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CONTROL OF ANTHOCYANIN SYNTHESIS IN *PETUNIA HYBRIDA* BY MULTIPLE ALLELIC SERIES OF THE GENES *AN1* AND *AN2*

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ABSTRACT

A mutable allele of the *An1* locus in *Petunia hybrida* has given rise to a multiple series of stable derivative alleles. Anthocyanin concentration in mature flowers of these mutants ($an1^{+/p}/an1$) decreases from the wild-type red to the recessive white in a continuous series. Anthocyanin composition changes regularly: the ratio of peonidin to cyanidin is 3.5 for an $an1^{+/+}/an1$ and 1.2 for an $an1^{+/p5}/an1$ mutant. Analysis of anthocyanins during flower development indicates that these differences in composition are due to the specific state of the *An1* locus and not to anthocyanin concentration. Anthocyanin concentration in flowers of the allelic series for *An1* correlates with the activity of the enzymes UDP-glucose: flavonoid-3-*O*-glucosyltransferase and SAM: anthocyanin-3′-*O*-methyltransferase. The same correlations were found for members of a comparable allelic series at the *An2* locus. The possibility that the correlation between the enzyme activities is due to the occurrence of a multienzyme complex is discussed.

THE Anthocyanin 1 (*An1*) locus of *Petunia hybrida* is one of the loci involved in the conversion of dihydroflavonols into anthocyanins (WIERING 1974; GERATS *et al.* 1982). One of the alleles of the *An1* locus (see BIANCHI *et al.* 1978) is $an1^{+/p6s}$. This allele is characterized by the occurrence of smaller and larger spots and sectors in a color varying between that of the dominant red and the recessive white phenotype on a pale background (A. G. M. GERATS, P. VAN LIEROP, J. VAN DE LAAN and F. BIANCHI, unpublished results). These mutations also occur in the germ line, giving rise to self-colored flowering plants ($an1^{+/p}$) in the next generation. By selection, a virtually continuous series of color variants can be obtained. In this respect the *An1* system resembles the systems at the Pallida locus of *Antirrhinum majus* (FINCHAM and HARRISON 1967), the *A2* locus of maize (REDDY and PETERSON 1976) and the *an2-1* system of *P. hybrida* (CORNU 1977). In addition to *An1*, *An2* also controls the conversion of dihydroflavonols into anthocyanins (Figure 1). A difference in phenotype of *an1/an1* and *an2/an2* mutants is that flowers of the former always are white, whereas flowers of the latter can be slightly colored. FARCY

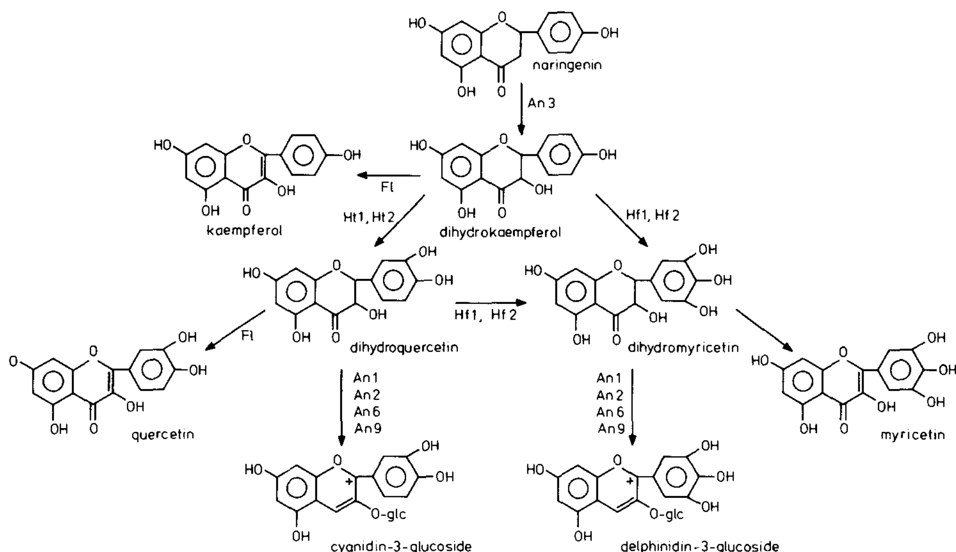

FIGURE 1.—Biosynthesis of anthocyanins and flavonols in *P. hybrida*. The genes mentioned are described in WIERING (1974).

