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Effects of Anthocyanin and Carotenoid Combinations on Foliage and Immature Fruit Color of *Capsicum annuum* L.

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Abstract

Shades ranging from violet to black pigmentation in pepper (*Capsicum annuum* L.) are attributed to anthocyanin accumulation. High-performance liquid chromatography and mass spectrometry analysis of violet and black fruit tissue identified a single anthocyanin that was determined to be delphinidin-3-p-coumaroyl-rutinoside-5-glucoside. Leaf tissue of a black-pigmented foliage genotype contained the same anthocyanin found in fruit but at a considerably higher concentration in comparison to violet and black fruit tissue. Fruit chlorophyll concentration was approximately 14-fold higher in black fruit in comparison to violet fruit that contained relatively little chlorophyll. β-carotene, lutein, violaxanthin, and neoxanthin carotenoid concentrations in black fruit were also significantly greater in comparison to violet fruit. High concentrations of delphinidin in combination with chlorophyll and accessory carotenoid pigments produced the characteristic black pigmentation observed in fruits and leaves of selected genotypes. Anthocyanins were accumulated in the outer mesocarp of violet and black fruit and in the palisade and mesophyll cells of black leaves. Consistent with chlorophyll content of respective genotypes, chloroplast density was greater in cells of black fruits. Utilizing *Capsicum* pigment variants, we determine the biochemical factors responsible for violet versus black-pigmented pepper tissue in the context of described pepper color genes.

Horticultural crops have been selected and bred for a number of characters that enhance their visual appeal and suitability for various market applications. Color is a key component that influences a consumer's initial perception of product quality. Color pigments are usually associated with flowers and fruits and in some cases, modified leaves associated with flowers.

Color is attributed to several pigments, including the chlorophylls, carotenoids, flavonoids, and betalains. Flavonoids can be further divided into copigments (colorless) and anthocyanins (colored). Chlorophylls are located within chloroplasts located in the cell cytoplasm. Carotenoids are contained within chromoplasts, whereas flavonoid and betalains are located within the cell vacuoles. Most flowers derive their color from one pigment source. The yellow through orange color of flowers are typically due to the carotenoid pigments, whereas blue to red colors are typically attributed to anthocyanins. Chlorophylls are responsible for green color. Griesbach (1984) demonstrated that new unusual colors could be produced through hybridizations that resulted in the 2 pigment classes occurring together. In the case of *Phalaenopsis*, red flower color was shown to be the result of mixing orange carotenoids with magenta flavonoids. The same phenomenon occurs in red *Sophronitis* flowers (Matsui and Nakamura 1988) and colored foliage (Lee 2002).

A wide range of functions for color pigments in plant tissues have been demonstrated or hypothesized. In flowers, they may function as attractants for pollinators and in seeds and fruits, aid in their dispersal (Harborne 1994; Schemske and Bradshaw 1999). They may also function as antioxidants as well as ultraviolet and visible light protectants in plant tissues (Gross 1991; Close and Beadle 2003).

Considerable diversity exists in *Capsicum* for fruit and leaf color (Stommel and Bosland 2006). Pepper fruit color is important for culinary product quality. In addition to fruit color, foliar pigmentation is also important for ornamental applications. Color of unripe pepper fruit varies from green to ivory, through varying shades of violet to nearly black. Green to ivory immature fruit color is attributed to a series of loci (Odland and Porter 1938). Ivory or sulfury white fruit color is conditioned by the *sulfur white*, sw_1 locus. Lettuce or yellow green color is attributed to the sw_2 locus that is dominant to sw_1 and recessive to dark green or cedar green color conditioned by sw_3. The sw_3 locus is dominant to sw_1.

Violet to black pigmentation in pepper tissues is attributed to anthocyanin accumulation and is influenced by an incompletely dominant gene, *Anthocyanin* (*A*) (Peterson 1959). A second gene, *modifier of A* (*MoA*), intensifies the purple color in the presence of *A* (Deshpande 1933). Violet to black pigmentation of unripe fruit provides ornamental interest for the lengthy maturation period that precedes fruit ripening and is also valued for culinary markets. Similar to green pepper fruit pigmentation, violet to black pigmentation of immature fruit is transient. Coincident with fruit ripening, orange to red carotenoids are accumulated and chlorophylls and anthocyanins are degraded.

