describes the lightness of the color, going from black (L* = 0) to perfect white (L* = 100); chromatic component a* takes a positive value for reddish colors and a negative value for the greenish ones; chromatic component b* takes a positive value for yellowish colors and a negative value for the bluish ones (Gonnet, 1998). An average of five measurements was used. Chroma (C*) and hue angle (*h*) were calculated based on the following equations: $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h = \tan^{-1}(b^*/a^*)$ (Gonnet, 1998; Gonnet and Hieu, 1992). After measuring the parameters of petal colors, the petals were dried in a FD-1T vacuum freeze dryer (Eastern Kingray Science and Technology, Beijing, China) at -45 °C for 24 h and conserved in vacuum dryer until extraction.

Sample extraction

Petals of each sample [≈50 to 100 mg dry weight (DW)] were extracted with 5 mL of acidic methanol solution (70 : 27 : 2 : 1; by volume, CH₃OH:H₂O:HCOOH:CF₃COOH) in a test tube and shaken in a QL-861 vortex (Kylin-bell Laboratory Instruments, Jiangsu, China) at 4 °C for 24 h (Hashimoto et al., 2000). Extractions were filtered continuously with a filter paper (15 mm) and Millipore disk (0.45 μm) before HPLC analysis. This experiment was performed three times for each sample.

High-performance liquid chromatography–diode array detection–electrospray ionization–mass spectrometry analysis of anthocyanins

QUALITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–DIODE ARRAY DETECTION ANALYSIS OF ANTHOCYANINS. Chromato-

graphic analysis was carried out on a HPLC (Dionex, Sunnyvale, CA) equipped with a P680 HPLC Pump, thermostatted Column Compartment TCC-100, and a Dionex photodiode array detector, DAD-100. The HPLC column was TSK gel ODS-80Ts QA (150 × 4.6 mm i.d.) (Tosoh, Tokyo) and was protected with a Transgenomic CARB Sep Coregel 87C Guard Cartridge (Transgenomic, Omaha, NE). The temperature was set at 35 °C. Injection volume was 10 μL. Flow rate was 0.8 mL·min⁻¹. The mobile phase consisted of 1.5% phosphoric acid water solution [1.5 H₃PO₄:98.5 H₂O (v/v)] as solvent A and phosphoric acid/formic acid/acetonitrile/water [1.5 H₃PO₄ : 20 HCOOH : 25 CH₃CN : 53.5 H₂O (by volume)] as solvent B (Sakata et al., 1995). The linear gradient was 20% B at 0 min, 70% B at 80 min, and then returned to 20% B in 10 min. Data were recorded on a computer with the Chromeleon software (Dionex). Chromatograms were acquired at 515 and 340 nm and photodiode array spectra were recorded between 200 to 800 nm. Anthocyanins were primarily identified by comparison with the data, which were obtained from the authentic samples.

Each peak was identified clearly by HPLC-ESI-MSⁿ (Agilent Technologies, Palo Alto, CA). The interface was an API electrospray (Agilent 1100 LC/MSD Trap VL) operating in positive ionization mode monitoring of the protonated molecular ions at the following operating conditions: gas (N₂) temperature = 350 °C, flow rate = 6.0 L·min⁻¹, nebulizer pressure = 241.3 kPa, octopole radiofrequency amplitude = 150 V, skim 1 voltage = 45.5 V, skim 2 voltage = 6.0 V, capillary exit = 123.9 V, and cap exit offset = 78.4 V. The temperature was set at 35 °C. The flow rate was 0.8 mL·min⁻¹ and the injection volume was 5 μL. The mobile phase was made up of solvent A (0.1% trifluoroacetic acid in water) and solvent B (acetonitrile). The gradient was 5% B at 0 min, 30% B at 40 min, and then returned to 5% B in 10 min. The ion trap mass

spectrometer scanned from m/z 100 to 800. The LC/MSD Trap software version 5.2 provided complete control over all instrumentation (Fu et al., 2006).

SEMIQUANTITATIVE ANALYSIS OF TOTAL ANTHOCYANINS AND TOTAL FLAVONES AND FLAVONOLS. The amount of total anthocyanins [TA (milligrams per 100 mg DW)] and total flavones and flavonols [TF (milligrams per 100 mg DW)] were measured semiquantitatively from a simple linear regression using Mv3G5G for TA and rutin for TF as standards at 515 and 340 nm, respectively. Copigmentation index (CI) was calculated by the formula: $CI = TF/TA$ (Wang et al., 2001a, 2001b, 2004; Zhang et al., 2007, 2008).

