The Effect of the *Ph 6* Gene on the Color of *Petunia hybrida* Vilm. Flowers

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ADDITIONAL INDEX WORDS. flower color, transposable element activator (Ac), anthocyanin

ABSTRACT. An *in vivo* system was developed to determine the effects of pH on naturally occurring pigment complexes within cells. The *in vivo* system was based on a transposable element activator (Ac) inserted into the *Ph6* gene. The transposable element activator (Ac) was crossed into two genetically marked *Petunia hybrida* lines expressing known flavonoid pigments. Plants expressing the transposable element activator (Ac) produced variegated flowers in which the background tissue was lighter in intensity than the sectors. Depending on the genetic background in which the transposable element is expressed, progeny with darker sectors that were also redder in color than the background tissue could also be obtained. The anthocyanin and copigment composition was the same for both of the differently colored sectors and background tissue, while the pH was lower by 0.4 unit in the redder sectors. It was suggested that the *Ph6* gene might be a regulatory gene that controls the expression of the pH and anthocyanin concentration.

Flower color is the result of a complex physical interaction among the flavonoid pigments (Brouillard, 1983). Within the cell, the flavonoid pigments responsible for color exist as anthocyanin–copigment complexes. At physiological pHs (≈ 3 to 6), anthocyanins are very faintly colored, whereas copigments are colorless. Within the complex, the copigments enhance the stability and intensity of the anthocyanin color. For example, a solution containing 10 mM cyanidin 3,5-diglucoside and 30 mM quercetin has an absorption eight times greater than a solution containing only the anthocyanin (Asen et al., 1972). The intensity of flower color is greatly influenced by the anthocyanin to copigment ratio.

In *Commelina communis* L., the anthocyanin–copigment complex contains six anthocyanin, six copigment and two magnesium molecules (Kondo et al., 1992). The hydrophobic interactions between the aromatic rings of the anthocyanin and copigment molecules result in color. The various residues that are attached covalently to the different rings can modify the color by influencing the hydrophobic interactions.

The chemical environment affects the hydrophobic interactions in the anthocyanin–copigment complex (Brouillard, 1983). For example, the more acidic the pH, the redder the color of the complex (Jurd and Asen, 1966). There are only a few examples in the literature of the effects of pH in living cells in which the anthocyanin–copigment complex was identified. In one instance involving *Ipomoea tricolor* L. 'Heavenly Blue', the pH increased from 6.5 to 7.5 as the flowers opened and the color of the peonidin–quercetin complex changed from $\lambda_{\max}$ of 547 to 592 (Asen et al., 1977).

The genetics of flower color is best known in *Petunia hybrida*. The first major study identified nine genes that were involved in the inheritance of flower color (Paris and Haney, 1958). As biochemical data became available, these genes were assigned specific functions in the biosynthetic pathway (Wiering and deVlaming, 1984; Holton and Cornish, 1995). Besides the anthocyanin structural genes, six genes (*Ph1*, *Ph2*, *Ph3*, *Ph4*, *Ph5*, and *Ph6*) were identified that influenced flower color by modifying the pH (Chuck et al., 1993; deVlaming et al., 1983). In the presence of

the dominant alleles of the *Ph* genes, the pH is lower and the color is redder. The *Ph1* and *Ph2* genes only affect flower color. The other genes are pleiotropic affecting other processes as well (i.e., fertility). All of the genetic differences in the pH of flowers could be explained by the action of only the *Ph1* and *Ph2* genes (Griesbach, 1996).

In *Petunia*, a transposable element activator (Ac) was used to tag the *Ph6* gene (Chuck et al., 1993). In plants homozygous for the inserted transposable element, the flowers were variegated producing dark sectors on a light background. When studying whole-plant Ac-induced mutants and revertants, it was found that when the *Ph6* gene was inactivated, the pH of flowers increased by 0.32 units. It was also noted that in certain genetic backgrounds there was also a color difference between the sectors and background. The sectors were redder. No biochemical analysis was performed to determine either the cause of the color difference or the actual pH of the mutant background and revertant sectors in variegated flowers. This project was undertaken to determine the biochemistry and genetics of the color difference and pH between the sectors and background tissue in flowers expressing an Ac-tagged *Ph6* gene. It was believed that the difference in color was due to a change in the pH.

