

Temperature-sensitive anthocyanin production in flowers of *Plantago lanceolata*

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Flower color in the weedy perennial *Plantago lanceolata* is phenotypically plastic. Darker flowers are produced at cooler ambient temperatures, and circumstantial evidence suggests that this is adaptive. The goal of this project was to investigate the chemical basis for the color plasticity. To test the hypothesis that increased anthocyanin production at low temperatures underlies the plasticity, extracts of *P. lanceolata* flowers produced at warm and cool temperatures were analyzed using UV/visible spectrophotometry coupled with mass spectrometry. Mass spectrometry allowed us to compare relative abundances of individual anthocyanins. Seventeen anthocyanins, derived from both cyanidin and delphinidin branches of the anthocyanin biosynthetic pathway, were detected. Most of these significantly increased in abundance under cool conditions. Genotypes differed significantly in anthocyanin levels and in their sensitivity to temperature change. Genotypes that showed greater floral color plasticity tended to show also greater temperature sensitivity with respect to anthocyanin production. Data suggest that the temperature regulation of the anthocyanin biosynthetic pathway occurs both upstream and downstream of the divergence of the cyanidin and delphinidin branches. The degree of temperature sensitivity, i.e. phenotypic plasticity, appears to be controlled downstream, whereas the overall temperature effect appears to be controlled upstream.

Introduction

Recently, Lacey and Herr (2005) showed that for the weedy perennial, *Plantago lanceolata* L. (Plantaginaceae), floral reflectance in the visible and near-infrared regions of the spectrum is phenotypically plastic. In other words, floral reflectance in these regions is not fixed, and it can be modified by the environment. Reflectance in the visible portion of the spectrum determines floral color. Individual plants adjust floral color/reflectance in response to ambient temperature. In spring and autumn when it is cool in North Carolina, *P. lanceolata* individuals produce darkly pigmented (maroon to black) spots on multiple floral parts (Lacey and Herr 2005). Because the tiny flowers are tightly clustered into spikes (the inflorescence type), the whole spike appears dark. In summer, plants produce flowers that lack or have much diminished pigmentation, and the spikes are much lighter and more reflective. This phenotypic plasticity in reflectance/color provides plants with partial thermoregulatory capability because the degree of reflectance influences internal floral temperature. Darkly colored spikes, produced under cool temperatures, absorb more solar radiation, thus warming flowers and developing seeds. Lightly colored spikes absorb

Abbreviations – ANOVA, analysis of variance; HPLC-MS, high-performance liquid chromatography with mass spectrometry; MANOVA, multivariate analysis of variance.

less radiation, thus cooling flowers and developing seeds.

Thermoregulatory capability is genetically variable (Lacey and Herr 2005). In North Carolina, in addition to the genotypes that are phenotypically plastic for reflectance, i.e. temperature sensitive, one also occasionally finds genotypes that are non-plastic, i.e. temperature insensitive. Only the plastic genotypes darken floral color at cool temperatures.

To determine the chemical basis for the temperature-sensitive change in reflectance in the visible portion of the spectrum, i.e. change in color, we initiated a study of the pigments, other than chlorophyll, produced by *P. lanceolata* flowers. We suspected that we would discover anthocyanins, which are widespread in angiosperms. In a number of plant species, low temperature has been observed to increase the anthocyanin levels and the expression of several structural genes in the anthocyanin biosynthetic pathway (Bednar et al. 2005, Christie et al. 1994, Dela et al. 2003, Islam et al. 2005, Leyva et al. 1995, Oren-Shamir and Levi-Nissim 1997, Rabino and Mancinelli 1986, Ratsek 1944, Shaked-Sachray et al. 2002, Shvarts et al. 1997b).

We tested the following two hypotheses (1) anthocyanin production in *P. lanceolata* increases at low temperatures and (2) anthocyanin production and phenotypic plasticity in production are genetically variable. Also, we sought to determine which anthocyanins are most temperature sensitive and most genetically variable, and we examined the relationship between temperature-sensitive plasticity in anthocyanin production and plasticity in floral color.

Anthocyanins, glycosides of anthocyanidins (Fig. 1), consist of an anthocyanidin core (the aglycone) substituted with various sugar or acid substituents (glycones) (Harborne and Baxter 1999). Many anthocyanins have the same anthocyanidin core. Previous studies examining the effect of temperature on anthocyanin production have measured anthocyanidin, rather than anthocyanin, quantities. We approached our study differently by using mass spectrometry. This technique allowed a comparison of relative quantities of individual anthocyanins, end products of the anthocyanin biosynthetic pathway, rather than anthocyanidins, which are intermediates in the pathway. Thus, our data provide new insights about the general location of temperature-sensitive steps in the pathway. Current understanding about temperature regulation of this pathway is negligible.

Materials and methods

Experimental genotypes and growing conditions

Twelve *P. lanceolata* genotypes collected from several natural populations in North Carolina were used for the experiments. These genotypes were assigned to one of three floral color plasticity groups: HIGH, MEDIUM and LOW. Ambient temperature strongly influences floral color in the HIGH color plasticity group of genotypes. Ambient temperature moderately influences floral color in the MEDIUM group of genotypes. Floral color in the low-plasticity genotypes is temperature insensitive. There were four genotypes in each plasticity group.