Statistical analysis

Cluster analysis was performed using SPSS (version 13.0 for Windows; SPSS, Chicago). Hierarchical cluster analysis among 15 accessions was performed based on the squared Euclidean distance by SPSS considering the color parameters (i.e., L^* , a^* , b^* , C^* , and h), the content of individual anthocyanins [i.e., Pn3G5G, Pn3G5Ara, Pn3G, Cy3G5G, cyanidin 3-glucoside-5-galactoside (Cy3G5Gal), Cy3G, Pg3G5G, and pelargonidin 3-glucoside-5-galactoside (Pg3G5Gal)] (each relative percentage of total peak area), and TA and TF values as variables. The between-group linkage cluster method was used to calculate the distance of each cluster. The graphical representations were performed using Sigmaplot (version 10.0 for Windows; SPSS).

Results

Identification of flower colors by CIELAB color coordinates

RHSCC values of plain petals ranged from 2 D (yellow) to 78 D (pink), including white (Table 1). The flower colors of all the accessions were distributed on the CIELAB color coordinates: $L^* = 30.2$ in *P. intermedia* to 92.6 in *P. mlokosewitschii*, $a^* = -15.3$ in *P. mlokosewitschii* to 44.3 in *P. daurica*, $b^* = -15.1$ in *P. anomala* to 23.5 in *P. mlokosewitschii*, $C^* = 8.8$ in pink *P. lactiflora* to 45.1 in *P. daurica*, and $h = -24.5$ in *P. anomala* ssp. *veitchii* to 132.7 in white *P. lactiflora* (Table 1). The color parameters of *P. mlokosewitschii* with yellow flowers was vastly different from other samples, and this characterization facilitated distinguishing it from other wild accessions (Figs. 2 and 3).

Chromatographic anthocyanin identification

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-DIODE ARRAY DETECTION TOGETHER WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-MASS SPECTROMETRY ANALYSIS OF ANTHOCYANINS. Five major anthocyanins (Pn3G5G, Pn3G, Cy3G5G, Cy3G, Pg3G5G) captured at 515 nm were the same as those reported previously by Hosoki and Seo (1991). However, Pg3G, which was detected in trace amounts by Hosoki and Seo (1991), was not detected in our research. Three rare anthocyanins, including Cy3G5Gal (Fig. 4, peak 1), Pg3G5Gal (Fig. 4, peak 3), and Pn3G5Ara (Fig. 4, peak 6), were detected in wild herbaceous peony species and subspecies for the first time.

No shoulder at 290 to 340 nm was observed in the ultraviolet absorption spectra in peaks 1, 3, and 6; thus, it indicated that no aromatic acids were involved in (Fossen and Andersen, 1998). The position of attachment of the sugars of peaks 1, 3, and 6 were preliminarily identified by their

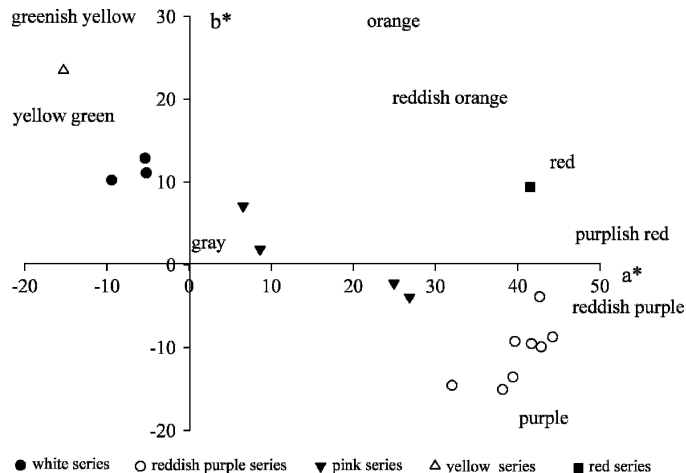


Fig. 2. Two-dimensional scattered picture of nine wild herbaceous peony species (15 accessions) in genus *Paeonia* based on the color parameters of a^* and b^* .

ultraviolet-visible characteristic spectral properties (Harborne, 1958a, 1958b).

The absorption spectra did not have the distinct shoulder to the main absorption peak in the 410 to 450 nm, which indicated that they contain sugars in the 5-position (Asen and Budin, 1966; Harborne, 1963). The ratio of absorption at 440 nm to the absorption at maximum visible absorption of three anthocyanins was calculated and compared with the control standards of tree peony (Tables 2 and 3). The results indicated that all anthocyanins contain sugars in both 3- and 5-positions (Francis, 1982).