and CORNU (1979) investigated anthocyanin composition in mature flowers of 40 *an2-n* mutants, which were derived from the *an2-1* unstable system and synthesized anthocyanin at levels intermediate between that of the recessive and the dominant alleles. Mutants producing low amounts of anthocyanins contained relatively more 3-glycosylated anthocyanins. When anthocyanin production was increased, the contribution of more highly substituted anthocyanins (Figure 2) increased. They concluded that the diverse *an2-n* alleles show regulation-like effects on subsequent modification reactions in *P. hybrida*. It should be noted, however, that a low anthocyanin concentration in itself may limit the enzyme reaction velocities, thereby explaining the deficit of more highly substituted and methylated anthocyanins in *an2-n* alleles with low anthocyanin production. To obtain more information about the control of both anthocyanin synthesis and modification we investigated $an1^{+/p}$ alleles for their quantitative and qualitative anthocyanin production, in mature flowers as well as during flower development. Furthermore, we determined activities of the UDP-glucose: flavonoid-3-*O*-glucosyltransferase (UFGT) and of *S*-adenosyl-L-methionine (SAM): anthocyanin-3′-*O*-methyltransferase (OMT) in extracts of flowerbuds of plants containing $an1^{+/p}$ or *an2-n* alleles.

MATERIALS AND METHODS

Plant material: The originally selected $an1^{+/p}$ mutants all produce cyanidin-3-glucoside as a main pigment. Five of these mutants were crossed to the line W20, a standard inbred line. Flowers of the progeny are expected to contain peonidin-3RGac5G as a main pigment. Cuttings of some *an2-n* mutants were transported from Dijon to Amsterdam as plantlets. They were grown in the greenhouse, beside the $an1^{+/p}$ mutants. All *an2-n* mutants have the general formula *an2-n/an2* and produce petunidin-3RGac5G as the main pigment (FARCY and CORNU 1979). In Table 1 the genes and alleles of interest in the present investigation are listed.

Anthocyanin extraction: Anthocyanins from mature flowers were extracted from one piece (di-

