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Floral Anthocyanins from Two Monkeyflower Species with Different Pollinators

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Abstract—*Mimulus lewisii* and *M. cardinalis* are closely related, but *M. lewisii* is pink-flowered and pollinated by bumblebees while *M. cardinalis* is red-flowered and pollinated by hummingbirds. Pollinator preference is believed to be due in part to the difference in flower color between these two species. Ten anthocyanins are characterized from *M. lewisii* and *M. cardinalis* petal lobes by high performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ES/MSMS). Total anthocyanin concentration in the petals of *M. cardinalis* is approximately twice as high as that in *M. lewisii*, and that this difference can be accounted for by the presence of two pelargonidin biosides, probably rhamnohexosides, unique to *M. cardinalis*. Cyanidin biosides comprise 33% of all the anthocyanins in *M. lewisii*, but only 2.4% of those in *M. cardinalis*. © 1997 Elsevier Science Ltd

Introduction

Pollination of flowers by animals depends on a number of traits that govern floral attractiveness (Waser and Price, 1981). Flower visitation often occurs in response to visual signals (Kevan, 1983), making color one of the most important attractive floral traits (Sprengel, 1793; Waser and Price, 1981; Harborne and Grayer, 1993). Animals in different pollinator guilds may possess considerably different visual sensitivity spectra (Levin, 1978; Kevan, 1983; Scogin, 1983). Thus, evolutionary changes in coloration may result from natural selection to increase attractiveness to the visual system of the most effective pollinator.

Section *Erythranthe* of the genus *Mimulus* (monkeyflower) contains six species; one species is bee-pollinated and five are hummingbird-pollinated. The bee-pollinated species, *M. lewisii*, has pale pink flower petals and a pair of yellow nectar guides that emerge from the corolla tube. This color pattern is visible to bees and characteristic of bee-pollinated plants (Daumer, 1958; Kevan, 1983). The hummingbird-pollinated *M. cardinalis* occurs sympatrically with *M. lewisii* in some parts of the Sierra Nevada range of California. *Mimulus cardinalis* has vivid red flowers that are highly attractive to hummingbirds, yet are not conspicuous to bees, which have poor color vision at red wavelengths.

Anthocyanins, which contribute to the red and pink colors of *Mimulus* flowers, consist of a flavylium cation conjugated to sugars, organic acids, or other substituents. The

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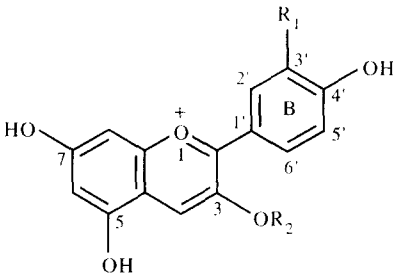

FIG. 1. STRUCTURES OF THE AGLYCONES PELARGONIDIN AND CYANIDIN. R_1, R_2 = H: pelargonidin $[C_{15}H_{11}O_5]^+$, MW 271 (**1**). R_1 = OH; R_2 = H: cyanidin $[C_{15}H_{11}O_6]^+$, MW 287 (**2**).

colors of anthocyanins depend largely upon the hydroxylation pattern on ring B of the flavylium cation, also referred to as anthocyanidin or aglycone (Fig. 1). The most primary chromophores are the brick-red pelargonidin (**1**), which is hydroxylated at the 4′ position, and the pink cyanidin (**2**), which is hydroxylated at the 3′ and 4′ positions (Fig. 1). Sugars linked to the flavylium cation by a glycosidic bond generally affect the color rather little.

We have characterized, both qualitatively and quantitatively, the anthocyanins in *M. lewisii* and *M. cardinalis* petal lobes by high performance liquid chromatography (HPLC) and electrospray tandem mass spectrometry (ES/MSMS). Since anthocyanin pigments control in part the hue and color intensity of flowers, knowledge of the qualitative and quantitative differences in anthocyanin pigmentation between *M. lewisii* and *M. cardinalis* should point directly to genetic differences which allow their pollinators to recognize and discriminate between them in nature.

