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Research article

Flower color patterning in pansy (*Viola* × *wittrockiana* Gams.) is caused by the differential expression of three genes from the anthocyanin pathway in acyanic and cyanic flower areas

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ABSTRACT

The petals of pansy (*Viola* × *wittrockiana* Gams.) 'Mengdie' exhibit a cyanic blotched pigmentation pattern. The accumulation of anthocyanins, cyanidin and delphinidin, was detected in the upper epidermal cells of the cyanic blotches.

In order to elucidate the mechanism by which cyanic blotches are formed in pansy petal, the expression level of genes involved in anthocyanin synthesis was measured and compared between cyanic blotches and acyanic areas of the flower. The use of primers in conserved regions allowed the successful isolation of six cDNA clones encoding putative anthocyanin enzymes from pansy petals. The clones isolated encoded chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS).

The transcription patterns of seven genes (*VwCHS*, *VwCHI*, *VwF3H*, *VwF3′H*, *VwDFR*, *VwF3′5′H*, and *VwANS*) in cyanic blotches and acyanic areas of the petals at seven stages of flower development were determined by real-time quantitative PCR. Transcription of *VwF3′5′H*, *VwDFR* and *VwANS* was significantly increased in cyanic blotches at stages III–V of flower development, implicating these genes in the pigmentation of *Viola* × *wittrockiana* Gams. petals.

1. Introduction

Petal blotches are groups of pigmented cells that occur in a defined region of the petal, which can affect the behavior of pollinators and the ornamental value of flowers (Moeller, 2005; Eckhart et al., 2006; Shang et al., 2011). Previous studies have shown that flower blotches can develop as a result of anthocyanin accumulation. For example, cyanidin-based glycosides, which accumulate abundantly at the basal petal, cause cyanic blotches in the petals of the Xibei tree peony (*Paeonia suffruticosa*) (Zhang et al., 2007). In *Oncidium*, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, delphinidin-3-*O*-diglucoside and cyanidin-3-*O*-glucoside are the most common anthocyanins found in petal and sepal blotches (Hieber et al., 2006; Chiou and Yeh, 2008).

Anthocyanins are a class of flavonoid compounds responsible for pink, orange, red, scarlet, purple, blue and cyanic flower coloration in many plant species. The anthocyanin pathway, which is a branch of the flavonoid pathway, has been elucidated in lots of other plants as well (Holton and Cornish, 1995; Grotewold, 2006; Katsumoto et al., 2007). A simplified anthocyanin pathway is illustrated in Fig. 1 (Tanaka et al., 2005; Yuan et al., 2013). Malonyl-CoA and 4-coumaroyl-CoA are the precursors for the formation of flavonoids. Dihydroflavonols are formed by the consecutive action of CHS, CHI and F3H and can be further converted to leucoanthocyanidins by DFR. Additional hydroxyl groups in the B-ring of the intermediates may be introduced by F3′5′H and F3′H.

Abbreviations: ANS, anthocyanidin synthase; AT, acyltransferase; cDNA, complementary DNA; CHI, chalcone isomerase; CHS, chalcone synthase; Ct, cycle threshold; DAD, diode-array detector; DFR, dihydroflavonol 4-reductase; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; GT, glycosyltransferase; MT, methyltransferase; RACE, rapid amplification of cDNA ends; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; RT-qPCR, real-time quantitative PCR.

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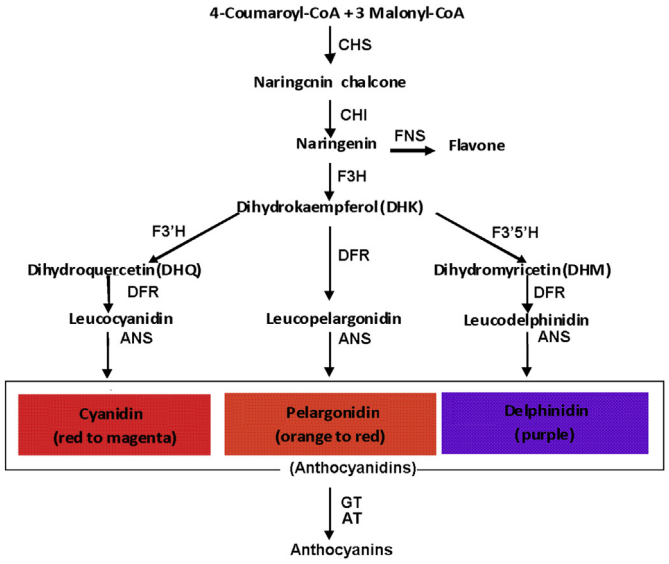

Fig. 1. Simplified anthocyanin pathway. The enzymes that catalyze each step are indicated by the following abbreviations: chalcone synthase (CHS); chalcone isomerase (CHI); flavanone 3-hydroxylase (F3H); flavonoid 3′-hydroxylase (F3′H); flavonoid 3′,5′-hydroxylase (F3′5′H); flavone synthase (FNS); dihydroflavonol 4-reductase (DFR); anthocyanidin synthase (ANS); glycosyltransferase (GT); acyltransferase (AT).

Leucoanthocyanidins carrying one, two or three hydroxyl groups in the B-ring are converted to pelargonidin, cyanidin and delphinidin respectively. Almost all anthocyanidins undergo several modifications, including glycosylation, acylation, and methylation, catalyzed by glycosyltransferase (GT), acyltransferase (AT) and methyltransferase (MT), respectively (Tanaka et al., 2005; Yuan et al., 2013).