In addition to immature fruit, gradations in anthocyanin pigmentation may be observed in other *Capsicum* plant parts as well. Foliage and stem color varies from green to varying shades of green/violet to nearly black. In contrast with fruit tissue, anthocyanin pigmentation is normally stable through plant development in other plant organs of those genotypes that exhibit anthocyanin accumulation.

Selected breeding of *Capsicum* accessions has produced new breeding lines and cultivars with a diverse array of fruit and foliage pigmentation (Stommel and Griesbach 1993, 2004, 2005). In this study, we conduct an anatomical characterization of pigmented tissues and determine the biochemical factors responsible for violet versus black color forms in the context of described pepper color genes.

Materials and Methods

Plant Material

Fully expanded leaves and full-size immature fruit were obtained from field-grown plants of the true-breeding *Capsicum annuum* L. genotypes G05C69-12 (black fruit/black foliage) and G05C74-12 (violet fruit/green foliage) selected from the United States Department of Agriculture, Agricultural Research Service, Beltsville, Maryland pepper breeding, and genetics program (Stommel and Griesbach 1993, 2004, 2005). Standard pedigree breeding methodology was utilized in developing these breeding lines that are descended from parent stock used for breeding the recently released cultivar "Black Pearl" (Stommel and Griesbach 2005). Plants were grown at the Beltsville Agricultural Research Center, Beltsville, MD, in Keyport fine loam soil using a completely randomized design. Plants were spaced at 0.3-m intervals in single rows on polyethylene-covered raised beds, with beds positioned on 1.5-m centers with trickle irrigation. Pest control and fertilizer regimes followed standard horticultural practices for pepper production in Maryland (University of Maryland 2007).

Color and pH Determination

Fruit and leaf color of 10–15 tissue samples from the respective breeding lines was determined using Munsell notation (Munsell 1912; Nickerson 1946). The Munsell color system is based on rigorous measurements of human subjects' visual responses to color. It is a color space that specifies colors based on 3 color dimensions, hue, value (or lightness), and chroma (saturation). The Munsell Book of Color (Munsell Color Services, New Windsor, NJ) was used instead of the Royal Horticultural Society's Colour Charts because it is not possible to interpolate between color chips using the Royal Horticultural Society's Colour Charts (Griesbach and Austin 2005).

It was previously determined that the pH of an epidermal peel suspension accurately reflected the pH of a single cell (Stewart et al. 1975). Therefore, the upper epidermis was stripped and the epidermal strips from a single fruit were combined. Strips were then ground into a suspension with distilled water and the pH of the suspension measured with a micro pH meter (Sentron 501; Sentron, Inc., Federal Way, Washington, DC). The pH measurements were recorded as a mean of 5 replicates, each replicate representing the pooled tissue collected from a single fruit.

Pigment Analysis

Analytical high-performance liquid chromatography (HPLC) (Griesbach et al. 1991) and mass spectroscopy were used to identify the anthocyanin pigments in immature violet and black pepper fruit and black foliage. Anthocyanins from 3 fruit samples derived from separate plants were extracted in acidified methanol (1% HCl). Aglycones were characterized using extracts that were acid hydrolyzed at 100 °C in 3 N HCl for 1 h. Isolated compounds were characterized along with known standards by HPLC, R_fs, UV, and Vis spectral analysis and by the products of controlled acid or base hydrolysis (Harborne 1967; Mabry et al. 1970). HPLC characterization of acid hydrolyzed compounds was performed on a 7.8 × 300 mm column of 5 μ Bondapak C18 using a 20-min linear gradient of 0% to 15% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid and held at 15% (v/v) for an additional 20 min. Flow rate was 1.0 ml min^{-1}, and detection was by absorption at 540 nm. Anthocyanidins were characterized by coelution with known standards (Griesbach et al. 1991). Nonacid hydrolyzed extracts were separated as described above by HPLC using a 30-min linear gradient of 0% to 10% (v/v) acetonitrile in aqueous 1.5% (v/v) phosphoric acid and 15% (v/v) acetic acid followed by a 10-min linear increase to 20% (v/v) acetonitrile and held at 20% (v/v) acetonitrile for an additional 20 min. Flow rate was 1.0 ml min^{-1}, and detection was by absorption at

540 nm. Anthocyanins were characterized by coelution with known standards (Asen et al. 1970). The amount of anthocyanin was determined by measuring the peak area of 3 replicates using the Maxima software (Waters Corporation, Milford, MA) and known concentrations of standards.