The chemical structure of anthocyanins was identified by comparison with the MS/MS data of the authentic samples. In the HPLC chromatograms of Chinese wild species, *P. anomala* and *P. anomala* ssp. *veitchii* (Fig. 4; Tables 2 and 3), peaks 1 and 2, shared the same MS data [$(M)^+$ m/z 611, MS/MS m/z 449, and 287 (cyanidin) $^+$]. Peaks 3 and 4 also shared the same MS data [$(M)^+$ m/z 595, MS/MS m/z 433 and 271 (pelargonidin) $^+$]. All of them contained an anthocyanidin plus

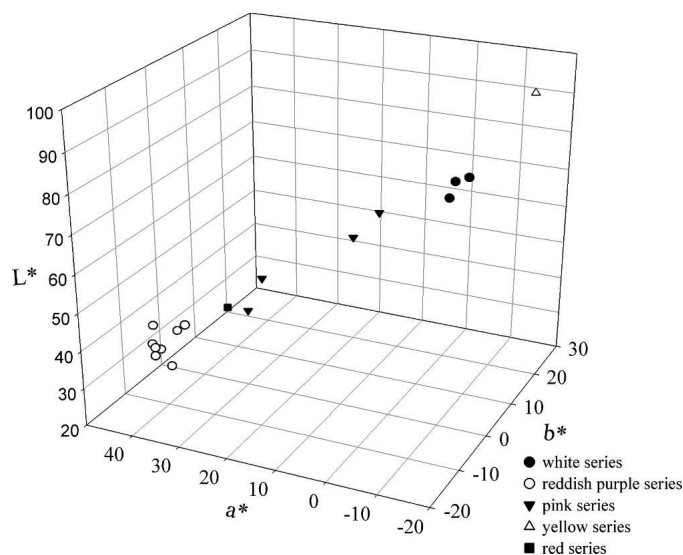


Fig. 3. Three-dimensional picture of nine wild herbaceous peony species (15 accessions) in genus *Paeonia* based on the color parameters of L^* , a^* , and b^* .

two hexoses. By comparing with the control standards of tree peony (Wang et al., 2001a), peaks 2 and 4 were identified as the 3,5-diglucosides of cyanidin and pelargonidin, respectively. Peaks 1 and 3 exhibited the same MS data as peaks 2 and 4 but shorter retention time (Tables 2 and 3). Considering the slight difference on retention time between them, peaks 1 and 3 should be two anthocyanins with higher polarity (Nicoué et al., 2007). Thus, peaks 1 and 3 were possibly considered to be Cy3G5Gal and Pg3G5Gal (Wu and Prior, 2005a, 2005b). However, the complete identification of chemical structure is now underway.

A unique peak (Fig. 4, peak 6) was detected from most European wild species and subspecies that was not detected in any Chinese accessions. Its ultraviolet-visible spectral chromatography showed the λ_{\max} at 514.1 nm, which was very close to those of Pn3G5G and Pn3G (513.5 and 515.5 nm, respectively). It exhibited molecular ions at m/z 595 and a major fragment at

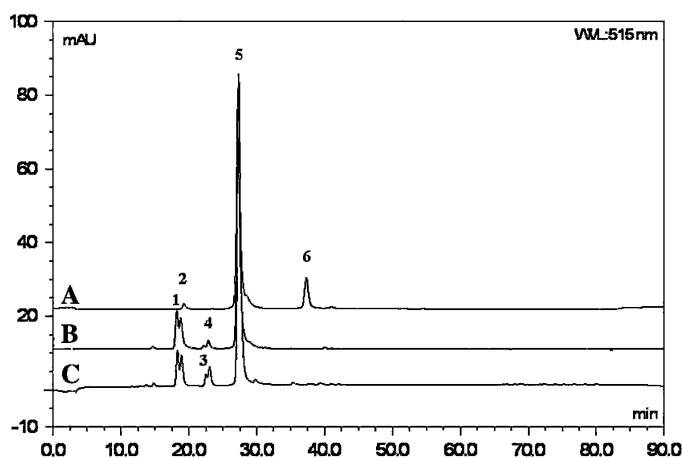


Fig. 4. Typical high-performance liquid chromatograms of anthocyanins in genus *Paeonia* captured at 515 nm: (A) *P. daurica* × *P. mascula* (H02222), (B) *P. anomala* ssp. *veitchii*, (C) *P. anomala*. Peak identification is shown in Tables 2 and 3.

