

# Genetics and Anthocyanin Analysis of Flower Color in Mexican Petunia

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**ABSTRACT.** The genetics and anthocyanins responsible for flower color were studied in *Ruellia simplex* Wright (mexican petunia). An F<sub>2</sub> population with 153 individuals segregating for four flower colors was developed from a cross between a maternal individual with white corolla with purple throat (WP) and a paternal individual with pink corolla (PK). All the F<sub>1</sub> generation had fully purple flowers (P). The F<sub>2</sub> generation segregated 94 P:30 PK:24 WP:5 WPK (WPK is a new color combination of white corolla limb and pink throat). These data were separated into groups for corolla limb color and for throat color. The ratio for corolla limb color segregated 94 P:30 PK:29 W, which fits a 9:3:4 recessive epistasis interaction ( $P = 0.22$ ). The data for corolla throat segregated 118 P:35 PK, which fits a 3:1 ratio ( $P = 0.54$ ). High-performance liquid chromatography mass spectrometry analyses were performed to elucidate the anthocyanins responsible for the four obtained flower colors. We found that delphinidin derivatives conferred purple corolla color, whereas pelargonidin derivatives were responsible for the pink corolla color. Purple corolla throat color was the result of delphinidin derivatives, whereas the pink color was the result of peonidin derivatives.

There are ≈350 species of *Ruellia* L. (Acanthaceae) that are perennial herbs, subshrubs, or shrubs with mostly tropical and subtropical distributions (Tripp and McDade, 2014). A chromosome number of  $2n = 2X = 34$  appears to be widespread in this large genus (Daniel et al., 1984, 1990; Daniel and Chuang, 1993). The most commonly found *Ruellia* species in southeastern U.S. gardens is *Ruellia simplex* (britton's petunia, mexican petunia, or mexican bluebell). Scientific names for this plant that have been used throughout the botanical and horticultural literature include *R. brittoniana* Leonard, *R. coerulea* Morong, *R. malacosperma* Greenm., and *R. tweediana* Griseb. These names have been reduced to synonyms of the oldest name and thus that with priority, *R. simplex* (Ezcurra and Daniel, 2007). The wild form of this species is found in sunny areas on periodically inundated soils in Mexico, the Antilles, and central western South America (Ezcurra and Daniel, 2007). It was introduced to Florida sometime before 1940 (Hupp et al., 2009) and is now a very popular landscape plant as a result of its low maintenance requirements and copious flowering (Gilman, 1999).

Flower colors are attributable primarily to flavonoid and carotenoid pigments and are inferred to serve to attract pollinators (Davies, 2004). Flavonoids are the most common flower color pigment, and the predominant flavonoid pigments are the anthocyanins. Anthocyanins are composed of an anthocyanidin and sugar moieties. They are the basis for most orange, pink, red, magenta, purple, blue, and blue–black floral colors. The common anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvinidin, named for the genera from which they were first isolated. Most anthocyanins are derived from just the following three basic anthocyanidin types: pelargonidin, cyanidin, and delphinidin (Schwinn and Davies, 2004). Cyanidin and delphinidin can be further modified by glycosylation, methylation, and acylation to form peonidin and malvidin, respectively (Holton and Cornish, 1995). Orange and pink colors tend to be based on pelargonidin derivatives, magenta colors on cyanidin derivatives, and purple and blue colors on delphinidin derivatives (Harborne, 1976). Flowers can accumulate mixtures of anthocyanin types, providing further variation in color. Other factors such as vacuolar pH and petal cell shape can also affect the flower color (Mol et al., 1998). The genetics of flower color are best known in *Petunia ×hybrida* Vilm, where nine major genes involved in the inheritance of flower color were initially identified (Paris and Haney, 1958). Based on biochemical data, these genes were assigned specific functions in the biosynthetic pathway (Forkmann, 1991; Holton and Cornish, 1995; Mol et al., 1998; Wiering and deVlaming, 1984). Additionally, genetic differences in flower color resulting from modifications

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in the pH were explained by genes *Ph1*, *Ph2*, and *Ph6* (Griesbach, 1996, 1998).