Materials and Methods

Plant material. Mimulus lewisii and *M. cardinalis* were propagated from seeds collected by Dr Robert K. Vickery Jr (University of Utah) from Yosemite National Park, California. *Mimulus lewisii* seeds came from Raisin Lake (seedlot 13357, elevation 2123 m) and *M. cardinalis* seeds from Wawona (seedlot 13363, elevation 1300 m). Seeds were germinated and plants were grown in the University of Washington Botany greenhouse.

Chemicals. Authentic standards cyanidin 3-glucoside chloride and pelargonidin 3-glucoside chloride were purchased from Apin Chemicals Ltd (Abingdon, Oxfordshire, U.K.); acetic acid, n-butanol, and HPLC grade methanol from Sigma Chemical Company (St. Louis, Missouri); hydrochloric acid 36.5–38.0% from J. T. Baker Inc. (Phillipsburg, New Jersey); and trifluoroacetic acid from Applied Biosystems (Foster City, California).

Extraction of anthocyanins. To obtain equal petal areas for sampling, one disk (diameter = 9 mm) was punched from each of the two lower lateral petals of one fresh flower. The two disks were extracted with 200 µl of methanol/0.1% HCl. To release the vacuolar compounds, the cells were disrupted by squeezing the disks with a close-fitting disposable pestle in a 1.5 ml microcentrifuge tube. After soaking for 10 min in the solvent, the cell debris was removed by centrifugation for 5 min at 12,000×*g*, and the supernatant was decanted for analysis.

Liquid chromatographic analysis. High performance liquid chromatography (HPLC) was carried out on a Beckmann 334 Gradient Liquid Chromatograph equipped with a Beckmann 163 Variable Wavelength Detector (Beckmann Instruments Inc., Berkeley, California). Separation was achieved on a C_{18} reversed-phase column (Alltima C_{18}, 5 µm, 250×4.6 mm i.d., Alltech, Deerfield, Illinois) by running a gradient of 0.1% aqueous trifluoroacetic acid (solvent A) and 0.1% trifluoroacetic acid in methanol (solvent B). The applied gradient, run at a flow rate of 2 ml min^{-1}, was started at 30% B for 5 min, was then increased to 46% B over 25 min and held for 5 min. Peaks were monitored at 510 nm, recorded and quantified by a Waters 740 Data Module (Waters, Milford, Massachusetts), and collected manually for MS analysis.

Relative concentration of each anthocyanin in M. lewisii *and* M. cardinalis. The concentration of each anthocyanin separated by HPLC, relative to the total anthocyanin concentration in *M. cardinalis* flowers, was estimated by collecting a pair of petal disks from five flowers of both species. Each of the ten pairs of disks was

extracted with methanol/HCl and chromatographed as above. Relative abundance of each compound was calculated by taking the mean area under the chromatographic peak and normalizing it to the mean total area under all anthocyanin peaks from *M. cardinalis*. For example, the relative abundance of compound A from *M. lewisii* was calculated as the mean area under peak A from five *M. lewisii* flowers (mean area = 466,975) divided by the mean total area under all anthocyanin peaks for *M. cardinalis* (mean area = 4,395,545, $n = 5$), giving the relative concentration value of 10.6% (Table 1). This method was used to compare directly the concentrations of each anthocyanin in the two species, and to show that *M. cardinalis* has a greater total concentration of anthocyanins in its flowers than does *M. lewisii*.

Electrospray tandem mass spectrometric analysis (ES/MSMS). Anthocyanins were analyzed in a positive ion mode on a Sciex API III triple quadrupole mass spectrometer fitted with a nebulization-assisted electrospray ionization source (PE Sciex, Thornhill, Ontario, Canada) by infusing the samples collected after HPLC separation with a syringe pump (Harvard Apparatus, model 11, Southnatick, Massachusetts) at a flow rate of $5\,\mu l\,min^{-1}$ through a 50 μm i.d. fused silica transfer line. Collision induced dissociation (CID) was performed by introducing argon at a thickness of 1.4×10^{14} atoms cm^{-2}. In a product ion scan, the precursor ions of the anthocyanin molecular ions $[M]^+$ were detected. The detected aglycone ions m/z 271 (pelargonidin) and m/z 287 (cyanidin) were then selected as product ions in the precursor ion scans.