Materials and Methods

Petunia hybrida 'ph6-m1' (Chuck et al., 1993) was crossed to *P. hybrida* 'Red' and *P. hybrida* 'Violet' (Griesbach, 1996). *Petunia hybrida* 'ph6-m1' contained the transposable element activator (Ac) inserted into the *Ph6* gene of *P. hybrida* 'V26'. *Petunia hybrida* 'V26' was homozygous dominant for the *F1*, *Ht1*, *Hf1*, *Mt1*, and *Mt2* genes and homozygous recessive for the *ph1*, *ph2*, *hf2*, *mf1*, and *mf2* genes (Ronald Koes, personal communication). *Petunia hybrida* 'Red' was homozygous dominant for the *F1*, *Ph6*, *Ph1*, *Ph2*, and *Ht1* genes and homozygous recessive for the *hf1*, *hf2*, *mt1*, *mt2*, *mf1*, and *mf2* genes (Griesbach, 1996). *Petunia hybrida* 'Violet' was homozygous dominant for the *F1*, *Ph6*, *Ht1*, *Hf1*, *Mt1*, *Mt2*, and *Mf1* genes and homozygous recessive for the *ph1*, *ph2*, *hf2*, and *mf2* (Griesbach, 1996).

An F₂ population was produced for both F₁s (*P. hybrida* 'ph6-m1' × *P. hybrida* 'Red' and *P. hybrida* 'ph6-m1' × *P. hybrida* 'Violet') and screened for flower variegation due to the excision of the transposable element activator (Ac). Variegated flowers had

Received for publication 19 Nov. 1997. Accepted for publication 23 Feb. 1998. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

sectors that were darker in intensity than the background tissue.

Variegated flowers were dissected, separating the sectors from the background tissue. Because of the small size of the individual sectors, all the sectors from a single variegated flower were combined before flavonoid (i.e., flavonols and anthocyanins) extraction. Flavonoids were extracted and characterized by high pressure liquid chromatography (HPLC), as previously described (Griesbach and Asen, 1990; Griesbach et al., 1991). Specific anthocyanins and flavonols were identified through coelution with known standards and were also quantified by HPLC with known standards. The specific flavonoids from the pooled tissue collected from a single flower were reported as the percentage of the total. The mean was determined from three replicates, each replicate representing the pooled tissue collected from the same single flower.

The pH of petal tissue was determined one day after flower opening. 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 for all of the sectors from a single flower were combined. The strips were then ground into a suspension and the pH of the suspension measured microspectrophotometrically as previously described (Stewart et al., 1975) and with a micro-pH meter (Sentron 501; Sentron, Inc., Federal Way, Washington). The pH measurements were reported as a mean of 10 replications, each replication representing the pooled tissue collected from the same single flower.

The amount of pigment per cell was quantified by confocal laser microscopy (LSM 410; Zeiss, New York, New York). The upper epidermal cells of the petal observed with a Plan-Neofluar 40×0.75 lens using the 568 nm band of a argon-krypton laser. The cells were optically sectioned and the sections digitally compressed into two dimensions. Image analysis (KS 300 Imaging System; Kontron Elektronik, Hamburg, Germany) was then used to determine the average density of a cell's two dimensional image. Density measurements were made in arbitrary transmission units and were reported as the mean of 50 determinations. The lower the transmission, the higher the concentration of pigment.

Results

GENETIC SEGREGATION. The parent expressing the transposable element (*P. hybrida* 'ph6-m1') produced variegated flowers in

which the sectors were darker in intensity than the background tissue. In the variegated flowers, the background tissue expressed the mutant *ph6ph6* phenotype, while the sectors expressed the revertant *Ph6Ph6* phenotype. As expected, neither *F*₁ hybrid (*P. hybrida* 'ph6-m1' × *P. hybrida* 'Red' or *P. hybrida* 'ph6-m1' × *P. hybrida* 'Violet') produced variegated flowers. Only plants that were recessive for the tagged *ph6* gene were expected to be variegated. Both *F*₁ plants were sib-mated to produce an *F*₂ population.