Flower morphology, flower size, and flower color in *Ruellia* are varied. Flower color ranges from white, cream, yellow, lavender, purple, pink, magenta, and red (Tripp, 2014). However, the range of flower colors in *R. simplex* is more limited. The wild-type and cultivars Purple Showers and the dwarf Purple Katie have purple flowers [Royal Horticultural Society (RHS) 87A (Royal Horticultural Society, 1995)]. Over the years, cultivars with the following other colors have become available: pink-flowered Chi Chi and Pink Katie (RHS 66D) and white-flowered Snow White and White Katie (RHS 155D). All purple and pink-flowered forms have a darker throat (either dark purple RHS 86B or dark pink RHS 74A, respectively). Additionally, in 2010, a mutant with white corolla and purple throat was found in cultivation in Vero Beach, FL (S.B. Wilson, personal communication), although not commercially available. Breeding efforts at the University of Florida have resulted in the recent release of non-invasive, sterile cultivars Mayan Purple, Mayan White (Freyre et al., 2012), and Mayan Pink (Freyre and Wilson, 2014).

In this study, we developed an F<sub>2</sub> population segregating for flower color in *R. simplex* from a cross of an individual with white corollas with purple throat and an individual with pink corollas. We were then able to elucidate the genetic control for flower color in this species. We also performed high-performance liquid chromatography mass spectrometry analyses to determine the anthocyanins responsible for four flower colors in *R. simplex*.

## Materials and Methods

### Parental plant material

The maternal parent was a true-breeding *R. simplex* mutant with white corollas and purple throat [WP (found in Vero Beach, FL)], whereas the paternal parent was true-breeding pink-flowered *R. simplex* 'Chi Chi' [PK (cultivar from Boynton Botanicals, Palm Beach, FL)]. Plants were maintained in greenhouses at the University of Florida, Gainesville. Plants were grown in 1-L pots with soilless mix (60% Canadian peatmoss, 40% perlite; Fafard 2P; Concord Fafard, Agawam, MA) and placed on benches in an insect-proof greenhouse. Plants were watered as needed with 0.15 g·L<sup>-1</sup> nitrogen using 20N-4.4P-16K water-soluble fertilizer (Everris Americas, Dublin, OH).

### Hybridizations

Hybridizations were conducted in July 2011. Fully expanded flower buds (within which the anthers had not dehisced pollen) on the maternal plant WP were emasculated by removing the corollas and attached anthers. Immediately afterward, the stigmas were hand-pollinated using pollen from anthers freshly collected from the paternal plant PK. The pollinated gynoecia were then tagged with a colored plastic string. A total of seven flowers was pollinated. When the fruit developed, they were enclosed with an empty tea bag secured with a paper clip to prevent loss of seeds during fruit dehiscence (fruit of Acanthaceae have explosive dehiscence). When fruit dehisced naturally, seeds were collected in a coin envelope. Seeds were counted and immature or damaged seeds were discarded. Seeds were maintained in a glass desiccator under room temperature until sowing.

**F<sub>1</sub>.** In Jan. 2012, F<sub>1</sub> seeds were sown ≈1 cm deep in a 20-row seeder tray (Landmark Plastics, Akron, OH) using pre-wetted soilless mix. The seed tray was placed in a polycarbonate mist house (with 30% light irradiance) for 15 d and received misting from 0800 to 1800 HR (5 s every 30 min). Temperature was maintained between 18 to 24 °C. The tray was then taken to the greenhouse and 15 d later seedlings were initially transplanted into six-packs and later into 10-cm substrate paper pots (Blackmore Co., Belleville, MI). When F<sub>1</sub> plants had more than two branches, two cuttings were pinched from each plant, stuck in 128-cell cutting trays with soilless mix, placed under mist in a research greenhouse for 15 d, and then into 10-cm substrate paper pots.