Results

HPLC analysis

Ten anthocyanin pigments (A–J) were isolated from *M. lewisii* and *M. cardinalis* petals by reversed phase HPLC separation of freshly prepared methanol/HCl extracts and quantified by integration of the peak area (Table 1). In *M. lewisii* petals, compounds A and F were the most abundant anthocyanins, with smaller amounts of D, E, G, I, and J. Compounds B, C, and H were not detected in *M. lewisii* petals. In *M. cardinalis* petals, the most abundant anthocyanins were compounds B and C. Additionally, compounds A, D, E, F, G, and H were present, whereas I and J were not detected. HPLC analysis of authentic standards showed that compound E coeluted with pelargonidin 3-glucoside and compound G coeluted with cyanidin 3-glucoside.

MS analysis indicated that compounds A–C each possess two substituents attached to the aglycone (see below). Anthocyanin glycosides acylated with hydroxycinnamic acids, such as caffeic acid, show distinct UV absorbance peaks between 310 and 335 nm (Harborne, 1958). To investigate the possibility of intramolecular copigmentation by acylation, individual UV/VIS absorbance spectra of compounds A–C were recorded. Because the concentration of these compounds in the eluates sampled after HPLC separation was too low to monitor absorbance spectra conventionally, eluates containing compounds A–C were analyzed by HPLC with integrated diode array detection. None of the compounds A, B, or C exhibited strong absorbance peaks in the range between 310 and 335 nm: E_{330}/E_{max} [%] (A): 28; E_{330}/E_{max} [%] (B): 13; E_{330}/E_{max} [%] (C): 15; E_{330}/E_{max} [%] pelargonidin caffeoyl 3,5-diglucoside: 48 (Harborne, 1958); E_{330}/E_{max} [%] authentic standard pelargonidin 3-glucoside: 11. Additionally, all three compounds showed distinct shoulders to the main peak in the visible area at 445 nm: E_{440}/E_{max} [%] (A): 33; E_{440}/E_{max} [%] (B): 42; E_{440}/E_{max} [%] (C): 43; E_{440}/E_{max} [%] authentic standard pelargonidin 3-glucoside: 39. These findings suggest that the analyzed compounds are exclusively derivatized in the 3-position of the respective aglycone and do not contain hydroxycinnamic acid moieties (Harborne, 1958). Thus, compounds A–C can be tentatively identified as anthocyanidin 3-biosides.

ES/MSMS analysis

Anthocyanins A–J that eluted after HPLC separation of *M. lewisii* and *M. cardinalis* petal extracts were individually collected and directly infused into the ES/MSMS, which was operated in a positive ion mode. The molecular ions $[M]^+$ were fragmented by CID

TABLE 1. ANTHOCYANIN COMPOSITION AND CONTENT OF *M. lewisii* AND *M. cardinalis*

Compound	HPLC retention time (min)	MSMS detected ions (m/z)	Aglycone	Substituent (R_2)	*M. lewisii* relative concentration (%)	*M. cardinalis* relative concentration (%)
A	15.7	623 $[M]^+$; 447; 271	pelargonidin	bioside	10.6	17.2
B	18.8	579 $[M]^+$; 433; 271	pelargonidin	deoxyhexoside	0.0	43.1
C	20.9	579 $[M]^+$; 433; 271	pelargonidin	deoxyhexoside	0.0	22.7
D	23.9	463 $[M]^+$; 287	cyanidin	monoside	5.1	0.8
E	28.5	433 $[M]^+$; 271	pelargonidin	hexoside	1.0	0.3
F	29.5	447 $[M]^+$; 271	pelargonidin	monoside	24.2	14.3
G	31.1	449 $[M]^+$; 287	cyanidin	hexoside	4.5	0.3
H	31.5	595 $[M]^+$; 449; 287	cyanidin	deoxyhexoside	0.0	1.3
I	32.5	491 $[M]^+$; 287	cyanidin	monoside	9.3	0.0
J	35.8	475 $[M]^+$; 271	pelargonidin	monoside	1.9	0.0
Total	—	—	—	—	56.6	100.0
Cyanidin 3-glucoside, standard	31.1	449 $[M]^+$; 287	cyanidin	3-glucoside	—	—
Pelargonidin 3-glucoside, standard	28.5	433 $[M]^+$; 271	pelargonidin	3-glucoside	—	—