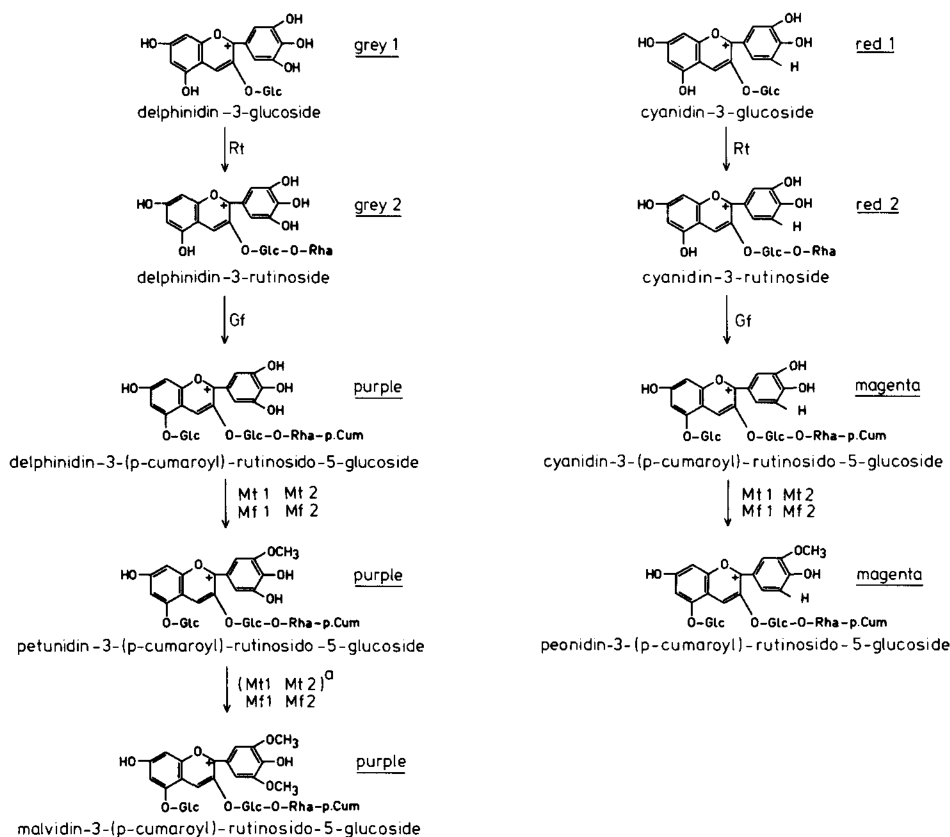

FIGURE 2.—Modifications of anthocyanins in *P. hybrida*. The genes mentioned are described in WIERING (1974).

TABLE 1

List of genes and their phenotypic effects in an appropriate background

Gene	Phenotypic effect
An1	Synthesis of anthocyanins in the flower
an1	No synthesis of anthocyanins in the flower
an1$^{+/p}$	Decreased anthocyanin synthesis in the flower
an1$^{+/+}$	Derivative allele, showing a seemingly wild-type activity
An2	Synthesis of anthocyanins in the flower
an2	No or little anthocyanin synthesis in the flower
an2-n	Decreased anthocyanin synthesis in the flower
Mt1, Mt2	Predominantly 3′-methylated anthocyanins (peonidin, petunidin)
Mf1, Mf2	Predominantly 3′-, 5′-methylated anthocyanins (malvidin)

ameter 12 mm) of each of the five petals, with methanol-HCl (0.5% v/v) for 30–60 min. Absorption was measured on a Zeiss PM QII spectrophotometer at 530 nm. For developmental curves corollas of buds and flowers were extracted in 3–5 ml of methanol-HCl (0.5% v/v). To 1 ml of the methanol extract, 0.75 ml of H_2O and 2 ml of chloroform were added, which resulted in a

Folch partition (FOLCH, LESS and SLOANE-STANLEY 1957). The upper phase of this partition contained the anthocyanins. Part of this upper phase was injected into an high performance liquid chromatograph, equipped with a Lichrosorb 10 RP18 column. Anthocyanins were eluted using a 20-min gradient of 10–25% methanol in 10% formic acid at a flow rate of 4 ml/min and a temperature of 45°. Anthocyanin concentration was calculated from the absorbence at 530 nm, using a millimolar extinction coefficient of 34. Retention times of the diverse anthocyanins were presented by SCHRAM, JONSSON and DE VLAMING (1983).

Enzyme extraction: Essentially, two methods of enzyme extraction were used. Five to ten flowerbuds were homogenized in 3 ml of extraction buffer (50 mM potassium phosphate, pH 7.5; 20 mM β-mercaptoethanol; 5% PVP) with a Braun homogenizer, and the homogenate was centrifuged at 40,000 × *g* for 20 min. The filtered supernatant was eluted over a polyclar AT column (10 × 0.5 cm) with elution buffer (10 mM potassium phosphate, pH 7.5; 4 mM β-mercaptoethanol). Fractions containing the bulk of the protein were pooled, and the pooled extract was used in all of the assays.

In the second method two flowerbuds were homogenized with a pestle and a mortar to which the following additions had been made: a little quartz sand, an excess of Dowex 1 × 2 and 1 ml of buffer (30 mM potassium phosphate, pH 6.5; 20 mM β-mercaptoethanol). The homogenate was centrifuged at 10,000 × *g* (Janetzki table centrifuge) for 10 min. The supernatant was used directly in all of the assays. In both methods all steps were performed at 0–4°.

Enzyme assays: Activity for UFGT was determined by incubating 50–90 μl of enzyme extract for 30–120 sec at 30° in a reaction mixture containing H_2O, potassium phosphate buffer (10 mM, pH 7.5), UDPG (1 mM) and delphinidin-chloride (20 μM) in HCl (5 mM) in a total volume of 200 μl. The reaction was started by the addition of delphinidin-chloride and terminated by the addition of 800 μl of chloroform-methanol, 2:1 (1% HCl, v/v).