On 15 May 2012 all substrate paper pots of the original F<sub>1</sub> plants were transplanted to a field at the Plant Sciences Research and Education Unit (PSREU) at Citra, FL. The field was fumigated for nematodes and weeds 3 weeks before planting and rototilled. Rows were formed by raising 10 cm off the ground and covered with silver plastic. Plants were transplanted on holes punched on center on the rows and spaced 50 cm apart. Each plant was top-dressed with ≈9 g of 15N-4P-10K controlled-release granular fertilizer (Scotts, Marysville, OH). Irrigation was through drip tapes under the rows and supplied as needed.

**F<sub>2</sub>.** Nine F<sub>1</sub> plants that had been kept in the greenhouse were potted up into 15-cm traditional standard plastic pots (Kord, Reidsville, NC), whereas the rest were discarded. The F<sub>1</sub> plants were grown to maturity in the greenhouse, and two to three fruit from self-pollination were harvested from each plant. Twenty seeds from each F<sub>1</sub> plant were sown as described previously on 14 Nov. 2012 to produce the F<sub>2</sub> generation. F<sub>2</sub> plants were maintained in the greenhouse in substrate paper pots until field transplant at the PSREU in Citra on 25 Apr. 2013 following the procedures described previously. Flower color was noted as each plant flowered.

### Sample extraction for anthocyanin analysis

Flowers for each of the four colors were collected from several F<sub>2</sub> plants. For each color, the corollas were separated from the rest of the flower and cut into small pieces using scissors. One gram of corolla tissue for each color was soaked in 10 mL of methanol (containing 3% acetic acid v/v) in a 30-mL glass test tube for 20 min. The test tubes were sonicated for an additional 10 min. The samples were transferred to 15-mL plastic tubes and centrifuged at 1107 g<sub>n</sub> for 10 min. The supernatant was transferred back to 30-mL glass tubes, capped, and placed in a freezer in the dark until further analysis.

### Total anthocyanins analysis

The extracts were diluted to appropriate concentration for analysis. The total anthocyanin content was measured using the pH differential spectrophotometric method described by Giusti and Wrolstad (2001). The extracts were diluted with 0.025 mol·L<sup>-1</sup> potassium chloride buffer, pH 1.0, and 0.4 mol·L<sup>-1</sup> sodium acetate buffer, pH 4.5, to the predetermined dilution factor. The samples were incubated at room temperature in darkness for 15 min and then the absorbance at 520 and 700 nm was measured using a spectrophotometer (DU 730 Life Science; Beckman Coulter, Fullerton, CA). The results were reported as milligrams of cyanidin 3,5-diglucoside equivalents per gram of fresh flowers. This was calculated using the formula: (A × MW)/(ε), where the absorbance (A) was

calculated as  $(A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$ , the molecular weight [MW (611)] was of cyanidin 3,5-diglucoside, and the molar absorptivity ( $\epsilon$ ) was of cyanidin 3,5-diglucoside (30175). Total anthocyanin means for each flower color were statistically compared using Tukey-Kramer honestly significant difference.

### High-performance liquid chromatography–diode array detector–mass spectrometry analysis

A high-performance liquid chromatography system (1200; Agilent Technologies, Palo Alto, CA) containing an autosampler, a binary pump, a column compartment, a diode array detector, a fluorescent detector, and a refractive index detector

was used for anthocyanin analysis. Separation of the anthocyanins was achieved using a 250 × 4.6-mm, 5- $\mu$ m column (Agilent Technologies). Elution was performed using mobile phase A (5% acetic acid aqueous solution) and mobile phase B (methanol). Ultraviolet-vis spectra were scanned from 180 to 600 nm on a diode array detector with a detection wavelength of 520 nm. A flow rate of 1 mL·min<sup>-1</sup> with a 50-min gradient was used as follows: 0 to 2 min, 5% B; 2 to 10 min, 5% to 15% B; 10 to 25 min, 15% to 25% B; 25 to 30 min, 25% to 30% B; 30 to 45 min, 30% B; 45 to 47 min, 30% to 70% B; and 47 to 50 min, 70% to 5% B followed by 5 min of re-equilibration of the column between samples. Electrospray mass spectrometry was performed with a high-capacity ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Column effluent was monitored in the positive ion mode. The following conditions for the mass spectrometer were used: nebulizer (310.3 kPa), dry gas (11.0 L·min<sup>-1</sup>), dry temperature (350 °C), ion trap (scan from m/z 150 to 1500), smart parameter setting compound stability (50%), and trap drive level (60%). The mass spectrometer was operated in Auto MS<sup>2</sup> mode to capture and fragment the most abundant ion in full scan mass spectra.