To assign a genotype to a color plasticity group, we measured percent reflectance (range = 500–580 nm) of a spike produced at low temperature and another spike produced at high temperature for each genotype. Reflectance was measured using a Shimadzu spectrophotometer with an integrating sphere. The sphere is enclosed in a module that can be completely closed, which allows one to measure percent reflectance of an opaque three-dimensional object (such as a spike) when a narrow beam of light (width = 5 nm) is directed toward the center of the spike (width >4 mm and length >1.4 cm). For each genotype, we determined the difference in spike reflectance at high and low temperatures at 10-nm intervals and then summed the differences. This sum was our measure of floral color plasticity. Sums for the genotypes ranged from 129 to 174% reflectance in the HIGH floral color plasticity group, from 102 to 115% for the MEDIUM group and from −31 to 84% for the LOW plasticity group. The 500–580 nm spectral range was chosen because (1) anthocyanins typically absorb light in this region of the spectrum; (2) this range spanned the region of greatest floral color plasticity, as measured in terms of reflectance (Lacey and Herr 2005); and 3) this range minimized potentially confounding effects of chlorophyll, carotenoids, and phytochrome, which absorb light mostly below 500 nm or above 580 nm.

Each genotype was cloned to produce six replicates. Replicates were allowed to grow vegetatively under short days (8 h day) for approximately 10 week, after which time they were moved to long-day chambers to induce flowering. Three replicate clones per genotype were moved to a high-temperature chamber (27°C 16 h day/22°C 8 h night). The other clones were moved to one of two low-temperature chambers (15°C 16 h day/10°C 8 h night). Replicates of genotypes were haphazardly placed in the chambers so that replicates of any one genotype did not grow all in the same location. PAR at plant height ranged from 264 to 419 $\mu mol\ s^{-1}\ m^{-2}$ from the side to the center of the high-temperature chamber. It ranged from 157 to 276 $\mu mol\ s^{-1}\ m^{-2}$ and from 299 to 388 $\mu mol\ s^{-1}\ m^{-2}$ from side to center of the two low-temperature chambers. All plants received 1/2-strength Hoagland's solution three times per week and were watered at least three times per week. Other than temperature and the additional watering of the plants in the high-temperature