m/z 301, indicating it was a peonidin derivative. The MS/MS data (m/z 463 and 433) showed the anthocyanin contained a hexose and a pentose. However, the ion abundance of (M-132)⁺ (m/z 463) was larger than that of (M-162)⁺ (m/z 433). It indicated that the secondary ion mass spectrum of the anthocyanin was easy to lose m/z 132. Generally speaking, 3- and 5-hydroxyls in anthocyanidins are common glycosylation sites and 5-position of sugar was the most susceptible to break (Cuyckens and Claeys, 2004). Taking into account that arabinose is the common pentose found in nature (Da Silva et al., 2007), peak 6 was tentatively identified as Pn3G5Ara (Fig. 4; Tables 2 and 3), although nuclear magnetic resonance (NMR) spectroscopy or other analytical methods are needed to determine the exact chemical structure.

Floral anthocyanin composition of nine wild herbaceous peony species (15 accessions)

Two anthocyanidins, including Pn and Cy, were detected in most accessions, whereas Pg was only observed in two Chinese species from Xinjiang Uygur Autonomous Region, China (*P. anomala* and *P. intermedia*) and one subspecies (*P. anomala* ssp. *veitchii*) from Gansu Province, China (Table 4). All the accessions were the Pn > Cy phenotype except for a vivid red species (*P. tenuifolia*), which had a high proportion of Cy (89.4%). No anthocyanidins were found in two white accessions (*P. obovata* and *P. daurica* ssp. *wittmanniana*). Most accessions had a high proportion of Pn; in other words, the metabolic pathway of Pn dominated in herbaceous peony species.

The petal anthocyanin composition of Chinese species (seven accessions) was quite different from that of European species (seven accessions). For the Chinese species and subspecies, the distribution patterns of petal anthocyanins were quite simple. Pn3G5G and Cy3G5G were the major anthocyanins in most accessions except for *P. intermedia*, which had maximum anthocyanins, including Pn3G5G, Pn3G, Cy3G5G, Cy3G, and Pg3G5G. In European accessions, Pn3G5G, Pn3G5Ara, and Cy3G5G accounted for high proportions except

Table 2. Anthocyanins identified in the petals of *Paeonia anomala* ssp. *veitchii*, *Paeonia anomala*, and *Paeonia daurica* × *Paeonia mascula* (H02222).

| Peak | λ_{\max}^z | $E_{440}/E_{\text{vis,max}}^z$ (%) ^z | M ⁺ (m/z) ^z | MS/MS (m/z) ^z | Identification | |
|------|--------------------|---|-----------------------------------|--------------------------|--|--|
| 1 | 265.4 ± 1.2 | 512.3 ± 0.3 | 19.1 ± 0.3 | 611 449 287 | Cyanidin-3- <i>O</i> -glucoside-5- <i>O</i> -galactoside | |
| 2 | 277.1 ± 0.8 | 512.4 ± 0.3 | 19.1 ± 0.3 | 611 449 287 | Cyanidin-3,5-di- <i>O</i> -glucoside | |
| 3 | 265.2 ± 0.1 | 345.3 ± 0.6 | 495.3 ± 0.8 | 25.3 ± 1.1 | 595 433 271 | Pelargonidin-3- <i>O</i> -glucoside-5- <i>O</i> -galactoside |
| 4 | 266.4 ± 0.5 | 273.2 ± 0.3 | 497.6 ± 0.1 | 26.6 ± 0.2 | 595 433 271 | Pelargonidin-3,5-di- <i>O</i> -glucoside |
| 5 | 247.9 ± 1.3 | 277.0 ± 0.3 | 513.5 ± 0.2 | 18.0 ± 0.1 | 625 463 301 | Peonidin-3,5-di- <i>O</i> -glucoside |
| 6 | 276.8 ± 0.3 | 514.1 ± 0.6 | 17.8 ± 0.1 | 595 433 463 301 | Peonidin-3- <i>O</i> -glucoside-5- <i>O</i> -arabinoside | |

^z λ_{\max} = maximum absorption wavelength (nm); E_{440} = the intensity at 440 nm; $E_{\text{vis,max}}$ = the intensity at maximum visible absorption; M⁺ = molecular ion; MS/MS = fragment ions of secondary ion mass spectrum.