The standard methyltransferase assay contained cyanidin-3RGac5G (60 μM) (in 5 mM HCl), SAM (0.5 mM) in 50 mM potassium phosphate buffer (pH 7.5), EDTA (5 mM), $MgCl_2$ (20 mM), potassium phosphate buffer (250 mM; pH 7.5) and 25 μl of enzyme extract in a total volume of 100 μl. The reaction was terminated after 15 min at 37° by the addition of 400 μl of chloroform-methanol, 2:1 (2% HCl, v/v).

Activity for glucose-6-phosphate dehydrogenase (G6PD) was measured spectrophotometrically. The assay contained Tris-HCl (127 mM, pH 8.0), $MgCl_2$ (6.6 mM), glucose-6-phosphate (2 mM), 50 μl of enzyme extract and NADP (0.75 mM) (not in the blank). The reaction was started by the addition of NADP, and the increase in absorption at 340 nm was followed for 5 min.

Activity for α-D-mannosidase was measured spectrophotometrically. The assay contained sodium acetate buffer (125 mM, pH 4.0), *p*-nitrophenyl-α-D-mannopyranoside (0.83 mM in H_2O), 50 μl of enzyme extract and H_2O in a total volume of 200 μl. The reaction was started by the addition of enzyme extract and terminated after 10 min at 30° by the addition of 800 μl of glycine-NaOH buffer (1 M glycine, pH 10.6). The extinction at 405 nm was measured within 2 hr after the reaction was carried out.

Activity for G6PD was measured in polyclar AT extracts as a reference enzyme; activity for α-D-mannosidase was likewise measured in Dowex extracts.

High performance liquid chromatography analysis: Part of the upper phase of a Folch partition was injected into a series 3B (Perkin Elmer) liquid chromatograph equipped with a Lichrosorb 10 RP18 column (24 × 0.5 cm). Detection occurred with an LC 75 (Perkin Elmer) wavelength detector at 530 nm; anthocyanins were eluted in a 7-min gradient of 20–50% methanol in 10% formic acid at a flow rate of 4 ml/min and a temperature of 45°. Under these conditions the retention times for delphinidin-3-glucoside and delphinidin aglucon are 2.9 and 4.7 min, respectively, as determined with appropriate standards.

Cyanidin-3RGac5G and peonidin-3RGac5G were detected using a 7-min gradient between 17.5 and 25% methanol in 10% formic acid. The retention times were 3.2 and 4.6 min, respectively, as determined with appropriate standards.

Protein assay: Protein content was measured using the Biorad protein assay (BRADFORD 1976), with bovine serum albumin as a standard protein.

RESULTS AND DISCUSSION

Anthocyanin synthesis in the an1$^{+/p}$ *alleles*: All *an1*$^{+/p}$ mutants were isolated in

heterozygous form: *an1*$^{+/p}$/*an1*. We obtained a very gradual series of mutant alleles, differing very little from one to the next in anthocyanin production. In the present study we investigated five *an1*$^{+/p}$ mutants that differed significantly in anthocyanin concentration of the mature flower. Table 2 gives peonidin-3RGac5G and cyanidin-3RGac5G concentrations and their ratio for mature flowers of the five mutants.

The ratio of peonidin to cyanidin increases from 1.2 in flowers of a very light-colored mutant to 3.5 in flowers of a dark-colored mutant. This indicates that when anthocyanin concentration increases in the mature flower the conversion of cyanidin to peonidin is increasingly efficient. The question remains whether this result reflects a kinetic effect, in which higher anthocyanin concentrations give rise to higher reaction velocities, or perhaps a genetic effect in which it is the state of the gene *An1* (and likewise *An2*) that determines the efficiency of the genes controlling subsequent biosynthetic steps.

A change in ratio during flower development of a dark-colored mutant would support the first hypothesis. In Table 3 the ratios for peonidin to cyanidin during flower development are given for two extreme mutants. The developmental curves show fixed ratios. These results indicate that the ratio of peonidin to cyanidin is determined by the state of the particular *an1*$^{+/p}$ allele and not merely by the anthocyanin concentration. The conclusion that the *an1*$^{+/p}$ alleles regulate the efficiency of the genes controlling biosynthetic steps is in agreement with the conclusion reached by FARCY and CORNU (1979) on the *an2-n* alleles.