In the 'ph6-m1' × 'Red' *F*₂ population, 105 of the progeny had nonvariegated flowers and 37 of the progeny had variegated flowers. The ratio of plants with nonvariegated flowers to plants with variegated flowers was 105:37. This ratio did not differ statistically from a 3:1 ratio which is expected for a single recessive gene (chi square = 0.11, *P* > 0.75). In the 'ph6-m1' × 'Violet' *F*₂ population, 110 of the progeny had nonvariegated flowers and 36 of the progeny had variegated flowers. This ratio fit a 3:1 ratio exactly (chi square = 0). A 3 *Ph6* : 1 *ph6ph6* ratio was expected in the *F*₂. Only those plants expressing the recessive phenotype should have produced variegated flowers.

All of the variegated flowers within the 'ph6-m1' × 'Violet' *F*₂ population were identical with sectors that were darker in intensity than the background tissue. In contrast, the variegated flowers in the 'ph6-m1' × 'Red' *F*₂ population were of two different types. In one type, the flowers had sectors that were darker in intensity than the background tissue. In the other type, the sectors besides being darker were also redder than the background tissue. Of the 37 progeny in the 'ph6-m1' × 'Red' *F*₂ population, 25 produced flowers with sectors that were darker and redder. The ratio of plants with redder sectors to plants with sectors the same color as the background was 25:12. This ratio did not fit the 3:1 ratio expected for a single gene (chi square = 0.65, *P* < 0.50) or the 9:7 (chi square = 1.76, *P* < 0.25), 12:4 (chi square = 1.32, *P* < 0.25) or 15:1 (chi square = 52.86, *P* < 0.001) ratios expected for two genes. The 25:2 ratio did fit the 45:19 ratio [(27+9+9) : (9+3+3+3+1)] expected for three genes (chi square = 0.13, *P* > 0.75). This ratio assumes that the phenotype is due to the expression one specific gene coupled with the expression of at least one other gene, of which there are two possible (i.e., *A-B-C-* or *A-B-cc* or *A-bbC-*).

pH. Preliminary pH measurements made with the microspectrophotometer and micro-pH meter were identical; therefore all further pH measurements were made with the micro-pH meter. In the all plants of the that produced variegated flowers in the 'ph6-

Table 1. Properties of the mutant background and revertant sectors of variegated flowers from the *F*₂ population of *Petunia hybrida* 'ph6-m1' × *P. hybrida* 'Red'. The value used to describe color was obtained from the Royal Horticulture Society's Colour Charts. The amount of pigment within an individual cell was measured in arbitrary transmission units as described in the text. A higher value indicates less absorption and therefore less pigment.

Plant	Color	Pigment	f/a ²	pH
8-1				
Mutant	Purple (64A)	128 (17) ³	1.06 (0.01)	5.8 (0.1)
Revertant	Magenta (78A)	14 (3)	1.00 (0.01)	5.4 (0.1)
8-8				
Mutant	Violet (90A)	95 (11)	1.18 (0.17)	5.7 (0.1)
Revertant	Purple (64A)	41 (3)	1.06 (0.11)	5.3 (0.1)
9-1				
Mutant	Pale violet (90C)	74 (10)	1.13 (0.13)	6.1 (0.1)
Revertant	Violet (90A)	31 (2)	1.16 (0.08)	6.0 (0.1)
9-2				
Mutant	Pale magenta (78C)	81 (12)	1.28 (0.20)	5.8 (0.1)
Revertant	Magenta (78A)	29 (5)	1.23 (0.19)	5.7 (0.1)

²f/a = ratio of flavonol to anthocyanin.

³Mean (standard deviation).

Table 2. Anthocyanin composition as percentage of total of the mutant background tissue and revertant sectors of variegated flowers from the F₂ population of *P. hybrida* 'ph6-m1' x *P. hybrida* 'Red'. All flowers contained petunidin and peonidin 3-rutinoside-5-glucosides acylated with either coumaric or caffeic acid. Plants 8-1 and 8-8 produced flowers in which the sectors were a different color than the background tissue; plants 9-1 and 9-2 produced sectors of the same color as the background.