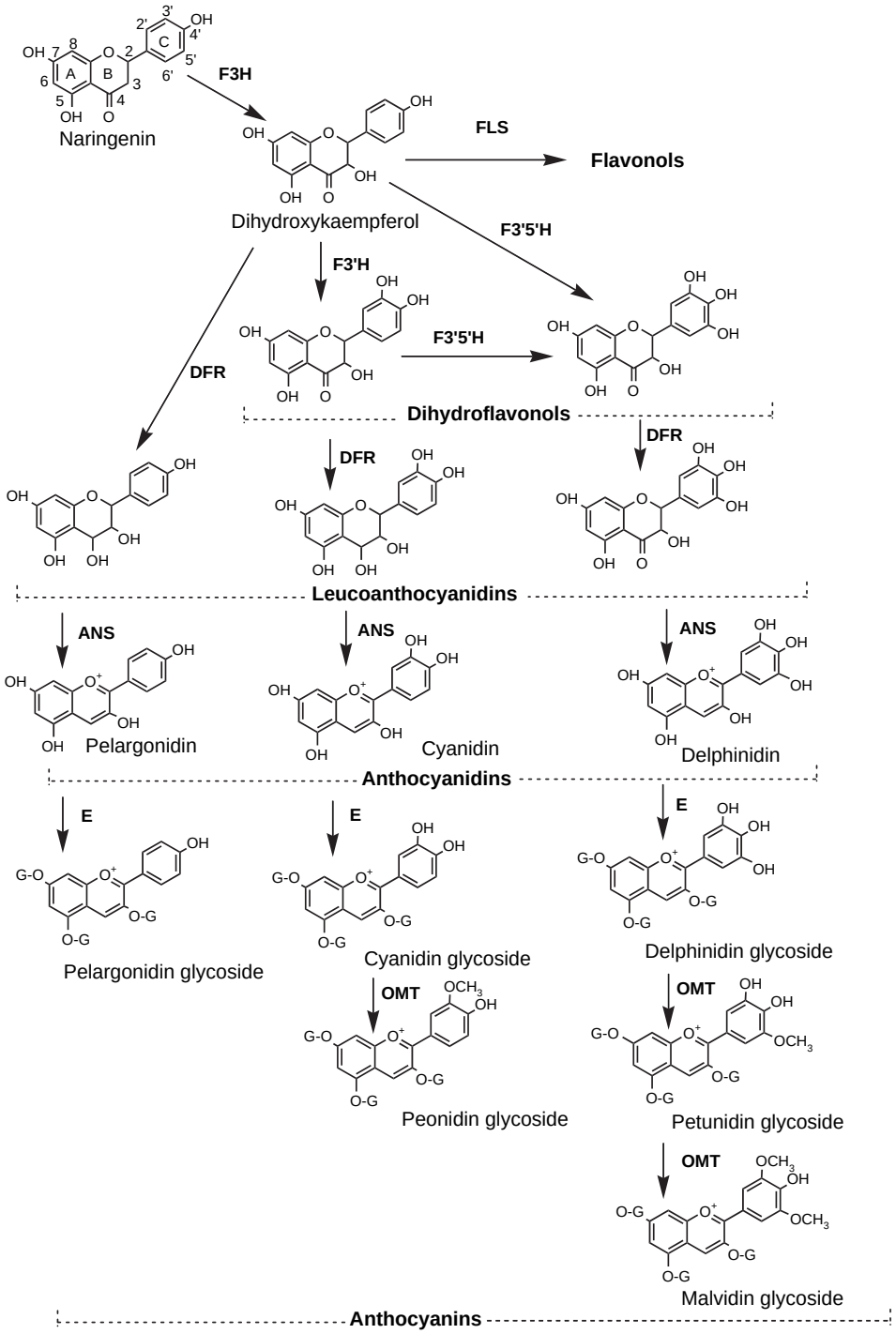

Fig. 1. Anthocyanin biosynthetic pathway in plants (Jonsson et al. 1983, Schijlen et al. 2004, Winkel-Shirley 1996, Winkel-Shirley 2001). The enzymes involved are as follows: flavone-3-hydroxylase (F3H), flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR), flavonoid-3′-hydroxylase (F3′H), flavonoid-3′,5′-hydroxylase (F3′5′H), anthocyanidin synthase (ANS) and *O*-methyltransferase (OMT). E represents the enzymes that convert anthocyanidins to various anthocyanins, which could include, but are not limited to, OMT, rhamnosyl transferase, UDP flavonoid glucosyl transferase and flavonoid-3-glucosyl transferase. The 'O-G' substituents on the 3, 5 or 7 carbons of the anthocyanins represent various *O*-glycosidic groups. Glycosidic substitutions at the 3 and 5 positions are most common.

chambers to prevent drought stress, all clones were treated in the same way.

Anthocyanin extraction

For each genotype growing in each temperature treatment, two to six replicate extracts were prepared from different batches of spikes collected on different days. Spikes were collected just prior to stigma emergence from the basal flowers (the first flowers to reach anthesis on a spike). To prepare the extracts, a modified version of a previously published method (Revilla et al. 1998) was used. We removed the floral parts from stem tissue and soaked the parts in methanol (1 g plant material per 10 ml methanol) for 24 h at −20°C. The liquid was removed, and floral parts were soaked again for 24 h at 4°C in a solution of 50:50 methanol:water. Solution volumes for both extractions were equal. Extracts were combined and stored at −20°C for 1–4 weeks prior to analysis. The anthocyanins of interest were observed to be stable [using high-performance liquid chromatography (HPLC) analysis] when stored under these conditions. On the day of analysis, extracts were centrifuged to remove particulate matter, and 10 μl of a 10^{-4} M solution of the alkaloid 1R,9S-β-hydrastine (Sigma Aldrich, Saint Louis, MO) in methanol was added to 1 ml aliquots of the extracts (for a final 1R,9S-β-hydrastine concentration of 10^{-6} M). The 1R,9S-β-hydrastine served as an internal standard for the analyses.

Preparation of standards

Standards of anthocyanins and anthocyanidins were used for method development and identification of unknown compounds from the *P. lanceolata* extracts. Stock solutions of peonidin chloride, malvidin chloride and malvidin 3-*O*-galactoside chloride (Sigma Aldrich); cyanidin chloride; petunidin chloride; peonidin glucoside chloride; malvidin glucoside chloride and pelargonidin diglucoside chloride (Indofine Chemical Co., Hillsborough, NJ) were prepared at concentrations of 10^{-2} M in methanol and were serially diluted to concentrations of 5×10^{-5} M prior to analysis. The standards were analyzed using the same conditions as the extracts.

Quantitative and qualitative analysis of *P. lanceolata* extracts

Extracts and standards were analyzed using high-performance liquid chromatography with mass spectrometric (HPLC-MS) detection or ultraviolet/visible spectrophotometric (HPLC-UV/VIS) detection. A binary pump high-performance liquid chromatograph (HP1100; Agilent, Palo Alto, CA) was used for the separations. It was coupled to an ion trap mass spectrometer with electrospray ionization source (LCQ Advantage; Thermofinnigan, San Jose, CA) or a photodiode array detector (HP1100; Agilent). Reversed phase HPLC separations were performed using a 50 × 2.1 mm C18 Column (Prevail packing; Alltech, Deerfield, IL). The mobile phase flow rate was 0.200 ml min^{-1}, and the injection volume was 10 μl. The solvent gradient used, which was a modified version of that reported previously for separation of anthocyanins (Tsao and Yang 2003), was as follows (where A = 6% acetic acid in nanopure water, B = HPLC grade acetonitrile): t = 0–20 min, 0% B–10% B; t = 20–35 min, 10% B–15% B; t = 35–45 min, 15% B–30% B; t = 45–50 min, 30% B–100% B; t = 50–60 min, 100% B and t = 60.1–70 min, 0% B. The mass spectrometer was operated with a sheath gas pressure of 20 arb, a spray voltage of 4.5 kV, a capillary temperature of 250°C, a capillary voltage of 3V and a tube lens offset of −60V.