### Results and Discussion

Four fruit were harvested from the seven crosses performed between WP × PK. Seeds were sown and 66 F<sub>1</sub> seedlings were obtained. A total of 55 plants flowered in the field in 2012, and all were found to have purple flowers. F<sub>2</sub> seeds were sown and a total of 155 seedlings germinated and were transplanted to the field in 2013. A total of 153 plants flowered and their flower color was noted. Segregation of flower color was observed in the F<sub>2</sub> generation. In addition to the purple, pink, and white with purple throat colors, a new combination of white with pink throat was recovered (Fig. 1). The segregation observed was 94 P:30 PK:24 WP:5 WPK.

Samples of each of the four distinct color patterns were used to determine the anthocyanin concentrations for each flower color. The tentative identification indicated that all the anthocyanins detected were diglucosides. Interestingly, it was found that the purple color had more than three times the amount of anthocyanin compared with the pink color (Table 1).

With the high-performance liquid chromatography–diode array detector–mass spectrometry analysis, the anthocyanin responsible for the purple color was tentatively identified as malvidin as a result of a combination of malvidin 3,5-diglucoside



Fig. 1. Flowers of the maternal *Ruellia simplex* individual with white corolla with purple throat, the paternal individual with pink corolla, one individual representing the F<sub>1</sub> population with purple corollas, and the F<sub>2</sub> population segregating for corolla color.

Table 1. Total anthocyanin content of *Ruellia simplex* with pink, purple, white with purple throat, or white with pink throat corolla color.

Corolla color	Total anthocyanins [mean ± SD (mg·g <sup>-1</sup> dry wt)] <sup>2</sup>
Purple	86.3 ± 3.4 a <sup>3</sup>
Pink	26.1 ± 6.6 b
White with purple throat	11.5 ± 0.1 b
White with pink throat	10.8 ± 1.1 b

<sup>2</sup>Mean of duplicate samples.

<sup>3</sup>Values with the same letter are not significantly different using Tukey-Kramer honestly significant difference ( $P < 0.005$ ).

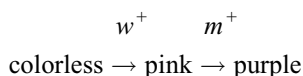
Table 2. Retention times and mass spectrometric (MS) data of anthocyanins in *Ruellia simplex* determined by high-performance liquid chromatography–diode array detector–mass spectrometry.

Flower color	Compound	Retention time (min)	Molecular		MS <sup>2</sup> (m/z)	Tentative identification
			wt	MS <sup>1</sup> (m/z)		
Purple	1	18.3	655	655 [M] <sup>+</sup>	493, <b>331</b> <sup>2</sup>	Malvidin 3,5-diglucoside
	2	23.3	741	741 [M] <sup>+</sup>	579, 493, <b>331</b>	Malvidin (malonylglucoside)-glucoside
Pink	1	16.2	595	595 [M] <sup>+</sup>	433, <b>271</b>	Pelargonidin 3,5-diglucoside
	2	17.6	681	681 [M] <sup>+</sup>	647, 519, 475, <b>271</b>	Pelargonidin (malonylglucoside)-glucoside
	3	22.1	681	681 [M] <sup>+</sup>	519, 433, <b>271</b>	Pelargonidin 3-(6'-malonylglucoside)-5-glucoside
	4	38.7	520	520 [M] <sup>+</sup>	433, <b>271</b>	Pelargonidin 3-(6'-malonylglucoside)
White with purple throat	1	17.1	655	655 [M] <sup>+</sup>	493, <b>331</b>	Malvidin 3,5-diglucoside
White with pink throat	1	17.7	625	625 [M] <sup>+</sup>	463, <b>301</b>	Peonidin 3,5-diglucoside