Plant	Petunidin		Peonidin	
	Coumaric (%)	Caffeic (%)	Coumaric (%)	Caffeic (%)
8-1				
Mutant	53.8 (3.6) ^y	13.9 (1.6)	23.0 (0.8)	9.3 (2.8)
Revertant	57.1 (3.8)	7.4 (2.5)	23.1 (2.2)	12.4 (4.2)
8-8				
Mutant	63.1 (7.8)	16.7 (1.9)	11.7 (1.3)	8.5 (1.1)
Revertant	54.8 (1.2)	14.4 (0.7)	22.3 (0.8)	8.4 (1.1)
9-1				
Mutant	62.2 (6.2)	13.3 (3.8)	12.5 (2.8)	12.0 (5.3)
Revertant	59.4 (6.7)	16.1 (2.7)	10.5 (1.2)	14.0 (5.8)
9-2				
Mutant	63.9 (3.4)	10.0 (4.1)	22.8 (0.8)	3.3 (1.0)
Revertant	60.3 (4.0)	12.1 (2.5)	22.6 (0.6)	5.0 (1.6)

^yPercentage of total (standard deviation).

m1' x 'Violet' F₂ population, the pH of the sectors and background tissue was the same. While in the 'ph6-m1' x 'Red' F₂ population, only those plants which produced variegated flowers in which the sectors were not redder than the background was there no difference in pH between the two tissues. In those plants in which the sectors were redder than the background, there was a pH difference between the two tissues. The redder sectors had a lower pH than the background tissue.

Four plants from the 'ph6-m1' x 'Red' F₂ population were selected for further analysis. Two of the plants produced sectors that were darker and redder than the background (Table 1, plants 8-1 and 8-8) and two of the plants produced sectors that were only darker than the background (Table 1, plants 9-1 and 9-2). Flowers of plant 8-1 had a purple background (RHS 78A) with magenta sectors (RHS 78A), while flowers of plant 8-8 had a violet background (RHS 90A) with purple sectors (RHS 64A). Flowers of plant 9-1 had a pale violet background (RHS 90C) with violet sectors (RHS 90A), while flowers of plant 9-2 had a pale magenta background (RHS 78C) with magenta sectors (RHS 78A).

PIGMENT CONCENTRATION. In all four of the selected 'ph6-m1' x 'Red' F₂ plants, the background tissue was less intense in color and produced less pigment than the sectors (Table 1). The greatest difference in the intensity was found in plant 8-1, where there was nearly a 10-fold change. In the other plants, the difference was ≈3-fold. The difference in the intensity of color was not due to a change in the ratio of anthocyanin to flavonol (Table 1). In addition, the difference in the intensity occurred irrespective of whether the sectors were redder than the background tissue.

PIGMENT COMPOSITION. All four of the 'ph6-m1' x 'Red' F₂ plants produced petunidin and peonidin (Table 2). The predominant anthocyanin was petunidin 3-coumaroyl-rutinoside-5-glucoside in the sectors and background tissue in all of the plants. This anthocyanin accounted for ≈60% of the total. There was significant variation in the relative concentration of the minor anthocyanins between the sectors and background tissue, but no significant variation in the relative concentration of the major anthocyanin in the two tissues. This small amount of variation would have little or no effect on the flower color.

The copigments found within the sectors and background tissue

Table 3. Flavonol composition as percentage of total of the mutant background tissue and revertant sectors of variegated flowers from the F₂ population of *P. hybrida* 'ph6-m1' x *P. hybrida* 'Red'. All flowers contained quercetin glucosides. Plants 8-1 and 8-8 produced flowers in which the sectors were a different color than the background tissue; plants 9-1 and 9-2 produced sectors of the same color as the background.

