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Anthocyanins in the "red" flowers of *Petunia exserta*

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Abstract

The percentage of individual anthocyanins in *Petunia exserta* flowers was 79% cyanidin-3-glucoside, 8% cyanidin-3-rutinoside, 7% pelargonidin-3-glucoside and 6% pelargonidin-3-rutinoside. The flower petal pH was 5.4. *Petunia exserta* should be very useful in breeding because its genetic background is different from the existing red-flowered garden petunias.

Keywords: Cyanidin glycosides; Pelargonidin; *Petunia exserta*; Solanaceae

1. Introduction

The genus *Petunia* was established by Jussieu in 1803 (Jussieu, 1803). He described *P. parviflora* and *P. nyctaginiflora* (= *P. axillaris* Lamarck), two very morphologically distinct species. The first monograph on *Petunia* was published in 1911 by Fries (Fries, 1911). He recognized 27 species of *Petunia* and established two subgenera: *Pseudonicotiana* and *Petunia*. Based upon cytotaxonomic studies, Wijsman and Jong (Wijsman & Jong, 1985) found that there were two groups of genetically distinct species and they split the genus. *Petunia axillaris* was conserved as the type species (Brummitt, 1989) and species with chromosome number 2n = 14 were kept in the genus *Petunia* Juss. Species with chromosome number 2n = 18 were transferred to the genus *Calibrachoa* La Llave and Lex (Wijsman, 1990; Stehmann & Semir, 1997) *Petunia* was estimated to have 12 species (Ando & Hashimoto, 1996), including *P. x hybrida* which is the cultivated garden petunia. *Petunia x hybrida* is not a true species, but a complex artificial hybrid derived from hybridization of *P. axillaris* and *P. integrifolia* Hooker (Sink, 1981).

Petunia axillaris is the only species with white flowers (Wijsman, 1982). All of the other species have purple flowers, except *P. saxicola* Smith et. Downs (Smith & Downs, 1966) and *P. exserta* Stehmann (Stehmann, 1987). *Petunia saxicola* is only known from its historic record and was not brought into cultivation. *Petunia exserta* was recently discovered and was found growing on sandstone towers in shady cracks within the rock. *Petunia exserta* has bright red flowers (RHS 45A). Although considerable information is known about anthocyanin biosynthesis and flower color biochemistry in *P. x hybrida* (Holton & Cornish, 1995; Fukui, Kusumi, Yoshida, Kondo, Matsuda & Nomoto, 1998), little is known about flower color biochemistry in the naturally occurring species. This study was undertaken to determine the anthocyanin pigment(s) and pH in *P. exserta* flowers.

2. Results and discussion

Analytical HPLC profiles (Fig. 1; Table 1) of *P. exserta* flowers contained four peaks with retention times (minutes $\pm t \times$ standard deviation) of 15.34 ± 0.89

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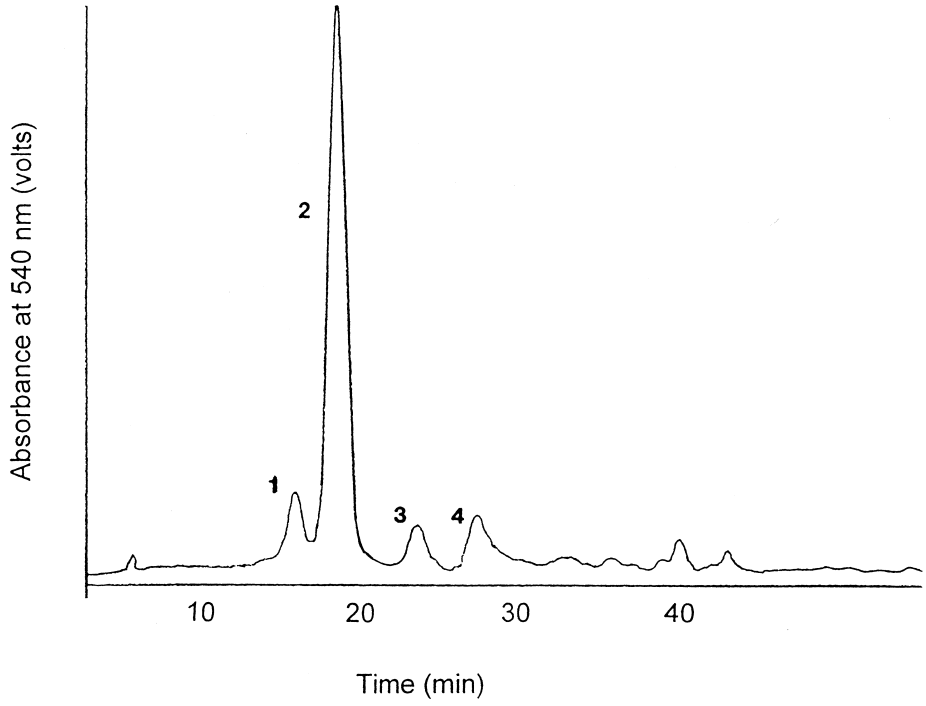

Fig. 1. Analytical HPLC profile of *P. exserta*.

