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Research article

Anatomical and biochemical analysis reveal the role of anthocyanins in flower coloration of herbaceous peony

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ABSTRACT

Herbaceous peony (*Paeonia lactiflora* Pall.) is particularly appreciated because of its elegant and gorgeous flower color, but little is known about the underlying mechanisms of flower coloration. In this study, three *P. lactiflora* cultivars 'Xuefeng', 'Fenyulou' and 'Dahonglou' with white, pink and red flower were selected as the materials. Their anatomical structures, cell sap pH and metal elements were investigated, and the colored pigment mainly distributed in palisade mesophyll was only found in 'Fenyulou' and 'Dahonglou', and their shape of epidermal cells, cell sap pH and metal elements were not the key factors deciding phenotype color. Moreover, the qualitative and quantitative analysis of flavonoids were performed, their total anthocyanin, anthoxanthin and flavonoid contents were decreased during flower development, and only anthocyanin content in 'Dahonglou' was always higher than that in 'Xuefeng' and 'Fenyulou'. Subsequently, three anthocyanin compositions were found, and peonidin 3,5-di-*O*-glucoside (Pn3G5G) was identified as the main anthocyanin composition. In addition, the full-length of flavonol synthase gene (*FLS*) was isolated with the GenBank accession number KM259902, and the expression patterns of eight flavonoid biosynthetic genes showed that only *PlDFR* and *PlANS* basically had the highest levels in 'Dahonglou' and the lowest levels in 'Xuefeng', and they basically displayed a descended trend during flower development especially *PlDFR*, suggesting that these two genes might play a key role in the anthocyanin biosynthesis which resulted in the shift from white to pink and red in flowers. These results would contribute to understand the underlying molecular mechanisms of flower coloration in *P. lactiflora*.

1. Introduction

Flower color is one of the most important features in ornamental plants, which not only affects their ornamental merit but also directly influences their commercial value. Until now, the studies on the flower color in ornamental plants are enormous, such as *Lycoris longituba* (He et al., 2011), *Rosa hybrida* (Schmitzer et al., 2012), *Paeonia suffruticosa* (Zhao et al., 2015a) and *Narcissus tazetta* (Li et al., 2015). And we have reviewed the recent advances on the development and regulation of flower color in ornamental plants, its development is influenced by many internal and external factors (Zhao et al., 2015b). Among these factors, pigment plays a major role, which can be generally divided into three groups, carotenoids, flavonoids and alkaloids according to their chemical structures, cellular localizations and biochemical synthesis pathways. And flavonoids have been extensively studied in many kinds of plants, and they are the decisive pigment presented in some flower colors (Tanaka and Brugliera, 2013; Li et al., 2015). Meanwhile, petal tissue structure contributes to the distinctively color phenotypes. In *Muscari latifolium*, the blue cells of the pale blue flowers are only located in the palisade mesophyll, while the location of pigment in the purple flowers are mainly in the lower epidermis and the sub-epidermal layer of the outer face of the tepal (Qi et al., 2013). And Noda et al. (1994) find that flower color intensity depends on the specialized cell shape, when magenta *Antirrhinum majus* is mutated to pink, conical epidermal cells become flat. In addition, vacuolar pH and metal ions are also related to the color development. Cheng et al. (2014) propose the lower pH value, the darker flower red color in *R. hybrida*. In *Hydrangea macrophylla*, aluminum plays an important role in red-to-blue color changes (Ito et al., 2009; Schreiber et al., 2011). Therefore, considering these factors will help to fully elucidate the complicated formation mechanism of flower color.

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Herbaceous peony (*Paeonia lactiflora* Pall.) is a traditionally famous flower in China and a high-grade cut flower in the euro-American countries, which contains nine flower color categories including white, pink, red, purple, black, blue, yellow, green and double color (Wang and Zhang, 2005). However, most of *P. lactiflora* cultivars still concentrate on white, pink, red and their derivative (Wu, 2006). And in *P. lactiflora*, the studies of flower color are focused on the chemical constituents, and the color is determined by flavonoids including anthocyanins and multiform glycosides of flavones and flavonols (Jia et al., 2008; Zhao et al., 2012b, 2014). Jia et al. (2008) analyze the anthocyanins of 41 *P. lactiflora* cultivars, 1 white *P. lactiflora* cultivar can not detect anthocyanins while peonidin 3,5-di-*O*-glucoside (Pn3G5G) is identified in the other 3 white *P. lactiflora* cultivars, deep purple or reddish purple cultivars contain 4-5 anthocyanins, whereas pink cultivars only contain cyanidin 3,5-di-*O*-glucoside (Cy3G5G) and Pn3G5G, and their contents are much lower than those of purple cultivars. Meanwhile, Zhao et al. (2012b) find a great deal of anthoxanthins in white and purplish-red *P. lactiflora* cultivars. All these informations provide a physiological and biochemical basis to understand the formation of white, pink and red flower colors in *P. lactiflora*. However, little is known about the effects of petal tissue structure, vacuolar pH and metal ions on white, pink and red flower colors in *P. lactiflora*, and the studies on the relationship between flavonoids and the changes of flower color during the flowering period are less. In order to investigate more precisely the causes of white, pink and red flower colors development in *P. lactiflora*, we firstly observed anatomical structures and determined cell sap pH as well as metal elements in relation to three *P. lactiflora* cultivars with different colors. Next, we performed the qualitative and quantitative analysis of flavonoids, and detected the expression patterns of the flavonoid biosynthetic genes. These results could determine the mechanism of three main colors development in *P. lactiflora*.