Plant	Flavonol (%) ^z			
	3-soph-7-glu	3,7-diglu	3-soph	3-glu
8-1				
Mutant	7.9 (4.5) ^y	47.1 (5.7)	39.3 (1.8)	5.7 (3.1)
Revertant	7.6 (2.5)	39.7 (3.3)	46.4 (3.0)	6.3 (4.0)
8-8				
Mutant	15.5 (9.6)	9.4 (1.7)	54.9 (3.9)	20.2 (9.7)
Revertant	8.6 (7.4)	17.7 (5.7)	28.1 (1.7)	45.6 (14.2)
9-1				
Mutant	23.6 (8.6)	15.2 (4.5)	50.6 (4.5)	10.6 (2.3)
Revertant	8.0 (0.4)	7.7 (2.2)	60.9 (0.2)	23.4 (2.7)
9-2				
Mutant	25.9 (13.4)	24.9 (4.2)	29.8 (4.8)	19.4 (4.5)
Revertant	19.1 (4.2)	24.4 (4.6)	28.9 (2.6)	27.6 (1.3)

^zSoph = sophoroside, glu = glucoside.

^yPercentage of total (standard deviation).

were all quercetin glycosides (Table 3). There was considerable variation in the glycosylation pattern of the quercetin between the sectors and background tissue; however, the differences in the glycosylation pattern of the copigment would not have had an affect on color (Asen, 1972).

Discussion

In this study, the effects of a single *Ph* gene on flower color was studied in detail. The *Ph6* gene influenced flower color in two independent ways. The *Ph6* gene influenced the intensity of pigmentation by effecting the total amount of the anthocyanins and flavonols that were produced (Table 1). In cells that were homozygous recessive for the *ph6* gene (i.e., mutant background tissue), there was 3- to 10-fold less pigment produced than in cells that were dominant for the *Ph6* gene (i.e., revertant sector tissue).

The *Ph6* gene could also have influenced the specific color of the sectors, making them redder than the background tissue. The redder color of the sectors was due to a change in the pH and not due to either the anthocyanin or copigment composition or concentration. The effect of the *Ph6* on the redder color of the sectors depended on the genetic background in which it was expressed. If the *Ph6* gene was expressed in the *P. hybrida* 'Red' genetic background, the pH of the sectors was 0.4 units lower than that of the background tissue (Table 1). While the *Ph6* gene had no effect on the pH if expressed in the genetic background of *P. hybrida* 'Violet'. The F₂ segregation data suggested that the influence of the *Ph6* gene on the pH required the expression of at least one additional gene of which there were two genes possible.

The additional genes required for the *Ph6* gene to affect pH were present in the *P. hybrida* 'Red' genotype and not in the *P. hybrida* 'Violet' genotype. The only genes that were expressed in the F₂ *P. hybrida* 'Red' hybrid and not in *P. hybrida* 'Violet' F₂ hybrid were *Ph1* and *Ph2*. Therefore, it appears that the change in pH requires the expression of the *Ph6* gene and at least one of the four *Ph1* or *Ph2* alleles, which is in contrast to the effect of the *Ph6* gene on pigment concentration where no additional genes are required for it effect.

These data suggest that the *Ph6* gene may be a regulatory gene that controls the expression of *Ph* and anthocyanin structural genes. Besides influencing the pH and flavonoid concentration, *Ph6* also effects seed coat development (Chuck et al., 1993). Anthocyanin regulatory genes (*An1*, *An2*, and *An11*) are known to control *Ph* and anthocyanin structural genes (Quattrocchio et al., 1994). In plants recessive for the *an1*, *an2*, and *an11* genes, the pH of the flower was 0.7 units higher. Genetic analysis suggested that the *An1* gene specifically controlled the *Ph4* gene. The *An1* gene encodes a basic helix-loop-helix transcription activator (Quattrocchio et al., 1993). The *An2* gene encodes a MYB-domain transcription activator (Quattrocchio, 1994). The *An11* gene encodes a WD-repeat protein (Vetten et al., 1997). It was suggested that the *An11* gene was a cytoplasmic component of a signal transduction cascade that modulates the function of the *An2* gene,

linking cellular signals with transcriptional activation (Vetten et al., 1997).

It is suggested that the *Ph6* directly controls flavonoid production and the *Ph1* and *Ph2* genes. In the revertant sectors, the *Ph6* gene becomes active and increases the production of anthocyanins and copigments which leads to an increase in the intensity of color. The *Ph6* gene also can turn on the expression of the *Ph1* or *Ph2* genes. Therefore if the revertant sector contains one or more *Ph1* or *Ph2* alleles, then the *Ph6* gene can turn on their expression which in turn lead to a reduction in the pH and a redder color.

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