and scanned for product ions that indicated the presence of common anthocyanin aglycones such as *m/z* 271 (pelargonidin), 287 (cyanidin), 301 (peonidin), 303 (delphinidin), 317 (petunidin), and 331 (malvidin). The corresponding substituents were determined by scanning the aglycone ions for precursor ions. Compound A, which was isolated from *M. lewisii* and *M. cardinalis* petals, showed a molecular ion $[M]^+$ (*m/z* 623) with two product ions *m/z* 447 and 271. The smallest of the product ions (*m/z* 271) revealed pelargonidin as the aglycone moiety. Selecting *m/z* 271 for precursor ion scans resulted in the detection of *m/z* 447 and 623, which indicated a glycosidic structure with two sugar moieties with identical masses (194 amu). Compounds B–J were analyzed analogously. The fragmentation patterns and structural properties of anthocyanins A–J are summarized in Table 1.

Qualitative and quantitative differences in anthocyanins between M. lewisii *and* M. cardinalis

Mimulus lewisii and *M. cardinalis* share five of the ten anthocyanins identified: A, D, E, F, and G (Table 1). *Mimulus lewisii* flowers contain two unique monosides, I and J, with aglycones cyanidin and pelargonidin, in relatively low concentration. The pelargonidin monosides A and F, common to both *Mimulus* species, are the most abundant anthocyanins in *M. lewisii*. *Mimulus cardinalis* flowers, on the other hand, possess two unique pelargonidin biosides (probably 3-rhamnoglucosides), B and C, that together exceed the total concentration of all anthocyanins in *M. lewisii* flowers. A third, less abundant bioside (likely another rhamnoglucoside), H, is also unique to *M. cardinalis*. Altogether, *M. cardinalis* has almost twice the total amount of anthocyanins as *M. lewisii* (100% vs. 56.6% relative concentrations; Table 1) accounting for the more intense color of *M. cardinalis* petals. Cyanidin biosides constitute only 2.4% of the anthocyanins extractable from *M. cardinalis*, but represent 33% (18.9/56.6) of all anthocyanins in *M. lewisii* (Table 1).

Discussion

Identification of anthocyanins from M. lewisii *and* M. cardinalis

Earlier comparative chromatographic and spectrophotometric studies on anthocyanins in the petals of *M. lewisii* and *M. cardinalis* flowers by Pollock *et al.* (1967) identified pelargonidin 3-glucoside, pelargonidin 3-rhamnoglucoside, the caffeoyl ester of pelargonidin 3-glucoside, cyanidin 3-glucoside, cyanidin 3-rhamnoglucoside, and the caffeoyl ester of cyanidin 3-glucoside in *M. cardinalis*. *Mimulus lewisii* petals were found to lack all pelargonidin glycosides and, additionally, some populations lacked the cyanidin 3-rhamnoglucoside. In contrast, our investigations indicate that the anthocyanins in flowers of both *M. lewisii* and *M. cardinalis* contain the aglycones pelargonidin and cyanidin glycosidically linked to deoxyhexosylhexosides, hexosides, and at least one unidentified substituent. Caffeoyl esters could not be detected in either species.

Ten anthocyanins from *M. lewisii* and *M. cardinalis* flowers were separated and quantified by HPLC and characterized as pelargonidin and cyanidin 3-monosides and 3-biosides according to UV/VIS spectra, chromatographic properties, and the masses of the molecules' fragments. ES/MSMS was used because of its power to analyze small quantities of polar, non-volatile, and thermolabile molecules, such as anthocyanins, due to high selectivity and sensitivity (Gläßgen *et al.*, 1992). During collision induced decay, the collision energy can be adjusted to cleave ester and glycosidic bonds only. Thus,

aglycones and their conjugates can be characterized by MSMS based on the mass/charge (m/z) values of product and selected precursor ions. However, ions with identical masses cannot be differentiated. Identification of a fragment m/z 176 as a precursor to the aglycone ion, for instance, indicates the presence of a substituent with a molecular weight of 194 amu that has lost a water molecule. It could represent a methylhexose, a uronic acid such as glucuronic or galacturonic acid, or a ferulic acid molecule. Precursor and product ion scans make it possible to determine which fragments are attached to one another, but it is not possible to resolve by which atoms and in which configuration the bonds are formed.