A HPLC-purified anthocyanin extract from pepper fruit was further characterized by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) on an Agilent MSD SL ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface, an Agilent HP series1100 HPLC, a Zorbax SB-C18 column (5 μm, 250 × 4.6 mm) and guard cartridge (Agilent Technol., Wilmington, DE), a diode array detector (DAD), and ChemStation software. The mobile phase consisted of 2 solvents: solvent A was 10% aqueous formic acid and solvent B was 10% formic acid in methanol. The mobile phase was comprised of 90% solvent A for 5 min, followed by a linear gradient to 70% solvent A at 15 min, then to 40% solvent A at 20 min, returning to 90% solvent A at 30 min, with a flow rate of 0.8 ml min^{-1}, and an injection volume of 50 μl. MS and ESI conditions were as follows: nebulizer, 60 psi; dry gas (N_2), 11 l min^{-1}; dry temperature, 350 °C; skimmer, 40 V; octopole RF amplitude, 200 Vpp; and capillary exit, 126 V. The ion trap mass spectrometer was operated in positive ion mode scanning from m/z of 100 to 800.

Chlorophyll and carotenoids in fruit tissue of violet and black immature-fruited materials were analyzed by HPLC. Triplicate fruit samples from separate plants were ground in a glass tissue grinder, then extracted twice with 80% (v/v) acetone in water and twice with 100% acetone (Adams and Demmig-Adams 1992). For each fruit sample, the 4 extracts were pooled, β-apo-8'-carotenal was added as an internal standard, and solvent was removed by evaporation under N_2. The sample was resolubilized in 5/94/1 (v/v) methyl-tert-butyl ether/methanol/H_2O with 0.05 M ammonium acetate and 0.05% triethylamine prior to HPLC analysis. Analysis was performed on an Agilent HP series 1100 HPLC equipped with a 3 μm reverse phase C_{30} column (250 mm length × 4.6 mm internal diameter), a 5 mm C_{18} Brownlee guard column, and a DAD set to 450 nm. The mobile phase consisted of methyl-tert-butyl ether (solvent A), methanol (solvent B), and water with 0.05 M ammonium acetate and 0.05% triethylamine (solvent C). Starting conditions were 5% A, 94% B, and 1% C, and solvent C remained at 1% throughout the run. A linear gradient was used to increase solvent A to 15% and reduce solvent B to 84% from 0 to 5 min, and these conditions were held for 5 min. Between 10 and 15 min, solvent A was increased to 70% (with B decreased to 29%), and these conditions were held until 20 min, when a linear gradient returned the mobile phase to the starting conditions over 2 min, and those conditions were held for 8 additional minutes until the end of the run. Compound identifications were determined by elution pattern relative to lutein (Khachik et al. 1992) and were confirmed with spectral characteristics and with comparison to elution times of authentic standards. Quantification of chlorophyll A, chlorophyll B, and β-carotene was performed using external standard curves for each compound. Quantification of xanthophylls was performed by comparing peak areas to those from an external standard curve for lutein because sufficient quantities of individual standards of other xanthophylls were not available for use in quantification.

Table 1. Spectrophotometric profiles and HPLC elution times of the anthocyanidin in known standards and pepper fruit and leaf tissue

Pigment	λ_{max}	$E_{440}/E_{Vis.max}$	Al^{+} shift	HPLC elution time (min)
Pelargonidin	270, 520	0.4	No	24
Cyanidin	277, 535	0.2	Yes	18
Peonidin	274, 532	0.3	No	28
Delphinidin	277, 546	0.2	Yes	13
Petunidin	276, 543	0.2	Yes	21
Malvidin	275, 542	0.2	No	31
Black fruit	275, 545	0.2	Yes	13
Violet fruit	277, 548	0.1	Yes	13
Black leaves	281, 541	0.1	Yes	13

Anatomical Analysis

Tissue cross sections were prepared from hand sections of fresh leaf and fruit tissue. Sections were mounted in water and examined with an inverted microscope (Ziess LSM 410) under differential interference contrast microscopy.