Table 3. Spectral characteristics and mass spectral data of the control standards identified from Chinese tree peony cultivar Qing Long Wo Mo Chi and Japanese tree peony cultivar Hohki.^z

| Peak | λ_{\max}^y | $E_{440}/E_{\text{vis,max}}^y$ (%) ^y | M ⁺ (m/z) ^y | MS/MS (m/z) ^y | Identification | |
|------|--------------------|---|-----------------------------------|--------------------------|--------------------------------------|--|
| 2 | 276.9 | 512.5 | 19.1 | 611 449 287 | Cyanidin-3,5-di- <i>O</i> -glucoside | |
| 4 | 266.8 | 273.2 | 497.6 | 26.6 | 595 433 271 | Pelargonidin-3,5-di- <i>O</i> -glucoside |
| 5 | 247.9 | 277.2 | 513.5 | 18.0 | 625 463 301 | Peonidin-3,5-di- <i>O</i> -glucoside |

^zWang et al., 2001a; Zhang et al., 2007.

^y λ_{\max} = maximum absorption wavelength (nm); E_{440} = the intensity at 440 nm; $E_{\text{vis,max}}$ = the intensity at maximum visible absorption; M⁺ = molecular ion; MS/MS = fragment ions of secondary ion mass spectrum.

Table 4. Mean petal anthocyanin values of nine wild herbaceous peony species (15 accessions) in the genus *Paeonia*.

| Species | Mean petal anthocyanin (% peak area) ^z | | | | | | | | | | | | | | | TA ^y (mg/100 mg DW) | TF ^y (TF/TA) | CI ^y (TF/TA) | |
|--|---|-----|--------|------|--------|------|------|--------|-----|----------|------|-----|-----------|--------|------|-----------------------------------|----------------------------|----------------------------|----------|
| | Pn | | | Cy | | | Pg | | | Aglycone | | | Glycoside | | | | | | |
| | 3G5G | 3G | 3G5Ara | 3G5G | 3G5Gal | 3G | 3G5G | 3G5Gal | 3G | Pn | Cy | Pg | 3G5G | 3G5Gal | 3G | | | | 3G5Ara |
| Chinese wild species | | | | | | | | | | | | | | | | | | | |
| <i>P. lactiflora</i> (pink) | 93.1 | — | — | 6.9 | — | — | — | — | — | 93.1 | 6.9 | — | 100.0 | — | — | — | 0.0306 | 2.1717 | 70.9407 |
| <i>P. lactiflora</i> (white) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 0.0000 | 2.5212 | ∞ |
| <i>P. obovata</i> (red) | 81.2 | — | — | 18.8 | — | — | — | — | — | 81.2 | 18.8 | — | 100.0 | — | — | — | 1.3720 | 1.3030 | 0.9497 |
| <i>P. obovata</i> (white) | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 0.0000 | 0.4193 | ∞ |
| <i>P. intermedia</i> | 69.1 | 4.4 | — | 23.4 | — | 2.6 | 0.3 | — | — | 73.6 | 26.1 | 0.3 | 92.9 | — | 7.1 | — | 1.3720 | 0.0282 | 0.0205 |
| <i>P. anomala</i> ssp. <i>veitchii</i> | 80.0 | — | — | 7.8 | 9.8 | — | 1.4 | 1.1 | 1.1 | 80.0 | 17.6 | 2.4 | 89.2 | 10.8 | — | — | 0.4198 | 1.1088 | 2.6415 |
| <i>P. anomala</i> | 89.1 | — | — | 4.3 | 3.3 | — | 0.8 | 2.5 | 2.5 | 89.1 | 7.6 | 3.3 | 94.2 | 5.8 | — | — | 0.7911 | 0.6520 | 0.8242 |
| European wild species | | | | | | | | | | | | | | | | | | | |
| <i>P. mascula</i> | 92.5 | — | 4.5 | 3.0 | — | — | — | — | — | 97.0 | 3.0 | — | 95.5 | — | — | 4.5 | 0.5667 | 0.7690 | 1.3569 |
| <i>P. daurica</i> ssp. <i>wittmanniana</i> | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 0.0000 | 0.4180 | ∞ |
| <i>P. daurica</i> subsp. <i>Wittmanniana</i> (flares) | 100.0 | — | — | — | — | — | — | — | — | 100.0 | — | — | 100.0 | — | — | — | 0.0134 | 0.1355 | 10.1422 |
| <i>P. mlkosewitschii</i> | 100.0 | — | — | — | — | — | — | — | — | 100.0 | — | — | 100.0 | — | — | — | 0.0037 | 2.7609 | 738.0199 |
| <i>P. mlkosewitschii</i> (flares) | 92.2 | — | 7.8 | — | — | — | — | — | — | 100.0 | — | — | 92.2 | — | — | 7.8 | 0.0221 | 1.1358 | 51.5046 |
| <i>P. officinalis</i> ssp. <i>humilis</i> | 92.8 | — | — | 7.2 | — | — | — | — | — | 92.8 | 7.2 | — | 100.0 | — | — | — | 1.0483 | 1.1968 | 1.1417 |
| <i>P. mascula</i> ssp. <i>russi</i> | 94.7 | — | 4.0 | 1.3 | — | — | — | — | — | 98.7 | 1.3 | — | 96.0 | — | — | 4.0 | 0.2316 | 1.0075 | 4.3503 |
| <i>P. daurica</i> | 92.3 | — | 6.5 | 1.2 | — | — | — | — | — | 98.8 | 1.2 | — | 93.5 | — | — | 6.5 | 1.3473 | 0.3896 | 0.2891 |
| <i>P. dauricax</i> × <i>P. mascula</i> | 85.6 | — | 12.6 | 1.8 | — | — | — | — | — | 98.2 | 1.8 | — | 87.4 | — | — | 12.6 | 1.4070 | 0.3033 | 0.2156 |
| North American wild species | | | | | | | | | | | | | | | | | | | |
| <i>P. tenuifolia</i> | 6.3 | 4.3 | — | 23.9 | — | 65.5 | — | — | — | 10.6 | 89.4 | — | 30.2 | — | 69.8 | — | 0.6073 | 0.0229 | 0.0377 |