(peak 1), 18.32 ± 0.78 (peak 2), 24.09 ± 0.72 (peak 3) and 28.31 ± 0.80 (peak 4). The four anthocyanins were purified by HPLC. Spectral properties (Table 2) of the purified anthocyanins indicated that they were not acylated. Base hydrolysis confirmed this fact.

Partial acid hydrolysis of peak 1 yielded another anthocyanin which co-eluted on HPLC with peak 2. Similarly, partial acid hydrolysis of peak 3 yielded another anthocyanin which co-eluted with peak 4. Partial acid hydrolysis of peaks 2 and 4 only yielded an aglycone. Complete acid hydrolysis of peaks 1 and 2 yielded cyanidin. Hydrolysis of peaks 3 and 4 yielded pelargonidin. Anthocyanidin identification was based upon co-elution on HPLC with known standards. This data suggested that peak 1 was a cyanidin-glycoside with 2 sugars; peak 2 was a cyanidin-glycoside with one sugar; peak 3 was a pelargonidin-glycoside with two sugars; and peak 4 was a pelargonidin-glycoside with one sugar.

Complete acid hydrolysis of peaks 1 and 3 yielded an equal amount of glucose and rhamnose. Hydrolysis of peaks 2 and 4 only yielded glucose. Sugar identifi-

Table 1
Analytic HPLC data of the anthocyanins and the flower pH from *P. exserta* and *P. x hybrida* "Red Magic"[a]

Species	Color	pH	Anthocyanin			
			Pg-gl	Pg-ru	Cy-gl	Cy-ru
P. x hybrida	45A	5.5 (0.2)	8.0 (0.7)	trace	57.8 (1.5)	34.2 (1.1)
P. exserta	45A	5.4 (0.1)	7.0 (0.8)	6.3 (1.6)	79.0 (2.2)	7.7 (0.7)

[a] Values are reported as the mean percentage of the total anthocyanin (standard deviation). Color is reported as an RHS Colour Chart chip. Abbreviations: Pg-gl = pelargonidin-3-glucoside; Pg-ru = pelargonidin-3-rutinoside; Cy-gl = cyanidin-3-glucoside; and Cy-ru = Cyanidin-3-rutinoside.

Table 2
Spectral properties of purified standards and anthocyanins and anthocyanidins from *P. exserta*[a]

Anthocyanin	λ_{max}	$\frac{E_{u.v.\ max}}{E_{vis.\ max}}$	$\frac{E_{440}}{E_{vis.\ max}}$
aPg	269, 520	80	34
Pg-3-gl	270, 506	64	38
Pg-3,5-gl	269, 504	45	21
Pg-3-ru	269, 510	64	38
aCy	275, 536	65	22
Cy-3-gl	274, 523	60	24
Cy-3,5-gl	273, 524	44	13
Cy-3-ru	281, 529	56	23
Peak 1	282, 527	56	24
Peak 2	281, 527	65	23
Peak 3	269, 509	64	39
Peak 4	269, 508	64	39
1 and 2 aglycone	275, 536	66	23
3 and 4 aglycone	270, 520	80	34

[a] Abbreviations: aPg = pelargonidin; Pg-3-gl = pelargonidin-3-glucoside; Pg-3-ru = pelargonidin-3-rutinoside; Pg-3,5-gl = pelargonidin-3,5-diglucoside; aCy = cyanidin; Cy-3-gl = cyanidin-3-glucoside; Cy-3,5-gl = cyanidin-3,5-diglucoside; and Cy-3-ru = cyanidin-3-rutinoside.

cation was based upon TLC with known standards. Based upon the anthocyanins in *P. x hybrida* (Holton & Cornish, 1995; Fukui, Kusumi, Yoshida, Kondo, Matsuda & Nomoto, 1998), these results suggested that peak 1 was cyanidin-3-rutinoside; peak 2 was cyanidin-3 glucoside; peak 3 was pelargonidin-3-rutinoside; and peak 4 was pelargonidin-3-glucoside. This was confirmed by their co-elution with known standards and by their spectral properties Table 2.

Analysis of flowers of *P. x hybrida* "Red Magic" Table 1 reconfirmed the anthocyanin composition and flower pH as previously reported(Griesbach, 1996; Muszynski, 1968; Griesbach, 1996; Griesbach, Asen & Leonhardt, 1991) *Petunia exserta* flowers were the same color and had the same anthocyanin composition as *P. x hybrida* "Red Magic" flowers Table 1.

Not all *P. x hybrida* plants that contain cyanidin-glycosides have red flowers (Griesbach, 1996; deVlaming Schram & Wiering, 1983; Griesbach, 1998). Similarly, not all petunias that have red flowers contain cyanidin-glycosides. The biochemical basis of these differences in the color was shown to be due to the pH of the flower (deVlaming Schram & Wiering, 1983; Griesbach, 1998). Both *P. x hybrida* "Red Magic" and *P. exserta* flowers had relatively low pHs (pH 5.5) as compared to other *P. x hybrida* plants Table 1. The pH of *P. x hybrida* flowers ranges from pH 5.2 to 6.5 (Griesbach, 1996; deVlaming Schram & Wiering, 1983).