Enzyme activities in the an1$^{+/p}$ *and* an2-n *alleles*: In the preceding section it was suggested that both *An1* and *An2* control the expression of the genes involved in substitution reactions of the anthocyanin skeleton. KHO, KAMSTEEG and VAN BREDERODE (1978) stated that *An1* and *An2* are both involved in the control of UFGT activity. We recently confirmed this suggestion and showed a five-to-15-fold reduction of UFGT activity in mutants homozygous recessive for *An1* or *An2* (GERATS *et al.* 1983). We investigated representatives of both allelic series for their UFGT activity. In addition, the OMT activity was measured. To avoid negative influence of gross environmental changes and of gross changes in protein amount, the activity of a reference enzyme was measured. It appeared that within cultivars the ratio of UFGT activity to the activity of the reference enzyme gave a valuable refinement of the measurement of UFGT activity (GERATS *et al.* 1983). G6PD was used as a reference enzyme in the polyclar AT extracts. In the Dowex extracts, G6PD activity is almost completely abolished. In these extracts α-mannosidase was used as a reference. For the reference enzymes we assumed that (1) both show a similar general response to gross environmental changes as do UFGT and OMT and (2) both enzymes show no genetic variation in the mutants under investigation. The results are presented in Tables 4 and 5. In both allelic series we found a positive correlation between anthocyanin concentration in extracts of mature flowers and UFGT activity in extracts of opening flowerbuds. Furthermore, a positive correlation between anthocyanin concentration and OMT activity was found for both allelic series. Moreover, the ratio of UFGT activity and OMT activity is constant for both allelic series (Figure 3). Obviously, the UFGT and

TABLE 2

Mean anthocyanin content (A_{530}) and composition in mature flowers of $an1^{+/p}/an1$ *mutants in three experiments*

Genotype	A_{530}	cya3RGac5G[a]	peo3RGac5G[a]	Ratio[b]
$an1^{+/+}/an1$	18.8 ± 1.8	135.7 ± 19.1	472.5 ± 32.5	3.5
$an1^{+/p9}/an1$	6.2 ± 0.3	46.2 ± 12.0	100.1 ± 30.5	2.2
$an1^{+/p8}/an1$	1.8 ± 0.1	13.1 ± 1.8	26.4 ± 7.9	2.0
$an1^{+/p6}/an1$	0.7 ± 0.1	5.7 ± 1.2	9.5 ± 2.0	1.7
$an1^{+/p5}/an1$	0.3 ± 0.1	1.6 ± 1.2	1.9 ± 1.6	1.2

[a] In nanomoles per flower.
[b] Peonidin-3RGac5G to cyanidin-3RGac5G.

TABLE 3

Mean anthocyanin content (A_{530}) and ratio of peonidin-3RGac5G to cyanidin-3RGac5G during flower development for two $an1^{+/p}/an1$ *mutants in three experiments*

Genotype	Flowerbud length 20 mm	30 mm	40 mm	45 mm	50 mm	flower
$an1^{+/+}/an1$						
A_{530}	0.4 ± 0.1	1.9 ± 0.3	7.1 ± 0.5	7.4 ± 1.5	11.0 ± 0.1	17.9 ± 3.0
Ratio	4.1	5.0	3.4	4.1	3.5	3.8
$an1^{+/p5}/an1$						
A_{530}	0.1	0.1	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.8 ± 0.1
Ratio	ND	ND	1.6	1.3	1.4	1.8

ND = not determined.

TABLE 4

UDP-glucose: flavonoid-3-O-glucosyltransferase UFGT and SAM: anthocyanin-3′-O-methyltransferase OMT activity in some $an1^{+/p}/an1$ *mutants*

	UFGT		OMT		UFGT	
Genotype	SA[a]	Ratio[b]	SA[a]	Ratio[b]	OMT	A_{530}
$an1^{+/p9}/an1$[c]	27.3	14.7	1.34	0.72	20.4	6.2 ± 0.3
$an1^{+/p8}/an1$	20.9	10.1	0.90	0.43	23.2	1.8 ± 0.1
$an1^{+/p6}/an1$	16.1	6.7	0.94	0.39	17.1	0.7 ± 0.1
$an1^{+/p5}/an1$	13.8	5.5	0.79	0.32	17.5	0.3 ± 0.1

Protein in assay, 70–100 μg.
[a] SA: specific activity in nanomoles per milligram of protein per minute.
[b] Ratio: UFGT SA/G6PD SA, respectively, OMT SA/G6PD SA.
[c] In a nonrelated experiment we measured a UFGT SA of 31.3 for $an1^{+/+}an1$; the flower color of such a mutant is phenotypically indistinguishable from that of an *An1/an1* heterozygote.