Results

Color and pH Determination

Plants bearing violet colored fruit produced fruit that varied from light violet (Munsell hue value/chroma of 1.5P 3.5/7) to dark violet (1.5P 1.5/5). Conversely, black (5.6RP 2.0/0.5) fruit and black (5.6RP 2.0/0.5) foliage were scored in discrete color classes.

There was no statistically significant difference in the pH of violet and black fruit ($P = 1.00$; data not shown). Therefore, the color difference between the fruit was not the result of a change in pH.

Pigment Analysis

Only a single anthocyanidin was found in violet fruit, black fruit, and black leaves. This anthocyanin had the same HPLC elution time and spectra as delphinidin (Table 1). This agreed with the literature where the anthocyanidin in *C. annuum* fruit was determined to be delphinidin (Endo 1953). Characterization of the glycone using HPLC revealed a single anthocyanin compound from black fruit that comigrated with the standard delphinidin-3-p-coumaroyl-rutinoside-5-glucoside (Figure 1). Additional analyses demonstrated comigration of the delphinidin glycone from black fruit with the anthocyanin compound present in black foliage and violet fruit (data not shown). MS/MS analysis produced a parent ion with m/z 919, in accord with delphinidin-3-*p*-coumaroyl-rutinoside-5-glucoside. The MS/MS