The anthocyanins present in the extracts were identified using HPLC retention times, mass spectral fragmentation patterns and UV/VIS absorption spectra. UV/VIS absorption spectra were obtained with the photodiode array detector of the HPLC in the range of 200–900 nm. Such spectra were obtained for the chromatographic peaks corresponding to the most abundant anthocyanins in several of the plant extracts with high anthocyanin content. Further structural information about the anthocyanins was obtained using mass spectral fragmentation patterns. Even anthocyanins present in extracts at concentrations too low to be detectable by UV/VIS absorbance spectrophotometry could be identified using this method. The identities of the glycones were confirmed on the basis of characteristic ions in the MS^2 and MS^3 spectra, obtained using collisionally induced dissociation, with a collision energy of 35%. Aglycones were tentatively identified based on the masses of neutral losses that resulted from MS^2 fragmentation.

To accomplish relative quantification, selected ion chromatograms were integrated to obtain a peak area for each anthocyanin. Each area was divided by the peak area of hydrastine, the internal standard, to generate a relative peak area. Relative peak areas were used in statistical analyses to minimize errors caused by daily variation in instrument response. The relative standard deviation of standardized peak areas for analysis of replicate extractions of the same genotype using this method was in the range of 30%.

Precise determination of the concentration of each anthocyanin in the extracts was not possible because standards are not available for the anthocyanins studied. Instead, relative peak areas were used to compare anthocyanin content among *P. lanceolata* phenotypes

and genotypes. It is not possible to compare accurately the relative concentrations of different anthocyanins in a given extract based on relative peak areas. However, it *is* possible to compare the concentrations of *any single anthocyanin* among different plant extracts. The conclusions made in this article rely only on the latter comparisons.

Hydrolysis of *P. lanceolata* extracts

Hydrolysis was performed in the *P. lanceolata* extracts using a slight modification of a previously published method (Pazmiño-Durán et al. 2001). The solvent was removed from an aliquot (50 ml) of a *P. lanceolata* extract from a single genotype using evaporation under vacuum at 35°C. The remaining solid (151.5 mg) was then redissolved in 50 ml of 2 M hydrochloric acid. The sample was heated for 45 min at 100°C and cooled on ice, and the solvent was again removed using the same procedure. The remaining product was resuspended in 450 μl of 75:25 methanol:water and filtered to remove particulate matter using a microcentrifugal filter (Costar; Fisher Scientific, Pittsburg, PA) with a 0.20 μm nylon membrane. The filtrate was analyzed using the same HPLC/ESI-MS procedure described for the *P. lanceolata* extracts, and retention times for the anthocyanidins detected were compared with those of standards.

Statistical treatment of data

We used SAS multivariate analyses of variance (MANOVAS) and analyses of variance (ANOVAS) to test the hypotheses. MANOVA was used because multiple anthocyanins were detected in each individual extract. Anthocyanins are all related in the sense that they are products of the same biochemical pathway. Thus, it was important to examine first the effect of the independent variables, temperature and genotype, on the whole pattern of anthocyanin production. The peak area standardized to hydrastine was the measure of anthocyanin production. Genotype was treated as fixed because we chose for our experiment specific genotypes that spanned the known range of genetic variation in color plasticity in North Carolina. MANOVA produces multivariate *F* statistics for Wilks' lambda, Pillai's trace, Hotelling–Lawley trace and Roy's greatest root. Wilks' lambda is reported here. For all analyses, the conclusions drawn from the four statistics are identical. When the MANOVA results were found to be significant, we then examined the effects of temperature and genotype on each individual anthocyanin using ANOVA. This allowed us to determine which anthocyanins explained the temperature effect on the overall pattern and which anthocyanins showed genetic variation in anthocyanin levels and in temperature sensitivity. The difference between standardized peak areas at low and high temperatures was used as the measure of temperature sensitivity in anthocyanin production. A larger difference indicates greater sensitivity, i.e. plasticity, of a genotype.

Results

Identification of anthocyanins

We identified 17 anthocyanins in the floral extracts (Table 1). The anthocyanins are derived from four common anthocyanidins (structures in Fig. 1): 8 from cyanidin, 6 from petunidin, 2 from delphinidin and 1 from peonidin. All are from the cyanidin and delphinidin branches of the anthocyanin biosynthetic pathway.