We classified the anthocyanins detected in *M. lewisii* and *M. cardinalis* flowers as listed in Table 1. Based on the identification by Pollock *et al.* (1967) of pelargonidin and cyanidin 3-rhamnoglucosides (rutinosides), the deoxyhexosides B and C probably are isomeric pelargonidin 3-rhamnoglucosides that may differ in the positions of the glycosidic bond between the rhamnose and the glucose moieties. Likewise, the deoxyhexoside compound H is probably cyanidin 3-rhamnoglucoside. The analytical data for compounds E and G are in agreement with the Pollock *et al.* (1967) identification of pelargonidin and cyanidin 3-glucoside, respectively. This finding is further supported by congruent analytic data of compounds E and G, and the authentic standards pelargonidin and cyanidin 3-glucoside. None of the masses of the molecular ions $[A]^+$, $[D]^+$, $[F]^+$, $[I]^+$, and $[J]^+$ match the molecular weights of the anthocyanins identified by Pollock *et al.* (1967).

In contrast to Pollock *et al.* (1967), our investigations did not confirm intramolecular copigmentation due to the presence of caffeoylglucosides. MSMS analysis of caffeoylglucosides would have detected two ions with identical masses of 162 amu, which were not observed. As acylated anthocyanins are sensitive to acid hydrolysis, it is possible that cleavage of caffeoyl ester linkages occur during extraction with methanol/HCl. However, this reaction is unlikely to go to completion during a rapid extraction followed by immediate analysis as performed in these studies.

Ultimately, only supplementary experiments, such as nuclear magnetic resonance (NMR) analysis, can confirm the precise structure of the isolated anthocyanins A–J.

Qualitative and quantitative differences in anthocyanins between M. lewisii *and* M. cardinalis

The principal goal of this work was to determine differences in the content and composition of anthocyanins in *M. lewisii* and *M. cardinalis* flowers, rather than to elucidate the precise structural formulas of the individual pigments. *Mimulus cardinalis* has about twice the total anthocyanin content of *M. lewisii*, and essentially all of this difference can be attributed to the abundance of two anthocyanins unique to *M. cardinalis*: the probable pelargonidin rhamnoglucosides B and C (Table 1). Both *M. cardinalis* and *M. lewisii* flowers contain compound E, probably a pelargonidin 3-glucoside, that is presumably the biosynthetic precursor molecule to compounds B and C. We hypothesize that the major difference in anthocyanin pigmentation between the two *Mimulus* species depends upon the activity of a rhamnosyl transferase that converts the 3-glucoside E to the 3-rhamnoglucosides B and C. We further expect that the gene encoding this enzyme is differentially expressed in the two *Mimulus* species; i.e. active in *M. cardinalis* but not in *M. lewisii*. This hypothesis is supported by the observation that a third probable rhamnoglucoside, the cyanidin bioside H, is unique to *M. cardinalis* despite the fact that

its likely precursor, the cyanidin glucoside G, is found in both *M. lewisii* and *M. cardinalis*. In *M. lewisii* flowers, activity of rhamnosyl transferases is not apparent; instead, the 3-glucosides may be acetylated, possibly leading to the formation of unique cyanidin and pelargonidin 3-acetylglucosides (compounds I and J).

Using genetic maps made from hybrids between *M. lewisii* and *M. cardinalis* (Bradshaw *et al.*, 1995), along with the detailed information now available on the anthocyanin biosynthetic pathway in plants (Forkmann, 1993), it should be now possible to determine precisely the genetic mechanism responsible for differences in the abundance of specific anthocyanins and their effect on pollinator visitation.

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