OMT activities are both correlated with the expression of the genes *An1* and *An2*. These results confirm the results shown in Table 2: when the anthocyanin concentration is increased (in mature flowers of the different mutants), the amount of peonidin increases. Furthermore, these results are in agreement with the results, obtained by FARCY and CORNU (1979), using the *an2-n* alleles.

TABLE 5

UDP-glucose: flavonoid-3-O-glucosyltransferase UFGT and SAM: anthocyanin-3'-O-methyltransferase OMT in some an2-n/an2 *mutants*

Genotype	UFGT		OMT		UFGT/OMT	A_{530}
	SA[a]	Ratio[b]	SA[a]	Ratio[b]		
An2/an2	30.0	26.1	3.69	3.2	8.1	5.50
an2-157/an2	18.3	25.4	1.97	2.8	9.3	4.46
an2-160/an2	9.1	15.0	0.82	1.4	11.1	2.86
an2/an2	2.3	3.5	0.09	0.1	(25.5)	1.73

[a] SA: specific activity in nanomoles per milligram of protein per minute.
[b] Ratio: UFGT SA/α-mannosidase SA or OMT SA/α-mannosidase SA.

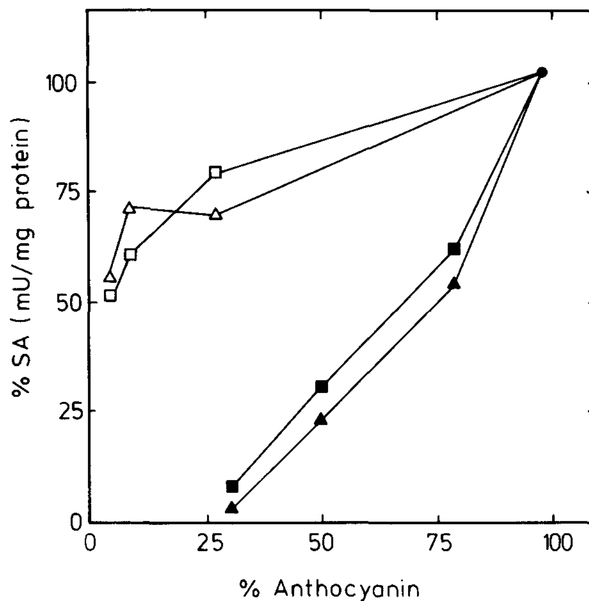

FIGURE 3.—Relation between UFGT and OMT activity in $an1^{+/p}/an1$ and *an2-n/an2* mutants. Anthocyanin concentration and enzyme activities of the darkest mutants ($an1^{+/p9}/an1$ and *an2-n/an2*, respectively) were taken as 100% (compare Tables 4 and 5). △,▲, methyltransferase; △,□, $an1^{+/p}/an1$ mutants; □,■, glucosyltransferase; ▲,■, *an2-n/an2* mutants.

Concomitant expression of genes involved in flavonoid biosynthesis has been reported for parsley cell cultures: upon illumination a group of enzymes was synthesized *de novo* (HAHLBROCK *et al.* 1971). DOONER (1983) discussed the coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. In our system, the expression of a particular gene (*An1* or *An2*) coincides with and controls the expression of other genes, either by transcriptional or translational or posttranslational control. In this context it is also interesting to note that enzymes involved in anthocyanidin modification are localized in the cytoplasm of the cell (HRAZDINA, WAGNER and SIEGELMAN 1978; JONSSON *et al.* 1983). It seems possible that these enzymes are incorporated in a multienzyme

complex. The strict correlation in expression of anthocyanidin-modifying enzymes supports this hypothesis.

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