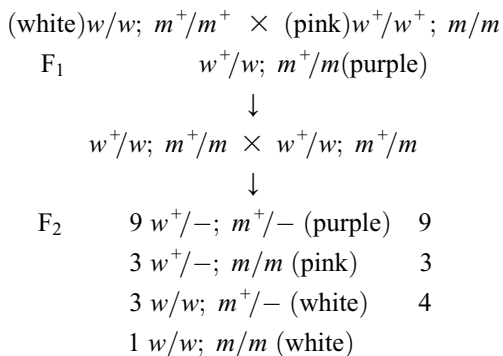
<sup>2</sup>Tons in bold were identified based on Sandhu and Gu (2010).

(Sandhu and Gu, 2010) and a second form tentatively identified as malvidin-(malonylglucoside)-glucoside, but the positioning of the glucosyl moieties could not be defined without further analysis. The anthocyanin responsible for the pink color was tentatively identified as pelargonidin, of which there were four different forms: pelargonidin 3,5-diglucoside, pelargonidin-(malonylglucoside)-glucoside, pelargonidin 3-(6'' malonylglucoside)-5-glucoside, and pelargonidin 3-(6'' malonylglucoside). Again, in the case of pelargonidin-(malonylglucoside)-glucoside, the positioning of the glucosyl moieties could not be defined without further analysis. Similar to the purple corolla, in the white with purple throat, the anthocyanin was also tentatively identified as malvidin 3,5-diglucoside. Interestingly, the anthocyanin in the white with pink throat was peonidin 3,5-diglucoside rather than a pelargonidin form (Table 2).

We hypothesize that there are separate genes involved for flower color determination in *R. simplex*, some expressed on the corolla limb and others on the corolla throat. This is not unusual, because in petunia, there are different loci that are phenotypically expressed either on the limb or the tube of the corolla, and they show complex epistatic relations (Tornielli et al., 2009). In *R. simplex* the segregation for corolla limb color 94 P:30 PK:29 W fits a 9:3:4 segregation ( $P = 0.22$ ), which is a modification of a 9:3:3:1 segregation resulting from recessive epistasis. This is similar to the segregation for flower color reported in *Collinsia parviflora* Lindl. [blue-eyed mary (Griffiths et al., 2012)]. The pathway for *R. simplex* is as follows:



The *w* and *m* genes are not linked. When plants homozygous for white and pink corolla limb are crossed, the F<sub>1</sub> and F<sub>2</sub> are as follows:



The *w* allele is epistatic on *m*<sup>+</sup> and *m*, so that *m*<sup>+</sup> and *m* can be expressed only in the presence of *w*<sup>+</sup>. Using the petunia anthocyanin pathway as a model (Holton and Cornish, 1995; Tornielli et al., 2009), *w*<sup>+</sup> and *m*<sup>+</sup> could possibly correspond to genes coding for or controlling the activities of F3H and F3'5'H, respectively, and purple malvidin would be formed. If the pathway is blocked at the second gene, dihydrokaempferol would be converted to pink pelargonidin. In the presence of *w*, naringenin would not be converted to dihydrokaempferol resulting in white color (Fig. 2A).

For the corolla throat, the segregation of color 118 P:35 PK fits a 3:1 segregation ( $P = 0.54$ ). The purple color is the result of a dominant gene possibly coding for or controlling F3'5'H