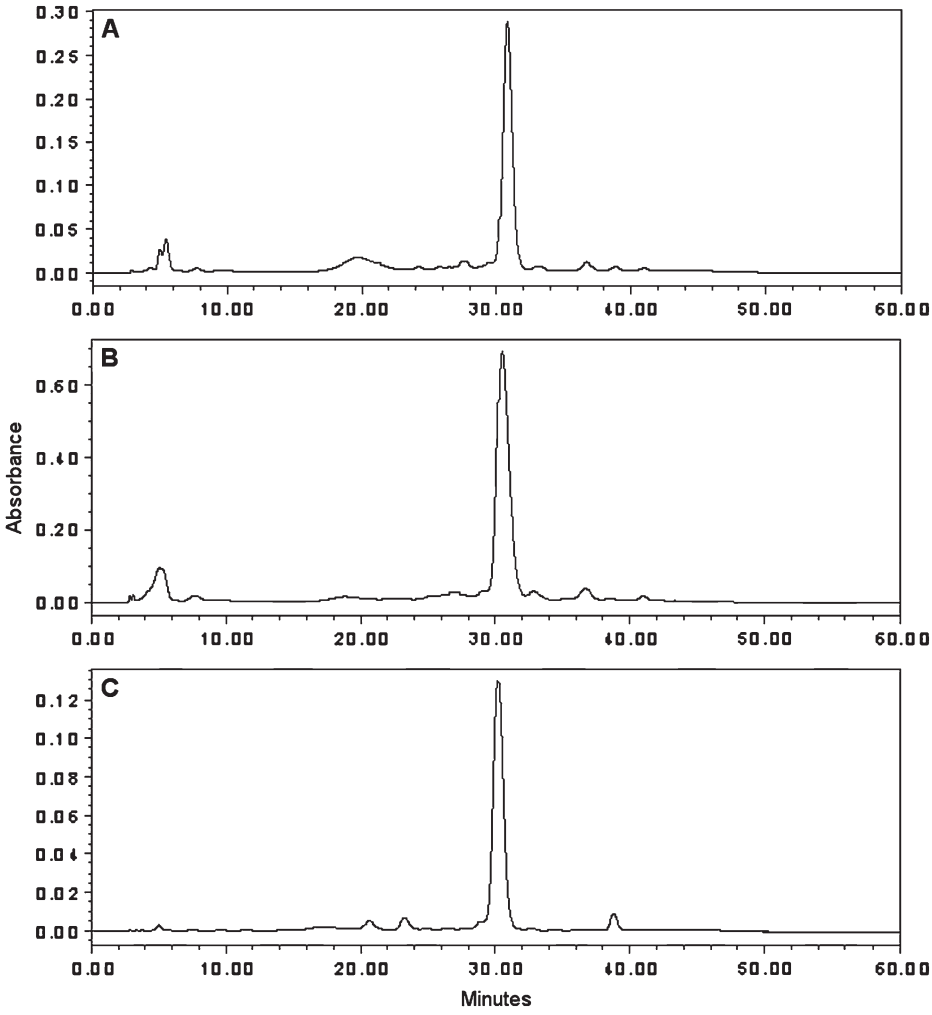

Figure 1. HPLC anthocyanin profiles representing (**A**) comigration of the delphinidin-3-p-coumaroyl-rutinoside-5-glucoside standard with anthocyanin from black *Capsicum annuum* L. fruit, (**B**) delphinidin-3-p-coumaroyl-rutinoside-5-glucoside standard (**C**) anthocyanin from black *Capsicum annuum* L. fruit.

product ions also support the compound identification, with a fragment of m/z 303 (in accord with delphinidin), one with m/z 757 (showing loss of a hexose minus water from the parent compound), and one with m/z 465 (showing an additional loss of a rhamnose and a p-coumaroyl group minus water, and in accord with delphinidin retaining a hexose group). Sadilova et al. (2006) recently identified the same compound in pepper fruit.

The difference in the delphinidin concentration between black (208 μg anthocyanin/g fresh weight) and violet fruit (90 μg/g) was not statistically significant ($P = 0.333$). The reason was the high variability between samples. Different fruits on the same plant varied in color intensity based on their age and exposure to light. Fruits that were shaded by leaves were less intensively pigmented than those that were unshaded. Delphinidin concentration was more than 7-fold greater in black leaves (1525 μg/g) in comparison to black fruit. Preliminary analysis of green fruits and leaves demonstrated that these tissues lacked colored anthocyanins (data not shown). The chlorophyll concentration (chlorophyll A + B) in black fruit (9.85 μg/g; Table 2) was approximately 14-fold higher in comparison to violet fruit (0.68 μg/g; Table 2). This difference was statistically significant ($P < 0.001$). As expected, the chlorophyll concentration was orders of magnitude higher in leaves (data not shown). Concentrations of the carotenoids β-carotene, lutein, violaxanthin, and neoxanthin in black fruit were also significantly greater in comparison to violet fruit (Table 2). The total concentration of the carotenoids detected in black fruit (30.16 μg/g) was approximately 7-fold greater than that in violet fruit (4.35 μg/g).

Anatomical Analysis

Light microscopy revealed a clear colorless exocarp and a 2 to 5 cell–layered region where anthocyanin accumulated in the vacuoles of black (Figure 2A) and violet-pigmented (Figure 2C) fruit. Color intensity was greatest in the smaller

Table 2. Chlorophyll, carotenoid, and xanthophyll content of black and violet *Capsicum annuum* L. fruit

Pigment	Black fruit (μg/g fresh wt)[a]	Violet fruit (μg/g fresh wt)[a]
Chlorophyll A	6.28 (1.39) a[b]	0.49 (0.09) b
Chlorophyll B	3.57 (0.61) a	0.19 (0.32) b
B-carotene	0.43 (0.06) a	0.06 (0.01) b
Lutein	14.23 (3.89) a	1.51 (0.19) b
Violaxanthin	9.89 (3.04) a	2.10 (0.09) b
Neoxanthin	3.57 (0.48) a	0.16 (0.28) b
Antheraxanthin	1.06 (0.65) a	0.36 (0.16) a
Zeaxanthin	0.98 (0.92) a	0.16 (0.04) a

[a] Mean (standard deviation).

[b] Means followed by the same letter are not significantly different according to the Tukey Test ($P \leq 0.05$).

outer mesocarp cells but more dilute and diffuse in the larger inner mesocarp cells. Consistent with chlorophyll content of respective genotypes, chloroplast density was greater in cells of black fruit (Figure 2B) relative to that observed in violet fruit (Figure 2D).

Cross sections of green (data not shown) and black-pigmented pepper leaves revealed a typical dicot dorsal–ventral leaf structure with a single-layered upper epidermis with protective cuticle and lower epidermis and a central mesophyll consisting of an upper palisade parenchyma layer and a central spongy parenchyma (Dutta 1997) (Figure 2E). The palisade mesophyll cells of black leaves contained a large number of chloroplasts typical of green leaves, but in contrast with green leaves also contained anthocyanin within the vacuoles (Figure 2F). Black leaves also exhibited anthocyanins in vacuoles of the spongy mesophyll cells that were adjacent to the lower epidermis.