The *P. lanceolata* anthocyanins absorbed in the 500–550 nm range (e.g. cyanidin glycoside a in Fig. 2A), in agreement with the known standards and the literature values for anthocyanins (Tsao and Yang 2003). Collisionally induced dissociation caused fragmentation of the bonds between the sugar substituent (the glycone) and the anthocyanidin core (the aglycone). Ions corresponding to successive neutral losses of sugars were observed in the MS^2 fragment spectra of the anthocyanins (e.g. cyanidin glycoside a in Fig. 2B). The masses of the fragments for the aglycones matched those of the standards (e.g. cyanidin glycoside a in Fig. 2C). Also, multiple neutral losses of water (m/z 18) and carbon monoxide (m/z 28) resulted from fragmenting the delphinidin and cyanidin aglycones in the *P. lanceolata* anthocyanins. This pattern of losses matched the losses from fragmenting cyanidin and delphinidin standards (e.g. cyanidin glycoside a in Fig. 2C). Such fragments are expected for all anthocyanidins substituted only with hydroxyl groups (i.e. delphinidin and cyanidin). Fragmentation of anthocyanidins substituted with methoxyl and hydroxyl groups (i.e. peonidin) resulted in an initial loss of 15 daltons (for the methyl group on the methoxide), followed by a cascade of neutral losses corresponding to the losses observed for the peonidin standard. Thus, the fragmentation patterns of the *P. lanceolata* anthocyanins matched those of standards and the fragmentation patterns of anthocyanins in other plant species (Flamini 2003; Bednar et al. 2005).

Hydrolysis of the anthocyanins further verified the cyanidin aglycones. Chromatographic analysis of the hydrolyzed anthocyanins showed a characteristic peak for cyanidin, for which both the MS^2 fragment spectra and the chromatographic retention time matched those of the standard. The other anthocyanidins were not observed after hydrolysis, presumably because their concentrations were too low for detection.

Table 1. Assignment of glycones and aglycones for anthocyanins from *P. lanceolata* flowers. caf, caffeic acid; cou, coumaric acid; fer, ferulic acid; gal, galactose; glu, glucose; gluc, glucuronic acid; mali, malic acid; malon, malonic acid; n.i., not identified; rha, rhamnose; t_R, retention time.

Name	t_R (min)	Anthocyanin mass	Aglycone mass	Glycone mass and tentative assignment
Cyanidin glycoside a	21	611	287	162 (glu/gal/caf), 162 (glu/gal/caf)
Cyanidin glycoside b	23	449	287	162 (glu/gal/caf)
Cyanidin glycoside c	29	697	287	162 (glu/gal/caf), 248 (mal + glu/gal)
Cyanidin glycoside d	33	783	287	599 (n.i.)
Cyanidin glycoside e	38	463	287	176 (gluc/fer)
Cyanidin glycoside f	41	449	287	162 (glu/gal/caf)
Cyanidin glycoside g	43	535	287	248 (malo + glu/gal or mali + xyl/arab)
Cyanidin glycoside h	45	449	287	162 (glu/gal/caf)
Delphinidin glycoside a	36	713	303	410 (n.i.)
Delphinidin glycoside b	38	465	303	162 (glu/gal/caf)
Peonidin glycoside a	43	477	301	176 (gluc/fer)
Petunidin glycoside a	34	479	317	162 (glu/gal/caf)
Petunidin glycoside b	35	625	317	146 (rha/cou), 162 (glu/gal/caf)
Petunidin glycoside c	41	625	317	146 (rha/cou), 162 (glu/gal/caf)
Petunidin glycoside d	42	479	317	162 (glu/gal/caf)
Petunidin glycoside e	44	565	317	248 (malo + glu/gal or mali + xyl/arab)
Petunidin	36	—	317	—

Influence of temperature and genotype on anthocyanin levels

Overall, anthocyanin levels were significantly higher in flowers produced at cool temperature (Fig. 3; MANOVA main effects of temperature: Wilks' lambda F value = 39.9, $P < 0.0001$). When tested individually, all but 4 of the 17 anthocyanins showed increased levels at cool temperature (ANOVAs, $P < 0.05$). Four, derived from petunidin, were temperature insensitive (ANOVAs $P > 0.05$). The probability that these temperature-insensitive anthocyanins would all be derived from petunidin by chance is very small [$P = 0.0063$, given the observed distribution of anthocyanidins (i.e. eight from cyanidin, six from petunidin, etc.)].

Genotypes differed significantly in overall anthocyanin levels (MANOVA main genotype effects: Wilks' lambda F value = 7.41, $P < 0.0001$), and genotypes differed when each anthocyanin was examined individually (ANOVAs: for every anthocyanin, $P < 0.05$). The relative difference in anthocyanin levels produced at warm and cool temperatures, i.e. the temperature-sensitive plasticity in anthocyanin production, varied significantly among genotypes (MANOVA genotype by temperature interaction: Wilks' lambda F value = 3.94, $P < 0.0001$). Whereas some genotypes greatly increased anthocyanin levels in response to a drop in temperature, other genotypes were unresponsive to temperature change. These genotype-specific differences were detected in 8 of the 17 anthocyanins (ANOVAs, $P < 0.05$). Of these eight, six are derived from cyanidin, one from delphinidin and one from peonidin (shown along x-axis in Fig. 4). None is derived from petunidin. The probability of this happening by chance is very low. Given the observed distribution of 17 anthocyanins, the probability that 6 would be derived from cyanidin and that 1 each would be derived from petunidin and delphinidin by chance alone is 0.0023. The more general probability of observing that 7 of the 8 are derived from the cyanidin branch (and only one from the delphinidin branch) of the biosynthetic pathway is 0.0039.