^zPn = peonidin; Cy = cyanidin; Pg = pelargonidin; 3G5G = 3,5-di-O-glucoside; 3G = 3-O-glucoside; 3G5Ara = 3-O-glucoside-5-O-arabinoside; 3G5Gal = 3-O-glucoside-5-O-galactoside.

^yTA = total anthocyanins; TF = total flavones and flavonols; CI = copigmentation index.

for *P. officinalis* ssp. *humilis* and *P. daurica* ssp. *wittmanniana*, which contained no Pn3G5Ara in petals at all. On the contrary, the North American species *P. tenuifolia* had a different anthocyanin composition. Although Pn3G5Ara was not observed, Cy3G and Pn3G were clearly detected in this species.

Paeonia mlokosewitschii and *P. daurica* ssp. *wittmanniana* (Hong and Zhou, 2003) had red flares on the base of petals, which made them double-colored petals. *Paeonia mlokosewitschii* was a yellow-flowered species with red veins on the edge of petals, whereas the main color of *P. daurica* ssp. *wittmanniana* was white and no anthocyanin was detected in its petals. Although both the flare colors and TA values (0.0221 mg/100 mg in *P. mlokosewitschii*; 0.0134 mg/100 mg in *P. daurica* ssp. *wittmanniana*) of the two accessions were quite similar, there were some slight differences in the anthocyanin composition of the flares. Anthocyanins in the flares of *P. mlokosewitschii* were Pn3G5G and Pn3G5Ara, whereas only Pn3G5G was detected in *P. daurica* ssp. *wittmanniana*'s flares. Anthocyanins of both flares were peonidin types, indicating similar biosynthetic pathways.

CLUSTER ANALYSIS. *Paeonia mlokosewitschii* and *P. daurica* ssp. *wittmanniana* belonged to the double color series. Although both of them had clear red flares on the base of the petals, the major colors were yellow and white, respectively. As a result, only the anthocyanins from yellow and white parts were used for principal component analysis (PCA).

Five clusters (1, 2, 3, 4, and 5) were derived (Fig. 5). Each sample cluster had the same flower color and similar anthocyanin composition. Cluster 1 consisted of three white accessions, all of which had no anthocyanin in the petals. Cluster 2 consisted of one yellow species, *P. mlokosewitschii*, which contained only Pn3G5G in the red veins of petals. Cluster 3 consisted of eight accessions with a reddish purple color. The total anthocyanins amount of each accession in this cluster was relatively high, ranging from 0.4198 mg/100 mg DW in *P. anomala* ssp. *veitchii*

to 1.4070 mg/100 mg DW in *P. daurica* × *P. mascula* (Table 4). Cluster 4 consisted of two pink accessions, including *P. lactiflora* (pink) and *P. mascula* ssp. *russi*. Cy3G5G and Pn3G5G were the main anthocyanins in this cluster. The TA value was very low compared with cluster 3. Cluster 5 was composed of one red species, *P. tenuifolia*, and its anthocyanin composition was far different from those of other accessions.