2. Materials and methods

2.1. Plant materials

Three P. lactiflora cultivars with different colors (white: 'Xuefeng', pink: 'Fenyulou', red: 'Dahonglou') (Fig. 1) were selected from the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32°30′ N, 119°25′ E). These flowers were picked from April to May, which could be divided into four developmental stages including flower-bud stage (Stage 1, S1), initiating bloom stage (Stage 2, S2), bloom stage (Stage 3, S3) and wither stage (Stage 4, S4). Some petals were used immediately for the color indices measurement as well as anatomy observations, and the others were stored at −80 °C for cell sap pH, metal elements, flavonoid and gene expression analysis.

Fig. 1. Flowers of three *P. lactiflora* cultivars with different colors. (a) 'Xuefeng'. (b) 'Fenyulou'. (c) 'Dahonglou'. S1 (Stage 1) = flower-bud stage, S2 (Stage 2) = initiating bloom stage, S3 (Stage 3) = bloom stage, S4 (Stage 4) = wither stage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Color indices measurement

The color indices of fresh petals were measured with a TC-P2A chroma meter on the CIE (Beijing Optical Instrument Factory, China) using three color parameters including L^*, a^* and b^* values.

2.3. Anatomy observations

Microscopic observation of outer epidermal cells and transverse sections were performed using free-hand sections. The petals were firstly cross-sectioned, and an epidermal layer was subsequently peeled off which were placed on a glass slide with a drop of water. Moreover, they were immediately observed by a light microscope (Olympus CX31RTSF, Tokyo, Japan).

The shape of epidermal cells were observed by the environmental scanning electron microscopy (Philips XL-30 ESEM, Amsterdam, The Netherlands), and the specific operational method was referred to the report of Zhao et al. (2015a).

2.4. Cell sap pH determination

5 g petals of each sample were ground with liquid nitrogen and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to 10 mL centrifuge tube, and then a Sartorius PB-10 pH meter (Beijing Sartorius Scientific Instrument Corporation) was used to determinate its pH value.

2.5. Metal elements measurement

The petals of each sample were firstly put in oven for 10 min at 105 °C after washing with deionized water, and then dried to constant weight at 70 °C as well as ground into powder. Subsequently, 5 mL HNO_3, 3 mL ultrapure water and 2 drops of H_2O_2 were added to 0.5 g powder, which were decomposed with the microwave digestion instrument (MARS6, CEM, USA). After digestion, the solution was diluted with ultrapure water to 50 mL. On this basis, 9 mL ultrapure water was added to 1 mL analyzed solution, and its metal elements were measured using the Inductively Coupled Plasma Emission Spectrometer (ICP) (iCAP6300, Thermo Fisher, USA).

2.6. Qualitative and quantitative analysis of flavonoids

The petals of each sample (1.0 g fresh weight) were extracted with 6 mL of acidic methanol solution (70: 0.1: 29.9; v/v/v, CH_3OH: HCl: H_2O) at 4 °C for 24 h. Qualitative and quantitative analysis of flavonoids was performed using HPLC-ESI-MS^n (LCQ Deca XP MAX, Thermo) coupled with photodiode array and mass spectrometry detectors (HPLC-PDA-MS, Thermo company) with a three-dimensional quadrupole ion trap mass spectrometer. The HPLC column was TSK gel ODS-80Ts QA (4.6 mm × 250 mm) (Tosoh, Japan). The specific conditions were the same as the report of Zhao et al. (2014) with some modifications. Each peak area of anthocyanins and anthoxanthins detected under 525 nm and 350 nm was recorded. Malvidin-3,5-di-*O*-glucoside (Mv3G5G) and rutin were used as the references for the relative quantitative analysis of anthocyanins and anthoxanthins,