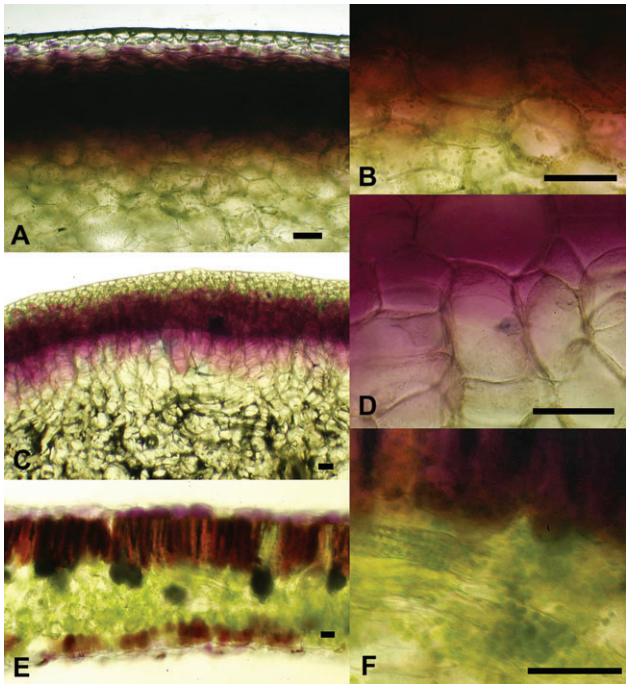

Figure 2. Cross sections of *Capsicum annuum* L. black (**A** and **B**) and violet (**C** and **D**) fruits and black foliage (**E** and **F**). The bar in each image is 75 μm.

Discussion

The difference between violet and black immature pepper fruit color was due to the concentration of chlorophyll and carotenoids. High concentrations of the delphinidin glycoside in combination with chlorophyll and accessory carotenoid pigments produced the characteristic black pigmentation observed in fruit and leaves of selected genotypes. Fruit classified as violet based on phenotype contained relatively little chlorophyll and carotenoids. Within the respective violet- and black-fruited variants, delphinidin concentration was variable and not significantly different between the 2 variants. Whereas intensity of violet pigmentation varied with delphinidin concentration, black color was scored discretely. Similar to other anthocyanin-pigmented species, anthocyanin pigment was preferentially accumulated in outer cell layers of pepper fruit and leaf tissues (Harborne 1988). Consistent with anatomical observations of chloroplast density, chlorophyll concentrations were significantly different between black and violet variants and the presence of higher chlorophyll concentrations imparted black pigmentation across all delphinidin concentrations evaluated in the black-fruited variant. The low-chlorophyll concentrations observed in violet tissues were insufficient to elicit black pigmentation.

In black fruit, consistent with tissues containing appreciable levels of chlorophyll, lutein, the major xanthophyll, represented approximately 45% of the total carotenoid present (Goodwin and Britton 1988). Significantly higher concentrations of β-carotene, lutein, violaxanthin, and neoxanthin observed in black fruit also contribute to overall fruit color. The addition of appreciable levels of orange to yellow carotenoids would be expected to shift fruit color toward yellow to brown hues. However, relative to observed carotenoid concentration, the nearly 700% higher delphinidin concentrations present in black tissues combined with green chlorophylls were the greatest determinants of black pigmentation.

Our observations on violet versus black pepper fruit illustrate the independence of the different pigment classes that contribute to color. Chlorophylls and anthocyanins are the end products of distinct biosynthetic pathways and so changes in production of the respective pigments may occur independently of each other. For example, green-leaved variants of *Sarracenia* L. may occur wherein the red flavonoids that are normally present in the leaves and flowers are absent (Sheridan and Mills 1998). The chlorophyll and carotenoids, however, are unaffected. In litchi fruit, the red color or lack of it has been shown to be due to chlorophyll levels (Wang et al. 2002, 2005). In poor-colored litchi fruit, high-chlorophyll levels were shown to mask anthocyanins present.