Floral blotches appear on petals as a result of differential expression of the genes required in anthocyanin biosynthesis. In *Oncidium*, up-regulation of the genes *OgCHI* and *OgDFR* results in increased anthocyanin production and pigmented spot formation in the yellow lip (Chiou and Yeh, 2008). In *Lilium* 'Sorbonne', expression of *LhCHSA*, *LhCHSB* and *LhDFR* is elevated within the spots located in the center of the petals, in comparison to the low expression levels in the margin (Yamagishi, 2010). In *Dendrobium moniliforme*, pigment accumulation in the base of the column has been established to be a consequence of preferential expression of *DmF3′5′H* (Whang et al., 2011). In *Clarkia gracilis*, precise spatio-temporal regulation of the expression of the anthocyanin genes F3′H, F3′5′H, *DFR1* and *DFR2* produces spotted petals (Martins et al., 2013).

Pansy (*Viola* × *wittrockiana* Gams.) is an important biennial plant of the family Violaceae. It is widely grown as a kind of main commercial flower in spring and its colorful white, yellow, red, purple, or orange flowers make it a popular landscaping plant. Some cultivars of pansy produce flowers with large cyanic blotches on the petals. The anthocyanin content of the cyanic blotched areas of pansy petals has been analyzed in previous studies. For example, purple blotches on the white flowers of the cultivar 'Mont Blanc' contain cyanidin-*p*-coumarylglycoside, while the purple blotches on the yellow flower of 'Rhinegold' contains delphinidin-3:5-*p*-coumaryl glucorhamnoside (Endo, 1959). Some genes or expressed sequence tags (ESTs), CHS, DFR, and ANS, have been isolated from a close relative of the pansy, *Viola cornuta* (Farzad et al., 2003). In this research, we sought to further elucidate the molecular mechanisms of blotch formation in the pansy, and thus to facilitate future molecular modification of the pansy to modulate pansy flower color.

The cDNAs of six genes (*VwCHS*, *VwCHI*, *VwF3H*, *VwDFR*, *VwF3′H*, and *VwANS*) involved in anthocyanin biosynthesis in pansy were isolated. Using real-time quantitative PCR, we analyzed the transcript levels of these six genes and F3′5′*H* (Katsumoto et al., 2007) in the cyanic blotches and acyanic areas of the petals at seven stages of flower development. These results provide new insight into the spatial and temporal patterns of anthocyanidin synthesis in pansy, and provide a basis for a broader understanding of the formation and pigmentation of petal cyanic blotches.

2. Methods

2.1. Plant materials

Viola × *wittrockiana* Gams. 'Mengdie' (yellow petals with purple blotches, Fig. 2) plants were grown at the horticultural farm of Hainan University (Latitude: 20.03°N, longitude: 110.33°E), Haikou, Hainan Province, China. Petal samples were collected from pansy flowers at seven different developmental stages (Fig. 2). The petal tissues were divided into two parts; cyanic blotches and acyanic areas, for total anthocyanin and anthocyanidins analyses and RNA extraction. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Observations of petal anatomy

Fresh cyanic and acyanic areas of pansy petals were cut into segments longitudinally (approx. 10 mm × 5 mm), and the upper epidermis and dorsal epidermis were separated with forceps. The sections were observed and photographed with a light microscope.

2.3. Determination of anthocyanidin content

Fresh petal samples (0.3 g) were ground in liquid nitrogen and submerged in 6 mL extraction solution of methanol containing 0.1% hydrochloric acid with gentle shaking at 4 °C for 24 h. Extracts were centrifuged for 15 min at 12,000 *g*. The supernatant, which contained the anthocyanin (Zhang et al., 2012), was hydrolyzed at 100 °C for 60 min and filtered through a 0.22-μm filter. A HyperSep C18 solid phase micro-extraction column was activated with 5 mL methanol containing 0.01% hydrochloric acid, 5 mL ethyl acetate, and 5 mL water containing 0.01% formic acid, successively. A 2-mL aliquot of the anthocyanidin extract was loaded onto the extraction column. Firstly proteins and sugars were eluted with 10 mL water containing 0.01% formic acid, then, polyphenols were eluted with 5 mL ethyl acetate, and finally anthocyanidins were eluted with methanol containing 0.01% hydrochloric acid.

Anthocyanidins were separated by high performance liquid chromatography (Alliance Separations Module 2695 (Waters, Milford, MA, USA) on a Welchrom-C18 column (5 μm, 4.6 × 250 mm). The mobile phase consisted of A, 0.5% hydrochloric acid in methanol and B, acetonitrile. Anthocyanidins were separated isocratically with 18% acetonitrile under the following conditions: flow rate, 1 mL min^{-1}; temperature, 30 °C, sample volume, 10 μL. The detection wavelength was 530 nm. Anthocyanidin elution was monitored by a 2487 Dual Wavelength Absorbance Detector (DAD) (Waters). Preliminary identification of anthocyanidins was conducted by comparison of UV–VIS spectra and elution order. To confirm the identity of detected compounds we employed a liquid chromatography/mass spectrometry-ion trap-time-of-flight system (LCMS-IT-TOF™, Shimadzu, Japan) coupled to a DAD system. The mass spectrometer was operated in positive ion mode. The mass scan range (*m*/*z*) was 200–400 amu.