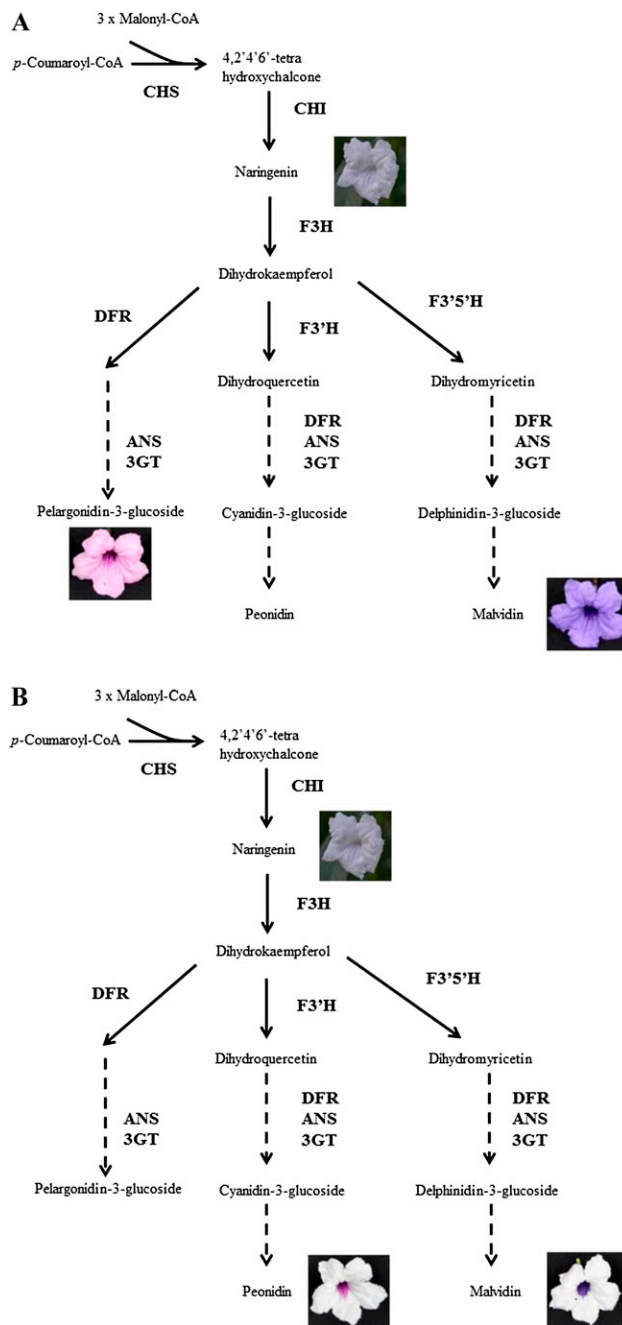


Fig. 2. Scheme of the flavonoid pathway in *Ruellia simplex*, simplified from the petunia flavonoid pathway, as in Holton and Cornish (1995) and Tornielli et al. (2009). CHS = chalcone synthase; CHI = chalcone flavanone isomerase; F3H = flavanone 3-hydroxylase; F3'H and F3'5'H = flavonoid 3' and 3'5' hydroxylase; DFR = dihydroflavonol 4-reductase; ANS = anthocyanin synthase; 3GT = anthocyanin 3-glucosyltransferase. Dashed lines indicate the presence of more than one intermediate, not shown in this scheme. (A) Anthocyanin pathway for the corolla limb color; (B) anthocyanin pathway for the corolla throat color.

activity, but this gene is only expressed in the tube. In the presence of the recessive allele, a second gene can be expressed, which could possibly be coding or controlling F3'H. This enzyme converts dihydrokaempferol to dihydroquercetin, which is an intermediate of cyanidin, which is later converted to peonidin conferring pink color (Fig. 2B).

In conclusion, the inheritance of flower colors in *R. simplex* can be explained by the inheritance of anthocyanin pigmentation, which is controlled by multiple genes that follow simple Mendelian genetics. The flavonoid pathway in *R. simplex* appears to be simpler than that of *Petunia*. The corolla limb color is determined by two genes that show recessive epistasis. Delphinidin derivatives confer purple corolla limb color, whereas pelargonidin derivatives are responsible for the pink corolla limb color. Another gene is responsible for the corolla throat color. In this case the dominant allele confers delphinidin derivatives and purple throat color, whereas the recessive allele confers peonidin derivatives and pink throat color.

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