Discussion

Anthocyanin composition of four Chinese wild species (seven accessions)

The anthocyanin composition of Chinese wild herbaceous peony species was very simple. A majority of accessions in this study only had Pn3G5G and Cy3G5G. Pg3G5G was only detected in *P. intermedia* and *P. anomala*, the exclusive wild species from Xinjiang Uygur Autonomous Region (Hong et al., 1994), and *P. anomala* ssp. *veitchii* from Gansu Province, China (Hong and Pan, 2004). The different distribution patterns of anthocyanins indicated different biosynthetic and metabolic pathways between wild species from Xinjiang Uygur Autonomous Region and those from other places in China. There were no pelargonidin types of anthocyanins detected in most accessions except for the three accessions mentioned previously. It indicated that the enzyme flavonoid 3'-hydroxylase (F3'H) was more competitive in the use of substrate than dihydroflavonol 4-reductase. F3'H catalyzed naringenin directly to synthesize eriodictyol and prevented the synthesis of dihydrokaempferol and sequentially interrupted the pathway of Pg synthesis (Nakayama et al., 1997; Schwinn et al., 1994).

Based on the original taxonomy of *Paeonia*, section *Paeonia* (Paeoniaceae), *P. anomala* in Xinjiang Uygur Autonomous Region, and *P. veitchii* Lynch in Gansu Province, China, were classified into two different species (Guo, 2002). However, Hong et al. (2001) made a taxonomic revision of *Paeonia*. *P. veitchii* was treated as the subspecies of *P. anomala*, namely *P. anomala* ssp. *veitchii*, for their similar blooming characteristics and variation in leaf shape and hairs on carpels among samples (Hong and Pan, 2004). According to our study of the anthocyanin compositions of these two accessions, we supported this revision because the unique anthocyanins of Cy3G5Gal (Fig. 4, peak 1) and Pg3G5Gal (Fig. 4, peak 3) had been detected in *P. anomala* and *P. anomala* ssp. *veitchii* indicating they belong to the same taxon.

Anthocyanin composition of four European wild species (seven accessions)

A new anthocyanin, Pn3G5Ara, was detected in European wild species (five accessions). However, it was not detected in any Chinese species and subspecies. According to the evolutionary theory of anthocyanins in previous research (Harborne, 1977), angiosperms in different climate areas have different anthocyanin metabolic pathways. Pelargonidin types only have appeared in very advanced tropical angiosperm families, whereas delphinidin types have occurred in temperate climates producing the delphinidin colors common in bee-pollinated families (Cooper-Driver, 2001; Zhao et al., 2005). Therefore, we assumed that the difference of anthocyanin composition between Chinese and European species may be caused by their different habitats and climatic types between the two zones, although further research is needed to study the evolutionary difference of structures and sugars.

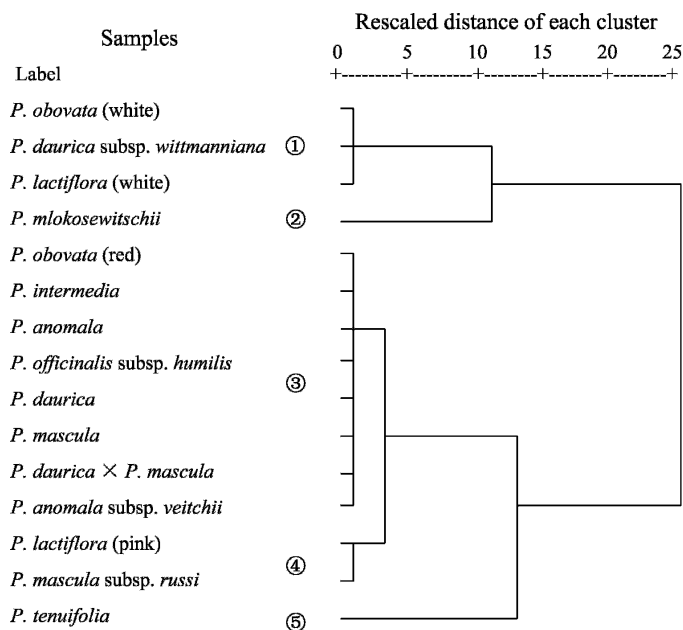


Fig. 5. Dendrogram of nine wild herbaceous peony species (15 accessions) in genus *Paeonia* by hierarchical cluster analysis. The cluster analysis was performed based on the Squared Euclidean distance by SPSS (version 13.0 for Windows; SPSS, Chicago, IL).