Because of the high number of anthocyanins showing genetic variation in temperature sensitivity, we could not statistically test if this sensitivity was related to floral color plasticity. The MANOVA test requires many more replicates genotypes than the number of anthocyanins showing genetic variation. However, when we looked at the temperature-sensitive plasticity in anthocyanin levels for each of the floral color plasticity groups, HIGH, MEDIUM, and LOW, we did see a trend toward a positive relationship (Fig. 4). The HIGH floral color plasticity group, whose flowers darken most at cool temperature, showed the greatest mean temperature sensitivity in anthocyanin level for all eight anthocyanins. For six of the eight anthocyanins, the LOW floral color plasticity group showed the least mean temperature sensitivity.

Discussion

Our data provide new insights about temperature regulation of anthocyanin production in plants. In previous studies, anthocyanin levels at high and low

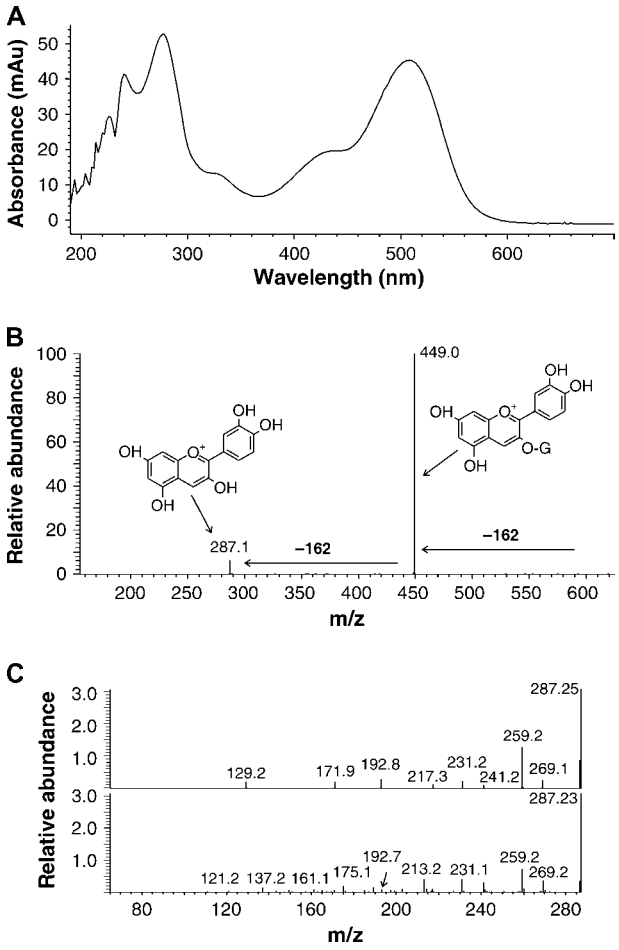

Fig. 2. Spectra used to identify cyanidin glycoside a from a *P. lanceolata* extract obtained from a genotype flowering under cool conditions. (A) Absorbance spectrum obtained using HPLC with spectrophotometric detection. Note the absorbance maximum in the 500–550 nm range, which is characteristic for anthocyanins (Tsao and Yang 2003). (B) MS^2 spectrum for cyanidin glycoside a (precursor ion m/z 610.9) from a *P. lanceolata* extract grown under cool conditions. Two peaks are observed, one at m/z 449.0 for the loss of the first sugar and the second at m/z 287.1 for the loss of both sugars. For both fragment peaks, the mass of the neutral loss is 162, which corresponds to a hexose (glucose or galactose). The fragment at m/z 287.1 corresponds to the aglycone (the anthocyanidin core). (C) Comparison of mass spectrum of fragments of the aglycone of cyanidin glycoside a from *P. lanceolata* (top), and the mass spectrum of fragments from standard cyanidin (bottom). The top spectrum was obtained with MS^3 analysis of the 287 fragment of the anthocyanin with m/z 611, and the bottom spectrum was obtained by MS^2 analysis of standard cyanidin (m/z 287). In both spectra, the collision energy was 35%. More of the lower abundance fragments are observed in the spectrum for the standard (bottom) because the standard produced a wider chromatographic peak (because of higher concentration) and provided 61 scans that could be averaged compared with only 7 scans averaged for the cyanidin from the extract.