Independence of pigment classes is also evident in mature pepper fruit. Color changes during fruit ripening are typically associated with chlorophyll loss, and/or anthocyanin loss, and/or carotene pigment formation. In pepper, retention of chlorophyll in mature fruit conditioned by the chlorophyll retainer gene (*cl*), combined with red (y^+) or yellow (*y*) carotenoid-mediated pigmentation, results in brown and olive green mature fruit color, respectively (Smith 1948, 1950). Retention of violet anthocyanin pigments in mature pepper fruit provides additional color gradation in combination with carotenoids that accumulate in ripening fruit (Stommel and Bosland 2006).

The results of our study are consistent with *Capsicum* anthocyanin and chlorophyll variants described in the literature. Although respective chlorophyll and anthocyanin variants have been described to account for ivory and black immature fruit color, an explanation for violet versus black fruit color is lacking. Very low chlorophyll levels and reduced anthocyanin content in violet fruit suggest the combined action of *sulfury white*, *Anthocyanin*, and *modifier of A* (*sw_1 A MoA*). Likewise, the relatively greater anthocyanin and chlorophyll concentrations in black fruit can be explained by the genotype *sw_1^+ A MoA*. A similar genetic mechanism may explain black to violet foliar pigmentation in pepper. Whereas the *A* locus accounts for the occurrence of anthocyanin pigmentation, the action of *MoA* explains variation in intensity of foliar anthocyanin pigmentation where color may range from black to varying degrees of blackish green as a result of variation in concentration of anthocyanin pigment overlaid on green foliage (Deshpande 1933). Violet hues similar to those observed in fruit are not evident in pepper foliage. It is plausible that lethality or close linkage between sw_1^+ and *A* prevents the occurrence of uniformly chlorophyll-deficient violet–colored foliage. Varying shades of violet foliar pigmentation can be observed in variegated pepper variants where anthocyanin is accumulated in chlorophyll-deficient tissue sectors.

Many factors influence the color of anthocyanins (Griesbach 2005). Although occurrence of a true blue hue is the exception in the plant world, "blue" typically refers to colors that tend toward bluish shades of lavender and violet such as those we have described for pepper color variants. The absence of blue-, lavender-, or violet-pigmented tissue is often attributed to their inability to produce blue delphinidin glycosides (Holton and Cornish 1995; Winkel-Shirley 2001). Although blue color is generally attributed to delphinidin, all anthocyanins with the exception of pelargonidin may produce blue color under appropriate conditions (Asen 1976). For example, flowers that contain cyanidin can be red as in *Rosa* L. hybrids (Asen et al. 1971) or blue as in *Meconopsis grandis* Prain. (Takeda et al. 1996).

The in vivo color of an anthocyanin not only depends on the specific anthocyanin but also on the presence of colorless copigments, metals, and pH (Griesbach 2005). The most important factor determining blue or red color is pH (Stewart et al. 1975; Brouillard 1988; Yoshida et al. 1995). Within the cell, anthocyanins occur bound to copigments in a chemical complex within the vacuole. The same anthocyanin can appear red or blue depending on the pH. As the pH becomes more alkaline, the color of a specific anthocyanin/copigment complex becomes bluer. We did not observe differences in pH between black and violet pepper tissues. In *Petunia*, there are 2 structural genes that are responsible for the vacuolar pH of flowers (de Vlaming et al. 1983; Griesbach 1996). These genes behave in a codominant manner with each allele contributing 0.5 units to the pH. Because there is no difference in the pH of black and violet pepper fruit, it may be possible through breeding to create peppers with true blue fruit by selecting parents with a more alkaline pH.

A number of black- to dark violet-pigmented black plants have been described and are increasingly popular for horticultural applications (Stommel and Griesbach 1993, 2005; Armitage 2002). Included among these are black pansies, cannas, coleus, heuchera, pearl millet, sweetpotato vine, taro, and others. Black flower coloration in *Lisianthius nigrescens* was attributed to delphinidin glycosides (Markham et al. 2004).

Violet and black color variants in *Capsicum* and other plant species have provided valuable opportunities to develop new like-pigmented landscape and garden plants. Characterization of these novel color forms, together with a wealth of information describing the flavonoid biosynthetic pathway, provides new opportunities to alter color and investigate regulatory aspects that affect color and pigment biosynthesis.

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