The morphological characters of *P. daurica* and *P. mascula* were similar. Recently, the discrimination of these two species had been determined by Hong et al. (2007). *Paeonia daurica* was shown to be clearly differentiated from *P. mascula* in the number of leaflets of the lower leaves. In 21 May 2002, Hong et al. (2007) found a special sample coded H02222 (*P. daurica* × *P. mascula*) in Amasya, Ladik, to Tasova, Turkey (985 m altitude). All characteristics showed it was similar to that of *P. daurica* except for the number of leaflets. Although most leaflet numbers were nine, there remained an exception of 10. This phenomenon was not consistent with *P. daurica*, which consistently had nine leaflets. Considering its location in a sympatric region of the two species, Hong et al. (2007) inferred it to be a hybrid between *P. daurica* and *P. mascula* (*P. daurica* × *P. mascula*) because it closely resembled *P. daurica*. In our study, we found the anthocyanin compositions of *P. daurica*, *P. mascula*, and H02222 (*P. daurica* × *P. mascula*) were identical except for total anthocyanin amount (Table 4). To verify if H02222 was from hybridization, we made an intersectional cross (*P. daurica* × *P. mascula*) and a reverse intersectional cross (*P. mascula* × *P. daurica*) in Apr. 2007 and obtained the resultant seeds. Results validated the cross-compatibility between *P. daurica* and *P. mascula*, although more information of these crosses should be observed and studied in the future.

Comparison on anthocyanin composition between herbaceous peony and tree peony

The tree peony and herbaceous peony belong to the same genus. They are partly similar in morphological, chiefly in the flower forms and colors. However, based on our investigation and measurements of flower colors, we found herbaceous peony lacks real vivid red color as compared with that of tree peony. Compared with the anthocyanin composition of tree peony, the most remarkable characteristic of herbaceous peony was that no Pg3G was detected in the petals. In addition, Pn3G5Ara, Cy3G5Gal, and Pg3G5Gal were unique anthocyanins in herbaceous peony. Based on our results, the mechanism of the formation of vivid red flower was quite different from tree peony to herbaceous peony, although the anthocyanin composition of them was similar. Hosoki et al. (1991) and Wang et al. (2001a) found that wild tree peony species lacked Pg3G, whereas Japanese vivid red cultivars lacked Cy3G and Cy3G5G but contained a large amount of Pg3G and Pg3G5G. Therefore, they assumed that the absence of Pg3G might be the reason for the shortage of red-flowered tree peony in China. Nevertheless, in herbaceous peony, we fortunately found a red wild species, *P. tenuifolia*, which had an obviously different anthocyanin composition. In general, Pn3G5G and Cy3G5G were the main anthocyanins in herbaceous peony and most accessions belonged to the Pn > Cy phenotype. The content of 3G-type pigments was much lower than that of 3G5G type. *P. tenuifolia* belonged to the Cy > Pn phenotype and the contents of Cy3G and Cy3G5G were up to 65.5% and 23.9%, respectively. The content of 3G type (69.8%) was much higher than that of 3G5G type (30.2%). The anthocyanin composition of *P. tenuifolia* was opposite to that of Japanese red tree peony cultivars. In other words, the former lacked Pg3G and Pg3G5G but contained a large amount of Cy3G and Cy3G5G in the petals. Taking into account that high content of Cy3G is responsible for the vivid red coloration of many red flowers such as *Camellia japonica* L. (Sakata, 1988), we hypothesized that the absence of Cy3G in the petals might be the reason for

the shortage of red-flowered herbaceous peony, although more samples should be examined to verify this conclusion.

The correlations between flower colors and different factors

Considering the anthocyanin compositions of all the species of herbaceous peony, we found that glycosylation and methylation were two major factors that influence flower colors.

Copigmentation effects, which were expected to occur when the TF/TA ratio exceeded 5 (Asen et al., 1971), were not observed in most herbaceous peony species (Table 4). As a result, copigmentation effect was not an important factor to influence the flower color of herbaceous peony. However, it is well known that petal colors are affected by many different factors. There is a lot of literature (Cooper-Driver, 2001) about the color characteristics of flowers in different species and different factors affecting the colors [pH, glycosylation, acylation, copigmentation, self-association, environment factors (light, temperature, soil, and so on)]. In general, the anthocyanins are mainly affected by the genetic factors, but the environmental factors have also certain effects. So further investigations are needed to make clear how different anthocyanins affect the final colors and the correlations between flower colors and various environment factors.

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