temperatures have routinely been compared by measuring the absorbance of hydrolyzed extracts (i.e. the anthocyanidins) in the 530 nm range (Chalmers et al. 1973, Faragher 1983, Kleinhenz et al. 2003, Rabino and Mancinelli 1986, Shichijo et al. 1993, Zhang et al. 1997). This method allows one to measure temperature-induced change in overall anthocyanin production. Our method provides more detailed information because it allows changes in production of single anthocyanins to be measured. Previous studies have shown an increase in total anthocyanin level with moderate temperature decline for vegetative structures (Christie et al. 1994, Kleinhenz et al. 2003, Rabino and Mancinelli 1986, Shichijo et al. 1993), flowers (Biran and Halevy 1974, Biran et al. 1973, Ratsek 1944, Shaked-Sachray et al. 2002, Shvarts et al. 1997a, 1997b) and fruits (Faragher 1983, Zhang et al. 1997). Our data show that in *P. lanceolata* flowers, levels of multiple anthocyanins increase with a moderate temperature decline.

The observed increases are not explained by the variation in light intensity among growth chambers used for the experiment. Photoinduction of anthocyanins has been demonstrated multiple times in laboratory and field experiments (Chalker-Scott 1999; Gould 2004; Hughes et al. 2005). Increasing UV, visible, and far-red radiation typically increases anthocyanins levels. These studies suggest that if light explained our results, we should have observed results opposite to what we did observe. This contradiction and previous work showing that floral color in *P. lanceolata* can be altered by changing temperature in a single growth chamber (Lacey and Herr 2005) support our conclusion that temperature, and not some other physical factor, explains our results.

The mechanisms underlying temperature sensitivity in anthocyanin production are poorly understood [e.g. reviews by Mol et al. (1996), Chalker-Scott (1999) and Gould 2004]. Our data suggest that temperature regulation occurs at multiple steps in the biosynthetic pathway. First, our data show that temperature, i.e. the MANOVA main temperature effect, influenced an equivalent number of anthocyanins produced along both the cyanidin and the delphinidin branches. This suggests that at least one temperature control is upstream to dihydrokaempferol, where the branching occurs (Fig. 1). This conclusion is consistent with genetic studies, showing that transcript levels of PAL, CHS, and CHI genes can increase at low temperature, which may lead to increased enzyme activity upstream (Christie et al. 1994, Leyva et al. 1995, Shaked-Sachray et al. 2002, Shvarts et al. 1997a). Second, temperature appears to control at least one step downstream of dihydrokaempferol and primarily in the cyanidin branch. This is suggested by the observation that, with one exception, genetic variation in

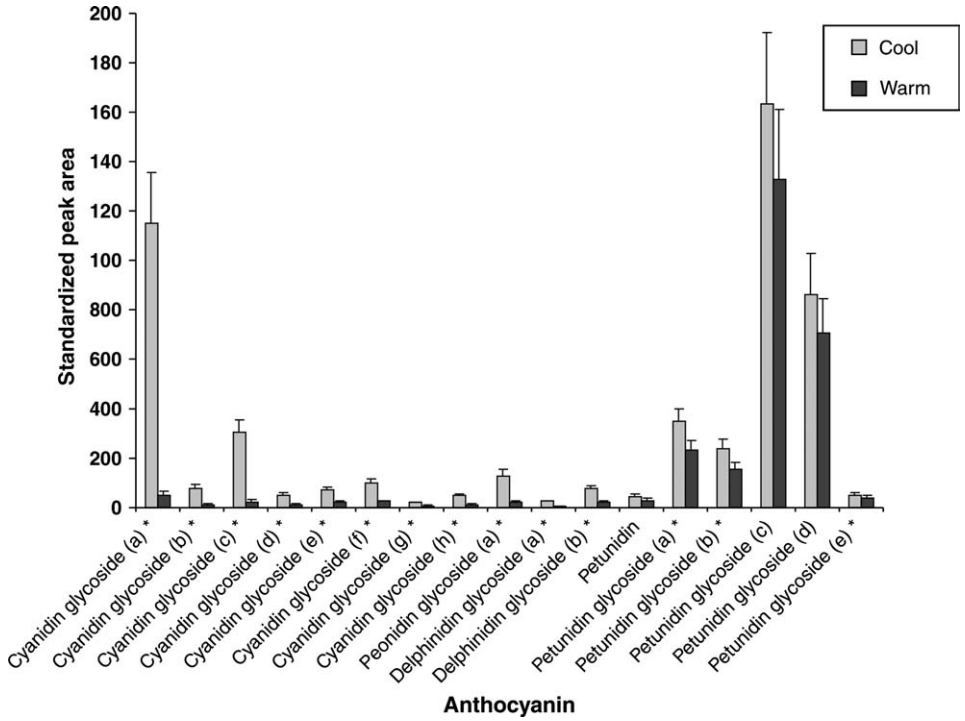

Fig. 3. Mean standardized abundances of anthocyanins detected in *P. lanceolata* flowers produced at warm (dark bars) and cool (light bars) temperatures. Mean values were calculated from two to four replicate extractions of 12 genotypes. Vertical bars represent 1 SE. Mass spectrometric data allow one to compare relative abundances within an anthocyanin but not between anthocyanins (*Materials and Methods*). The asterisk indicates a significant temperature effect.

temperature sensitivity of anthocyanin levels was detected in anthocyanins produced only along the cyanidin branch. The probability of this happening by chance is very low. The combined observations suggest that the genes controlling temperature sensitivity, i.e. phenotypic plasticity, differ from those controlling the overall temperature effect, and that for this species, the 'plasticity' genes are associated with the cyanidin branch. More generally, the data raise the question: are plasticity genes closer to the end products that incur a phenotypic change (in this case flower color) than are genes controlling an overall environmental effect? Such a location of plasticity genes would allow a plant to respond more quickly to an environmental change than if the controlling genes were farther upstream and would be advantageous if the plasticity is adaptive. Further research will be needed to address this question.