Even though the anthocyanin composition and pH in *P. exserta* were identical to that in *P. x hybrida* "Red Magic", red garden petunias are not red because of introgressed genes from *P. exserta*. The first red *P.* x *hybrida* cultivar ("Tango") was registered in 1950 and was the result of inbreeding of a mutation found in a commercial cultivar (Maatsch & Nolting, 1968; Weddle, 1976). Because *P. exserta* is not in the background of red garden petunias, it should be important in breeding.

Red flower color in garden petunias is due to the combination of cyanidin-glycosides and low pH (Griesbach, 1996; deVlaming Schram & Wiering, 1983). Petunias containing cyanidin-glycosides at high pH are not red (Griesbach, 1996). There is a tight linkage between the genes for pH and anthocyanin formation (Griesbach, 1996; deVlaming Schram & Wiering, 1983). Because of this linkage, the red-flowered cultivars are more inbred than cultivars with a different flower color. There is also considerable genetic similarity between the various red cultivars because they all originated from the same genetic mutation (Claude Hope, personal communication). Due to these factors, red-flowered cultivars are less vigorous than cultivars of other colors. In addition, it is very difficult to introduce red flower color in new breeding lines.

Petunia exserta should be very useful in breeding improved red-flowered cultivars, because of its different genetic background. Internal reproductive barriers in *Petunia* are weak and interspecific hybrids are easily produced (Watanabe, Ando, Iida, Suzuki, Buto et al., 1996). Fertile interspecific hybrids between *P. exserta* and *P. integrifolia*, *P. axillaris* and *P. x hybrida* have been easily produced (data not shown). These hybrids are currently being used to expand the gene pool of the garden petunia.

3. Experimental

3.1. Plant material

Plants of *P. exserta* and *P. x hybrida* "Red Magic" were grown during the summer at Beltsville, MD, USA in a greenhouse using standard horticultural practices. The 1986 edition of the Royal Horticultural Society (RHS) Colour Charts were used to denote flower color (Voss & Hale, 1998). Approximately 1 gm of fresh weight petal tissue was extracted with 1% (v/v) HCl–MeOH. Extracts were reduced to dryness at 40° under reduced pressure. The residue was dissolved in 1% HCl–MeOH and filtered.

3.2. HPLC analysis

Anthocyanins in each conc. extract were analyzed by high resolution HPLC (Waters Maxima 820 with 490E Detector) on a 7.8 × 300 mm column of μBondapak C18 using a 30 min linear gradient of 0 to 10% MeCN in aqueous 15% HOAc–1.5% H_3PO_4 , followed by a 10 min linear increase to 20% MeCN and finally held at 20% MeCN for an additional 10 min. Flow rate was 1.0 ml min^{-1} and detection was by absorption at 540 nm. Individual anthocyanins and anthocyanidins were characterized by co-elution with known standards from *Petunia x hybrida* (deVlaming Schram & Wiering, 1983) and *Eustoma grandiflorum* (Asen, Griesbach, Norris & Leonhardt, 1986). Individual anthocyanins were reported as the percentage of the total anthocyanin present. Each value was the mean of three replicates with each replicate from a different flower harvested on a different day.

3.3. Hydrolysis

The anthocyanins were purified through prep. HPLC on a μBondapak C18 column using the same procedure described for high resolution HPLC except H_3PO_4 was not used in aqueous solvent. The isolated anthocyanins were base hydrolyzed in aqueous 1% (w/v) KOH for 30 min at room temp. under N_2, acidified and the acyl moiety extracted with Et_2O (Asen, 1982).

The deacylated anthocyanins were partially hydrolyzed at 70° in EtOH–aqueous 10% (v/v) HCl (1:1) for the characterization of the intermediates formed (Asen & Budin, 1966). Complete acid hydrolysis was obtained at 100° after 2 h. Isolated compounds were characterized, along with known standards, by HPLC, visible and u.v. absorbance and by the products of controlled acid or base hydrolysis.

3.4. Spectral analysis

The major glycosides and their aglycones were characterized by diagnostic visible and u.v. spectrophotometry (Shimadzu Graphicord 240 spectrophotometer) (Harborne, Mabry & Mabry, 1975). Measurements were reported as the ratios of $E_{u.v.\ max}/E_{vis.\ max}$ and $E_{440}/E_{vis.\ max}$.

3.5. Determination of pH

It has been previously determined that the pH of crude petal cell sap is an accurate measurement of the vacuolar pH (Stewart, Norris & Asen, 1975). The pH of petal cell sap was determined one day after anthesis. The upper epidermis was stripped and ground into a suspension and the pH was measured with a micro-pH meter (Sentron 501). The pH measurements were reported as a mean of 10 replicates with each replicate representing a different flower.

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