Previous discussions concerning the adaptive significance of increased anthocyanin production at low temperature has focused almost exclusively on leaf and stem tissues [e.g. reviews by Onslow (1925), Chalker-Scott (1999) and Gould (2004)]. Some studies suggest that anthocyanins confer cold hardiness, and Chalker-Scott (1999, 2002) has suggested that anthocyanins function as osmoregulators, thereby increasing cold hardiness. However, the data provide conflicting results, and the adaptive significance in vegetative tissues is still unclear.

Our data in combination with data from Lacey and Herr (2005) and circumstantial evidence from studies of flowers in other species suggest that increased anthocyanin production at low temperature in flowers is adaptive because of the warming effect of anthocyanins. Anthocyanins are known to determine flower color in many species. Floral color influences internal floral temperature in *P. lanceolata* (Lacey and Herr 2005) and in several other species (Jewell et al. 1994, McKee and Richards 1998, Mølgaard 1989). Temperature influences pollen development, pollen tube growth and seed set in many species (Jacobsen and Martens 1994, Lankinin 2001, Stanton and Galen 1989, Stephenson et al. 1992, Webber et al. 2005). Plants that have the capability of adjusting internal floral temperature by modifying floral color should have a selective advantage over those that lack the capability in environments where ambient temperature fluctuates during the reproduction season. Such a situation exists for *P. lanceolata*, which can flower from spring through the summer into autumn.

To explore the adaptive significance of the thermoregulatory capability of *P. lanceolata* flowers, one would like to identify the mechanism underlying the capability and

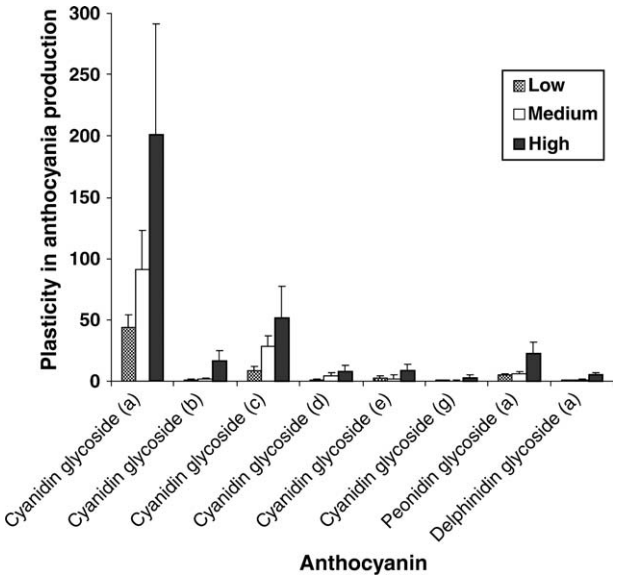

Fig. 4. Mean temperature sensitivity, i.e. plasticity, in anthocyanin production shown for LOW, MEDIUM and HIGH floral color plasticity groups in *P. lanceolata*. The only anthocyanins shown are those for which there were significant genotype-specific differences in temperature sensitivity. Mass spectrometric data allow one to compare relative abundances within an anthocyanin but not between anthocyanins (*Materials and Methods*).

also show that the underlying mechanism is genetically variable. Genetic variation is necessary for evolutionary change by natural selection. In *P. lanceolata*, the thermoregulatory capability is explained by temperature-sensitive changes in floral reflectance in the visible and near-infrared spectral regions. The relative importance of reflectance in the visible region probably increases on cloudy days, when solar irradiance in the visible region increases relative to irradiance in other regions (Gates 1980). Anthocyanins absorb light in the visible region, and our data show both that *P. lanceolata* flowers contain multiple anthocyanins and that temperature sensitivity in anthocyanin production is genetically variable. The low number of replicate experimental genotypes limited our ability to test statistically for a positive correlation between temperature sensitivity in anthocyanin production and floral color plasticity. However, our data do show a trend in that direction. Previous studies examining genetic variation in temperature sensitivity have examined only cultivars or inbred lines (Christie et al. 1994, Kakani et al. 2005, Kleinhenz et al. 2003). Our experiment shows that the temperature sensitivity of anthocyanin production is genetically variable in natural populations. Thus, natural genetic variation in anthocyanin production, the likely mechanism of thermoregulatory ability in the visible region, does exist.

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